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Alina Cequier Soler

Células madre mesenquimales en medicina regenerativa equina: importancia del equilibrio inmunomodulación - inmunogenicidad en las terapias celulares alogénicas

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CÉLULAS MADRE MESENQUIMALES EN MEDICINA REGENERATIVA EQUINA: IMPORTANCIA DEL EQUILIBRIO INMUNOMODULACIÓN - INMUNOGENICIDAD EN LAS TERAPIAS CELULARES ALOGÉNICAS

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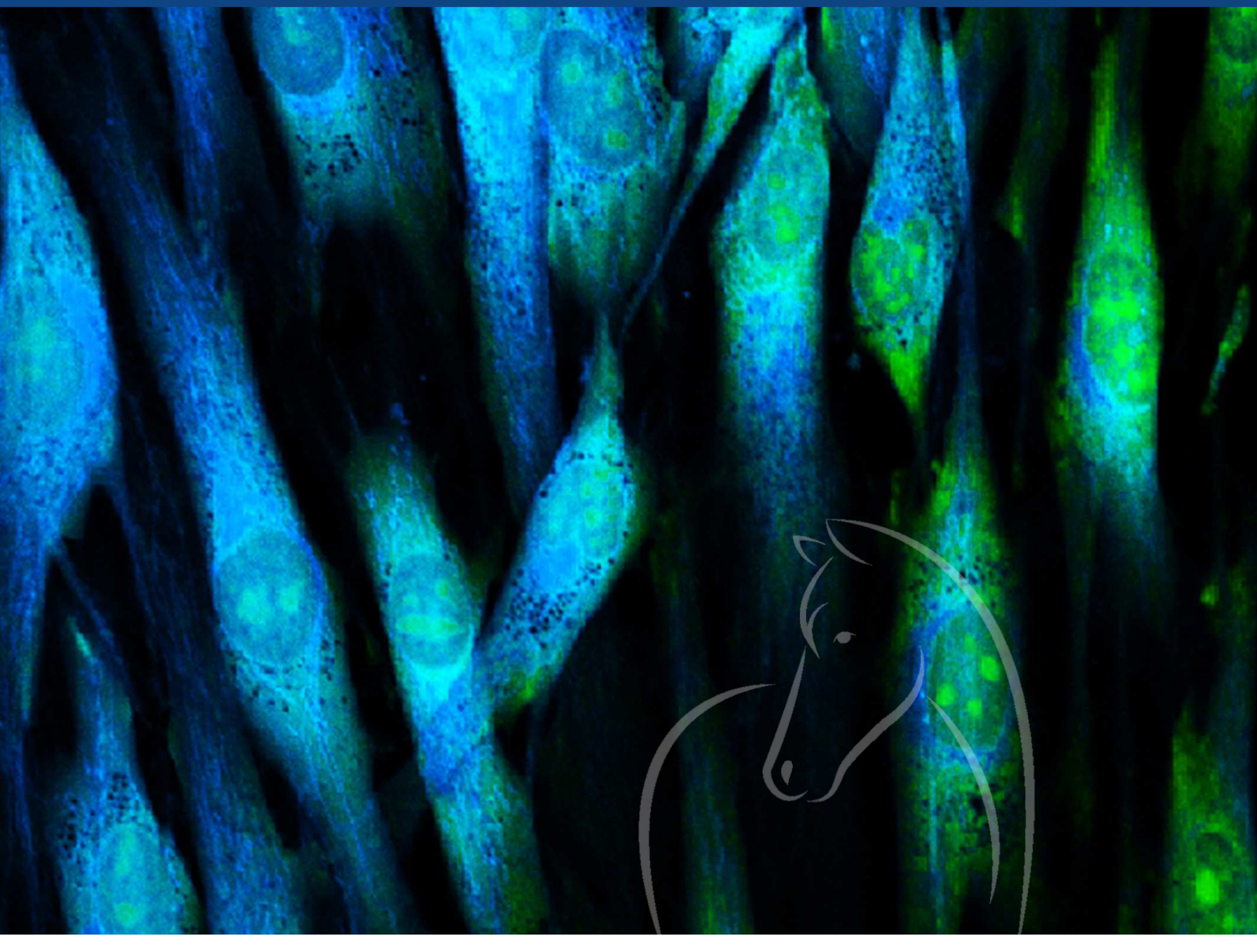
Universidad Zaragoza

TESIS DOCTORAL

Células madre mesenquimales en medicina regenerativa equina: importancia del equilibrio inmunomodulación - inmunogenicidad en las terapias celulares alogénicas

Alina Cequier Soler

Zaragoza, 2023



Laboratorio de Genética Bioquímica (LAGENBIO)

Departamento de Anatomía, Embriología y Genética Animal

Facultad de Veterinaria

Universidad de Zaragoza

Células madre mesenquimales en medicina regenerativa equina: importancia del equilibrio inmunomodulación - inmunogenicidad en las terapias celulares alogénicas

Memoria presentada por:

Alina Cequier Soler

Para optar al grado de Doctora por la Universidad de Zaragoza

Directores:

Clementina Rodellar Penella

Antonio Romero Lasheras

Zaragoza, julio 2023

Departamento de
Anatomía, Embriología
y Genética Animal
Universidad Zaragoza

Doña **Clementina Rodellar Penella**, Catedrática del Departamento de Anatomía, Embriología y Genética Animal de la Universidad de Zaragoza, y Don **Antonio Romero Lasheras**, profesor asociado del Departamento de Patología Animal de la Universidad de Zaragoza y subdirector de área de grandes animales del Hospital Veterinario de la Universidad de Zaragoza,

CERTIFICAN QUE:

La Tesis doctoral titulada "Células madre mesenquimales en medicina regenerativa equina: importancia del equilibrio inmunomodulación - inmunogenicidad en las terapias celulares alogénicas" que se recoge en la presente Memoria, de la que es autora Alina Cequier Soler, ha sido realizada bajo nuestra dirección y se corresponde con el Proyecto de Tesis aprobado por la Comisión Académica de Doctorado en septiembre de 2018, cumpliendo, por tanto, todos los requerimientos exigidos para que su autora pueda optar al Grado de Doctora por la Universidad de Zaragoza con la mención de "Doctor Internacional".

Y para que así conste, firman la presente en Zaragoza a 27 de julio de 2023.

Fdo. Clementina Rodellar Penella

Fdo. Antonio Romero Lasheras

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“Everything will be okay in the end.

If it's not okay, it's not the end.”

John Lennon

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ABREVIATURAS

A

ACS: suero autólogo condicionado

ADN/DNA: ácido desoxirribonucleico

AINE: antiinflamatorios no esteroideo

ARM: Alliance for Regenerative Medicine

ARN/RNA: ácido ribonucleico

AT-MSCs: células madre mesenquimales derivadas de tejido adiposo

B

β2M: beta-2 microglobulina

bFGF: factor de crecimiento fibroblástico básico

BM-MSCs: células madre mesenquimales derivadas de médula ósea

BSA: albúmina de suero bovino

C

CB-MSCs: células madre mesenquimales derivadas de sangre del cordón umbilical

CCL: ligando de quimiocina

CD: clúster de diferenciación

cADN: ADN complementario

CFSE: carboxyfluoresceína diacetato N-succinimidil éster

CFU-F: unidades formadoras de colonias con morfología fibroblástica

ci-MSCs: células madre mesenquimales prediferenciadas a condrocito

COX2: cyclooxygenasa 2

CPA: célula presentadora de antígeno

CT-MSCs: células madre mesenquimales derivadas de tejido del cordón umbilical

CXCL: quimioquina CXC ligando

CXCR: quimioquina CXC receptor

D

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: dimetil-sulfóxido

E

ELA: antígeno leucocitario equino

ELISA: ensayo de inmunoadsorción ligado a enzima

ESCs: células madre embrionarias

F

FAO: Food and Agriculture Organization of the United Nations

FBS: suero fetal bovino

FDA: Food and Drug Administration

FGF: factor de crecimiento fibroblástico

FITC: isotiocianato de fluoresceína

FSC vs SSC: Forward SCatter (eje X) versus Side SCatter (eje Y)

G

GAPDH: gliceraldehído-3-fosfato deshidrogenasa

H

HA: ácido hialurónico

HGF: factor de crecimiento hepatocítico

HLA: antígeno leucocitario humano

IDO1: indoleamina 2,3- dioxigenasa 1

IDO2: indoleamina 2,3- dioxigenasa 2

I

IFNγ: Interferón gamma

IGF1: factor de crecimiento insulínico tipo 1

IL: interleuquina

IL6: interleuquina 6

iNOS1: sintasa de óxido nítrico inducible 1

iNOS2: sintasa de óxido nítrico inducible 2

iPSCs: células madre pluripotentes inducidas

ISCT: Sociedad Internacional de Terapia Celular

ISSCR: International Society for Stem Cell Research

IV: intravenoso

L

LPS: lipopolisacáridos

M

MCP-1: proteína quimioatrayente de monocitos 1

MHC: complejo mayor de histocompatibilidad

MLR: mixed lymphocyte reaction

mRNA: RNA mensajero

MSC-C: células madre mesenquimales diferenciadas a condrocito

MSC-E: células madre mesenquimales estimuladas con citoquinas proinflamatorias

MSC-SE: células madre mesenquimales sin estimular

MSC: mesenchymal stem cell

MTT: bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil-2H-tetrazolio

N

NK: natural killer, células

NO: óxido nítrico

O

OA: osteoartritis

OMS: Organización Mundial de la Salud

P

PB-MSCs: células madre mesenquimales derivadas de sangre periférica

PBL: peripheral blood lymphocytes

PBMC: peripheral blood mononuclear cells

PBS: tampón fosfato salino

PCR: reacción en cadena de la polimerasa

PGE2: prostaglandina E2

PGF: factor de crecimiento placentario

PHA: fitohemaglutinina

PLGA: poliácido láctico-co-glicólico

PMN: polimorfonucleares, células

Poly (I:C): ácido poliinosínico-policitidílico

PPARγ: receptor activado por proliferadores de peroxisomas gamma

Prá: Pura Raza Árabe

PRE: Pura Raza Español, caballo

PRP: plasma rico en plaquetas

PSI: Pura Sangre Inglés

PWM: mitógeno pokeweed

R

ROS: especies reactivas de oxígeno

RT-qPCR: reacción en cadena de la polimerasa cuantitativa en tiempo real

RUNX2: factor de transcripción de diferenciación de los osteoblastos 2

S

SDF-1: factor 1 derivado de células estromales

ST: Standard bred horse

T

TCR: receptor de células T

TGF-β: factor de crecimiento transformante beta

Th: T efectoras (helper), células

TLRs: toll like receptors

TNFα: factor de necrosis tumoral alfa

tp-MSCs: células madre mesenquimales prediferenciadas a tenocito

Treg: T reguladoras, células

V

VCAM1: molécula de adhesión celular vascular 1

VEGF: factor de crecimiento vascular endotelial

W

WHO: World Health Organization

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RESUMEN / SUMMARY

RESUMEN

Las células madre mesenquimales (MSCs) equinas son de gran interés en el campo de la medicina regenerativa, especialmente para el tratamiento de lesiones musculoesqueléticas. Debido a sus propiedades reguladoras, ejercidas a través de la secreción de diferentes moléculas, pueden ser también de gran utilidad en el tratamiento de enfermedades inflamatorias e inmunomediadas. Además, la posibilidad de utilizar MSCs alogénicas ha aumentado su atractivo, permitiendo su aplicación en etapas tempranas de la enfermedad y cuando existen limitaciones para la terapia autóloga. Sin embargo, hay que tener en cuenta que las MSCs no son inmunoprivilegiadas y pueden generar respuesta inmune en el paciente alogénico, condicionando la efectividad de las terapias alogénicas. Existen diversos factores que pueden influir en dicho reconocimiento inmune de las MSCs alogénicas y, de hecho, algunas estrategias terapéuticas planteadas para potenciar su efecto pueden influir sobre las interacciones entre las MSCs alogénicas y el sistema inmune del receptor. Este sería el caso de la estimulación de las MSCs con citoquinas proinflamatorias, la administración de MSCs ya diferenciadas y la compatibilidad para el complejo mayor de histocompatibilidad (MHC) entre donante y receptor.

El objetivo principal de la presente Tesis Doctoral fue estudiar cómo la inflamación y la diferenciación condrogénica afectan al equilibrio entre la capacidad inmunomoduladora e inmunogénica de las MSCs equinas, teniendo en cuenta la influencia de la compatibilidad para el MHC entre donante y receptor y eligiendo la especie equina por su importancia como paciente y como modelo animal. Para ello, se valoró la respuesta inmune frente a las MSCs tanto a nivel celular y humoral como local y sistémico, utilizando ensayos *in vitro* e *in vivo*, para caracterizar de forma completa dicha respuesta.

En primer lugar, se revisó el estado actual, las aplicaciones y las limitaciones de la utilización de las MSCs equinas en diferentes patologías y su potencial traslación como modelo animal para el estudio de diferentes enfermedades en la especie humana, entre las que destacan la uveítis recurrente, las afecciones fibroproliferativas de la piel y el asma.

En los ensayos *in vitro* llevados a cabo en la presente Tesis Doctoral se analizó la capacidad inmunomoduladora e inmunogénica de las MSCs equinas sin estimular (MSC-SE), estimuladas con citoquinas proinflamatorias (MSC-E) y diferenciadas a condrocito (MSC-C) al exponerlas *in vitro* a linfocitos de sangre periférica (PBL) autólogos, alogénicos MHC-compatibles y MHC-incompatibles. La estimulación de las MSCs activó su potencial regulador, reduciendo la proliferación de linfocitos T citotóxicos, T colaboradores y linfocitos B. Sin embargo, las MSC-E, especialmente en condiciones de incompatibilidad, mostraron una activación moderada del perfil inmunogénico y estimularon la proliferación de las células T citotóxicas y colaboradoras, aunque estos

resultados fueron acompañados de una mayor activación de su perfil regulador. Tras la diferenciación condrogénica, las MSC-C equinas no perdieron su capacidad reguladora ni aumentaron su inmunogenicidad, aunque estimularon la proliferación de linfocitos B y mostraron una capacidad reducida para estimular los linfocitos T reguladores.

En el estudio *in vivo*, se administraron MSC-SE, MSC-E y MSC-C de forma autóloga, y en receptores MHC-compatibles y MHC-incompatibles. Las MSCs fueron encapsuladas en hidrogeles de alginato y se colocaron tres implantes subcutáneos con el mismo tipo de MSCs en las tablas del cuello de cada receptor. Los implantes se recuperaron a las 1, 3 y 6 semanas de su implantación para su análisis. Transcurrido un mes tras retirar el último de los implantes, se expuso nuevamente a cada animal al mismo tipo de MSCs en las tablas contralaterales del cuello para valorar el efecto de una segunda administración.

El análisis del perfil inmune de las MSCs recuperadas mostró que las MSC-C, tras ser administradas tanto en caballos MHC-compatibles como MHC-incompatibles, conservaban sus propiedades inmunomoduladoras a lo largo del tiempo. Además, la compatibilidad del MHC atenuó la respuesta inmune local inducida tras la administración, especialmente en las MSC-C y MSC-SE. Sin embargo, las MSC-SE administradas en caballos MHC-incompatibles mostraron un perfil inmunogénico más pronunciado y un menor perfil inmunomodulador, junto con una mayor respuesta inmunitaria local en comparación con el grupo MHC-compatible después de ambas administraciones. La administración de MSC-E indujo un reclutamiento local, temprano y pronunciado, de linfocitos, acompañado de una disminución en la expresión de genes relacionados con la inmunomodulación y un aumento de la expresión de genes relacionados con la inmunogenicidad.

La respuesta inmune celular sistémica y la generación de respuesta celular de memoria se evaluó reexponiendo *in vitro* los PBLs de los distintos receptores al tipo de MSCs que habían recibido de sus correspondientes donantes. Todos los tipos de MSCs MHC-incompatibles indujeron la proliferación de linfocitos T, pero este efecto fue más pronunciado y sostenido en el tiempo en las MSC-E, así como en las MSC-SE después de la segunda administración. Sin embargo, en situación MHC-incompatible, las MSC-E tuvieron menor efecto sobre los linfocitos B a lo largo del tiempo, siendo estos activados en mayor medida tanto por las MSC-C como por las MSC-SE.

En la valoración de la respuesta inmune humoral, los receptores autólogos y MHC-compatibles no desarrollaron anticuerpos contra el MHC del donante de MSCs, independientemente de tipo de MSCs que recibieron. Sin embargo, los caballos MHC-incompatibles desarrollaron aloanticuerpos después de la primera administración y su producción aumentó tras la segunda administración de las mismas MSCs. Aunque los tres tipos de MSCs indujeron el desarrollo de una respuesta inmune humoral en los receptores MHC-incompatibles, las MSC-C produjeron mayores niveles de aloanticuerpos, lo que podría relacionarse con la mayor activación de las células B mencionada anteriormente.

En conclusión, la exposición de las MSCs a citoquinas proinflamatorias activa el perfil inmunomodulador de las MSCs *in vitro*, aunque dicho perfil no se conserva *in vivo*. En cambio, prevalece un perfil inmunogénico que afecta negativamente a la capacidad inmunoevasiva de las MSC-E, sobre todo en condiciones de incompatibilidad para el MHC, lo que puede limitar su uso terapéutico.

En cuanto a la diferenciación de las MSCs en condrocitos, ésta no afecta a sus propiedades inmunomoduladoras, pero puede favorecer el desarrollo de mecanismos de memoria inmunológica. Además, en receptores incompatibles para el MHC, las MSCs desarrollan un perfil más inmunogénico, lo que se relaciona con una mayor respuesta inmunitaria local y una mayor producción de aloanticuerpos después de una segunda administración.

Además, la administración repetida de MSC-SE en caballos incompatibles para el MHC mostró una mayor respuesta inmunitaria local y humoral en comparación con la administración de MSCs compatibles, lo que estaría relacionado con un perfil más inmunogénico y menos inmunomodulador de dichas MSCs.

Los resultados obtenidos en esta Tesis Doctoral muestran la relevancia de la compatibilidad del MHC entre el donante y el receptor, y contribuyen a una mejor comprensión del efecto de la inflamación y la diferenciación en el equilibrio inmunomodulación - inmunogenicidad de las MSCs equinas. El conocimiento y control de estos factores constituyen potenciales herramientas para lograr terapias celulares alogénicas efectivas y seguras.

SUMMARY

Mesenchymal stem cells (MSCs) in horses have garnered significant interest in the field of regenerative medicine, particularly for the treatment of musculoskeletal injuries. Due to their regulatory properties, exerted through the secretion of various molecules, they may also be highly valuable for treating inflammatory and immune-mediated diseases. Additionally, the potential to use allogeneic MSCs has increased their appeal, allowing for application in early stages of the disease and when autologous therapy is limited. However, it is essential to consider that MSCs are not immunoprivileged and may elicit an immune response in allogeneic recipients, potentially impacting the effectiveness of allogeneic therapies. Several factors can influence the immune recognition of allogeneic MSCs, and certain therapeutic strategies aimed at enhancing their effects may influence the interactions between allogeneic MSCs and the recipient's immune system. Examples include priming MSCs with proinflammatory cytokines, administering pre-differentiated MSCs, and considering compatibility for the major histocompatibility complex (MHC) between donor and recipient.

The main objective of this Doctoral Thesis was to study how inflammation and chondrogenic differentiation affect the balance between the immunomodulatory and immunogenic capacity of equine MSCs, taking into account the influence of MHC compatibility between donor and recipient and choosing the equine species for its importance as both a patient and an animal model. To achieve this, the immune response to MSCs was evaluated at the cellular and humoral levels, both locally and systemically, using *in vitro* and *in vivo* assays to comprehensively characterize this response.

Firstly, the current status, applications, and limitations of using equine MSCs in various pathologies were reviewed, as well as their potential as an animal model for studying different human diseases, including recurrent uveitis, fibroproliferative skin disorders, and asthma.

In the *in vitro* assays carried out in this Doctoral Thesis, the immunomodulatory and immunogenic capacity of equine MSCs in basal conditions (MSC-naïve), stimulated with proinflammatory cytokines (MSC-primed), and differentiated into chondrocytes (MSC-chondro) were analyzed. They were exposed *in vitro* to autologous, MHC-matched allogeneic, and MHC-mismatched allogeneic peripheral blood lymphocytes (PBLs). Stimulation of MSCs activated their regulatory potential, reducing the proliferation of cytotoxic T lymphocytes, helper T cells, and B cells. However, MSC-primed, especially under MHC-mismatched conditions, showed a moderate activation of the immunogenic profile and stimulated the proliferation of cytotoxic and helper T cells, although these results were accompanied by a greater activation of their regulatory profile. Following chondrogenic differentiation, equine MSC-chondro did not lose their regulatory capacity

or increase their immunogenicity, although they stimulated B cell proliferation and showed reduced capacity to stimulate regulatory T cells.

In the *in vivo* study, MSC-naïve, MSC-primed, and MSC-chondro were administered in autologous, MHC-matched, and MHC-mismatched recipients. Mesenchymal stem cells were encapsulated in alginate hydrogels and three subcutaneous implants with the same type of MSCs were placed on the necks of each recipient. The implants were retrieved at 1, 3, and 6 weeks after implantation for analysis. After one month from removing the last implant, each animal was exposed again to the same type of MSCs on the opposite side of the neck to assess the effect of a second administration.

The analysis of the immune profile of the recovered MSCs showed that MSC-chondro, after being administered to both MHC-matched and MHC-mismatched horses, retained their immunomodulatory properties over time. Moreover, MHC compatibility attenuated the local immune response induced after administration, especially in MSC-chondro and MSC-naïve. However, MSC-naïve administered to MHC-mismatched horses exhibited a more pronounced immunogenic profile and a reduced immunomodulatory profile, along with a higher local immune response compared to the MHC-matched group after both administrations. The administration of MSC-primed induced an early and pronounced local recruitment of lymphocytes, accompanied by a decrease in the expression of genes related to immunomodulation and an increase in the expression of genes related to immunogenicity.

The systemic cellular immune response and the generation of cellular memory response were evaluated by re-exposing the PBLs of the different recipients to the type of MSCs they had received from their corresponding donors *in vitro*. All types of MHC-mismatched MSCs induced the proliferation of T lymphocytes, but this effect was more pronounced and sustained over time in MSC-primed, as well as in MSC-naïve after the second administration. However, in an MHC-mismatched situation, MSC-primed had a lesser effect on B cells over time, as these were more activated by both MSC-chondro and MSC-naïve.

Regarding the assessment of the humoral immune response, autologous and MHC-matched recipients did not develop antibodies against the MHC of the MSC donors, regardless of the type of MSCs they received. However, MHC-mismatched horses developed alloantibodies after the first administration, and their production increased after the second administration of the same MSCs. Although all three types of MSCs induced the development of a humoral immune response in MHC-mismatched recipients, MSC-chondro produced higher levels of alloantibodies, likely related to the increased B cell activation mentioned earlier.

In conclusion, exposing MSCs to proinflammatory cytokines activates their immunomodulatory profile *in vitro*, but this profile is not maintained *in vivo*. Instead, an immunogenic profile prevails, negatively affecting the immune evasive ability of MSC-

primed, especially under MHC-mismatched conditions, which could limit their therapeutic use.

As for the differentiation of MSCs into chondrocytes, it does not affect their immunomodulatory properties but may favor the development of immune memory mechanisms. Furthermore, in MHC-mismatched recipients, MSC-chondro develop a more immunogenic profile, which is associated with a greater local immune response and increased production of alloantibodies after a second administration.

It is also observed that there is a higher local and humoral immune response after repeated administration of MSC-naïve in MHC-mismatched recipients compared to the administration of matched MSCs, which is related to a more immunogenic and less immunomodulatory profile of these MSCs.

The results obtained in this Doctoral Thesis highlight the importance of MHC compatibility between donor and recipient and contribute to a better understanding of the effect of inflammation and differentiation on the immunomodulation-immunogenicity balance of equine MSCs. Knowledge and control of these factors constitute potential tools to achieve effective and safe allogeneic cellular therapies.

I. INTRODUCCIÓN

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1. ANTECEDENTES

El caballo ha evolucionado desde su uso como herramienta de trabajo en el campo a ser considerado un animal de compañía y atleta profesional en disciplinas deportivas olímpicas como la Doma Clásica, Salto de Obstáculos y Concurso Completo. La Food and Agriculture Organization of the United Nations (FAO) estima que actualmente hay una población mundial de 58 millones de caballos, con una creciente dedicación al ocio y deporte. España, con una historia estrechamente ligada a los caballos, es el cuarto país europeo con mayor cantidad de caballos, con más de 722.158 ejemplares censados y 188.286 explotaciones. Además, la industria equina es un sector económicamente importante que genera un impacto económico superior a los 7.392 millones de euros anuales, representando el 0,59% del PIB español (Green Oak Group y Deloitte 2022).

El papel que desempeña el caballo en las disciplinas deportivas es fundamental, por lo que las patologías que disminuyen su rendimiento son especialmente relevantes. Las patologías musculoesqueléticas son un desafío para la profesión veterinaria, ya que son una de las principales causas de disminución del rendimiento deportivo en los caballos debido a su dedicación al deporte (Knott et al. 2022). Por tanto, mantener la salud del sistema musculoesquelético del caballo es esencial para su rendimiento deportivo.

Sin embargo, las lesiones del sistema musculoesquelético tienen una limitada capacidad de reparación debido a la escasa vascularización de tejidos como tendones, ligamentos o cartílago (Voga et al. 2020). Este proceso de reparación es largo y puede prolongarse durante meses, conllevando costes económicos directos e indirectos. Además, el tejido cicatricial formado es más débil y menos elástico que el tejido original, lo que lo hace más propenso a recidivas (Smith et al. 2013).

Dada la limitada capacidad de reparación de estos tejidos, es importante buscar tratamientos que permitan mejorar su cicatrización. Los tratamientos convencionales utilizados en estas patologías tienen tiempos largos de recuperación y altas tasas de recidiva de lesiones, por lo que es necesario encontrar nuevas opciones de tratamiento (Ortved 2018).

En los últimos años, se ha dado una mayor importancia a las terapias biológicas avanzadas, también conocidas como medicina regenerativa, especialmente a las terapias celulares, para mejorar la reparación de tendones, ligamentos y cartílago articular en lesiones musculoesqueléticas (Ribitsch et al. 2021).

Cabe destacar que, además de su interés como paciente, el caballo tiene una gran relevancia como modelo animal. En este sentido, la Food and Drug Administration (FDA) ha señalado al caballo como el modelo animal más apropiado para evaluar los efectos de las terapias celulares en patologías articulares (Iacono y Merlo 2022) y también ha

sido propuesto como modelo para otras patologías como el asma (Lange-Consiglio et al. 2019) y la uveítis recurrente (Witkowski et al. 2016).

De entre las diferentes terapias celulares que pueden emplearse en la especie equina, destaca la utilización de células madre mesenquimales (MSCs, de sus siglas en inglés *mesenchymal stem cells*). Inicialmente, las MSCs se empezaron a utilizar por su capacidad para diferenciarse en diferentes tipos de células e integrarse en la arquitectura tisular, promoviendo la regeneración directa del tejido dañado. Sin embargo, en los últimos años se ha demostrado que su mecanismo terapéutico se basa en otras propiedades, que ejercen principalmente de forma paracrina, mediante la síntesis de diversas moléculas bioactivas con capacidad trófica, antifibrótica, quimioatrayente e inmunomoduladora (Gugjoo et al. 2019). El descubrimiento de estas propiedades ha supuesto un gran cambio en la visión que se tenía acerca de las MSCs, lo que ha permitido aumentar el rango de sus aplicaciones terapéuticas (Voga et al. 2020).

Otro factor que ha aumentado el atractivo de las MSCs es la posibilidad de su administración alogénica, permitiendo aplicarlas en fases tempranas de la patología y cuando existen limitaciones para la terapia autóloga (Colbath et al. 2020b). Tradicionalmente, las MSCs se consideraban "inmunoprivilegiadas", pero esta concepción ha ido cambiando al observarse que sí pueden producir cierto nivel de respuesta inmunitaria (Berglund et al. 2017b) cuando son utilizadas en animales incompatibles para el complejo mayor de histocompatibilidad (MHC, de sus siglas en inglés *major histocompatibility complex*). De este modo, la respuesta inmunitaria del receptor puede influir en la efectividad de las MSCs alogénicas, aunque en ciertas situaciones estas células son capaces de suprimir la respuesta inmunitaria y "pasar desapercibidas" (Ankrum et al. 2014). La relación entre la capacidad de inmunomodulación y la inmunogenicidad de las MSCs es compleja y el equilibrio entre ambas es fundamental para el uso seguro y eficaz de las MSCs alogénicas, por lo que los factores que influyen sobre dicho equilibrio requieren de un estudio en profundidad.

Entre los factores que pueden modificar este equilibrio a favor de un perfil más regulador o, al contrario, más inmunogénico, se incluyen, entre otros, la exposición a un entorno inflamatorio o el proceso de diferenciación. La baja expresión de moléculas inmunogénicas y la secreción de moléculas inmunomoduladoras son clave para la capacidad inmunoevasora de las MSCs (Berglund et al. 2017b). En la especie equina se ha demostrado que la exposición de MSCs a citoquinas proinflamatorias induce sus propiedades inmunorreguladoras, pero también puede aumentar su inmunogenicidad (Schnabel et al. 2014; Barrachina et al. 2017). Aunque las propiedades inmunomoduladoras han cobrado mayor protagonismo como mecanismo terapéutico de las MSCs, su capacidad de diferenciación también puede contribuir al desarrollo de estrategias terapéuticas (Van Hecke et al. 2021). Por ello, surge la cuestión de qué ocurre con las propiedades inmunes de las MSCs después de diferenciarse y cuál es la

repercusión en el equilibrio inmunomodulación-inmunogenicidad. El estudio de estas propiedades *in vivo* tras la administración de MSCs es complicado debido a la dificultad para recuperar las células una vez administradas en el animal. Por ello, el empleo de biomateriales que permiten recuperar y estudiar las MSCs resulta muy interesante para poder analizar los cambios en el perfil de las células con mayor detalle (Santos et al. 2019). Mediante estos análisis se puede conseguir una mejor comprensión de las propiedades de las MSCs y su potencial en terapias celulares.

De esta forma, nos encontramos ante diversas situaciones que pueden influir sobre el delicado equilibrio inmunomodulación-inmunogenicidad de las MSCs, aspecto clave para la administración segura y efectiva de MSCs alogénicas. Sólo conociendo las interacciones de las MSCs alogénicas con el sistema inmune del receptor será posible optimizar las diferentes estrategias terapéuticas, siendo el caballo una especie de gran importancia para la consecución de dichas terapias, tanto como paciente como modelo animal.

Con el objetivo de contribuir a la optimización del uso de las MSCs alogénicas como estrategia terapéutica, la presente Tesis Doctoral se centra en el estudio de las propiedades inmunomoduladoras de las MSCs en la especie equina, su inmunogenicidad y su interacción con el sistema inmune del receptor.

2. LA ESPECIE EQUINA COMO MODELO ANIMAL EN EL CAMPO DE LA MEDICINA REGENERATIVA

Además de su importancia como paciente, el caballo tiene un gran valor como modelo animal debido a sus similitudes con la especie humana en términos de fisiopatogenia y clínica de muchas enfermedades, lo que puede ayudar en la resolución terapéutica de enfermedades tanto humanas como veterinarias (Colbath et al. 2017b).

Cabe destacar que la FDA y la International Society for Stem Cell Research (ISSCR) recomiendan el uso de modelos animales de gran tamaño para evaluar la eficacia y seguridad de los tratamientos terapéuticos avanzados (Hurtig et al. 2011). Esto se alinea con el concepto de “One Health, One Medicine” propuesto por la World Health Organization (WHO) y que defiende que la salud humana, animal y ambiental constituyen un único sistema que requiere de un avance conjunto (Fortier et al. 2020).

Las patologías musculoesqueléticas, tanto inflamatorias como degenerativas, presentan múltiples similitudes entre ambas especies (Colbath et al. 2017b) y son uno de los campos de mayor interés en la aplicación de las terapias celulares tanto en medicina humana como en medicina veterinaria. Cabe destacar que el caballo no solo se considera modelo de estas enfermedades, sino que se ha demostrado que puede utilizarse como modelo animal en el tratamiento de diferentes patologías oftalmológicas (Spaas et al. 2011; Marfe et al. 2012; Davis et al. 2019), reproductivas (Rink et al. 2018; Navarrete et al. 2020) y respiratorias (Barussi et al. 2016), entre otras.

Actualmente, la resolución terapéutica para muchas de estas patologías no es siempre curativa, por lo que las investigaciones más recientes se están centrando en encontrar tratamientos basados en terapias biológicas avanzadas, destacando las MSCs. La biología y las propiedades de las MSCs equinas son más similares a las de las MSCs humanas en comparación con otros modelos animales, por lo que se considera la especie equina un buen modelo traslacional para la investigación en esta área (Carrade et al. 2012; Colbath et al. 2017b).

Aunque deben realizarse más estudios sobre estas enfermedades y terapias, el rápido avance conseguido en la especie equina como modelo animal en lo que respecta a la medicina regenerativa la convierte en una especie idónea para la investigación traslacional (Fortier et al. 2020; Ribitsch et al. 2020).

3. MEDICINA REGENERATIVA

La medicina regenerativa es una rama emergente de la medicina que tiene como objetivo restaurar o restablecer la estructura y función original de un órgano o tejido en caso de enfermedad, lesión y/o degeneración asociada a la edad o defectos congénitos (Mao y Mooney 2015; Ribitsch et al. 2020).

Es importante destacar que el concepto de reparación es distinto al de regeneración, ya que la reparación conduce a la restauración de la continuidad interrumpida sintetizando un tejido cicatricial con características distintas al tejido original. Por el contrario, la regeneración restaura esa continuidad interrumpida mediante la síntesis de tejidos u órganos con las mismas propiedades que el original (Golchin y Farahany 2019; Voga et al. 2020).

En las últimas dos décadas se han logrado grandes avances en esta materia, estando cada día más cerca de conseguir el objetivo de establecer terapias regenerativas. Sin embargo y debido precisamente al mayor conocimiento de los nuevos productos terapéuticos y sus mecanismos de acción, a día de hoy, se ha descubierto que su función no es tanto reemplazar o regenerar células o tejidos lesionados, sino promover los propios mecanismos de reparación del organismo para lograr una mejor reparación de los tejidos dañados (Glotzbach et al. 2011; Ribitsch et al. 2021).

Aunque actualmente se sigue utilizando el término de medicina regenerativa en muchos ámbitos y países, el término utilizado en la Unión Europea que engloba estos productos terapéuticos es el de terapias avanzadas (Pimenta et al. 2021). Dichas terapias aplicadas con el objetivo de conseguir reparar los tejidos lesionados abarcan la ingeniería de tejidos (basada en tejidos y células manipulados mediante ingeniería), la terapia génica (basada en ácidos nucleicos recombinantes), y la terapia celular (basada en células somáticas sometidas a ciertas manipulaciones, como el cultivo celular), así como las combinaciones entre los diferentes tipos (Reglamento (CE) Nº 1394/2007; Directiva 2001/83/CE). Dentro de la terapia celular se incluyen las MSCs, temática principal de

esta Tesis Doctoral, y que son también de gran interés para las estrategias de ingeniería tisular (Li et al. 2019).

3.1 Productos ortobiológicos

Cuando las terapias avanzadas se aplican con la finalidad de promover la reparación de tejidos musculoesqueléticos, los productos utilizados reciben el nombre de ortobiológicos (Schroeder et al. 2020; Kruel et al. 2021).

Los productos ortobiológicos generalmente proceden del propio paciente (autólogos) y pueden contener células y/o factores de crecimiento y proteínas bioactivas con capacidad de mejorar el proceso de cicatrización de una lesión. El objetivo de estos productos es promover la reparación del tejido y fomentar la restauración de su estructura y función originales, de forma que el tejido reparado sea lo más similar posible al sano y disminuya el riesgo de recidivas (Stafford et al. 2020). A pesar de que el uso de estos productos puede promover la reparación de tejidos lesionados, es importante destacar que los programas de rehabilitación y ejercicio controlado siguen siendo fundamentales para lograr el éxito en los tratamientos de patologías del aparato locomotor (Ortved 2018).

Actualmente, existen diferentes productos ortobiológicos, la mayoría de ellos en fase de investigación. Debido a la falta de estandarización de los protocolos terapéuticos empleados en diversos estudios, resulta difícil la evaluación de muchos de los resultados observados (Ribitsch et al. 2021) y, aunque se ha demostrado el potencial terapéutico en alguno de ellos, otros carecen todavía de suficiente evidencia contrastada acerca de su efectividad, que avalaría su uso en determinadas patologías.

La clasificación de estos productos atendiendo a la celularidad de los mismos se muestra en la **Figura 1**. Se diferencian dos grupos principales: acelulares y celulares (que a su vez se dividen en función de su contenido en células progenitoras).

En el caso de los productos acelulares se encuentran el suero autólogo condicionado y la solución proteica autóloga cuyo mecanismo de acción se basa en un alto contenido en proteínas anabólicas y antiinflamatorias. Por otro lado, los productos celulares se dividen de acuerdo con su contenido en células progenitoras como son las MSCs, la fracción vascular estromal derivada del tejido adiposo, el concentrado de aspirado de médula ósea y las células madre pluripotentes inducidas (iPSCs, de sus siglas en inglés *induced pluripotent stem cells*). Dentro de los celulares también se encuentran los productos que no contienen células progenitoras como el plasma rico en plaquetas, la fibrina rica en plaquetas y el implante autólogo de condrocitos (Mateu 2021).

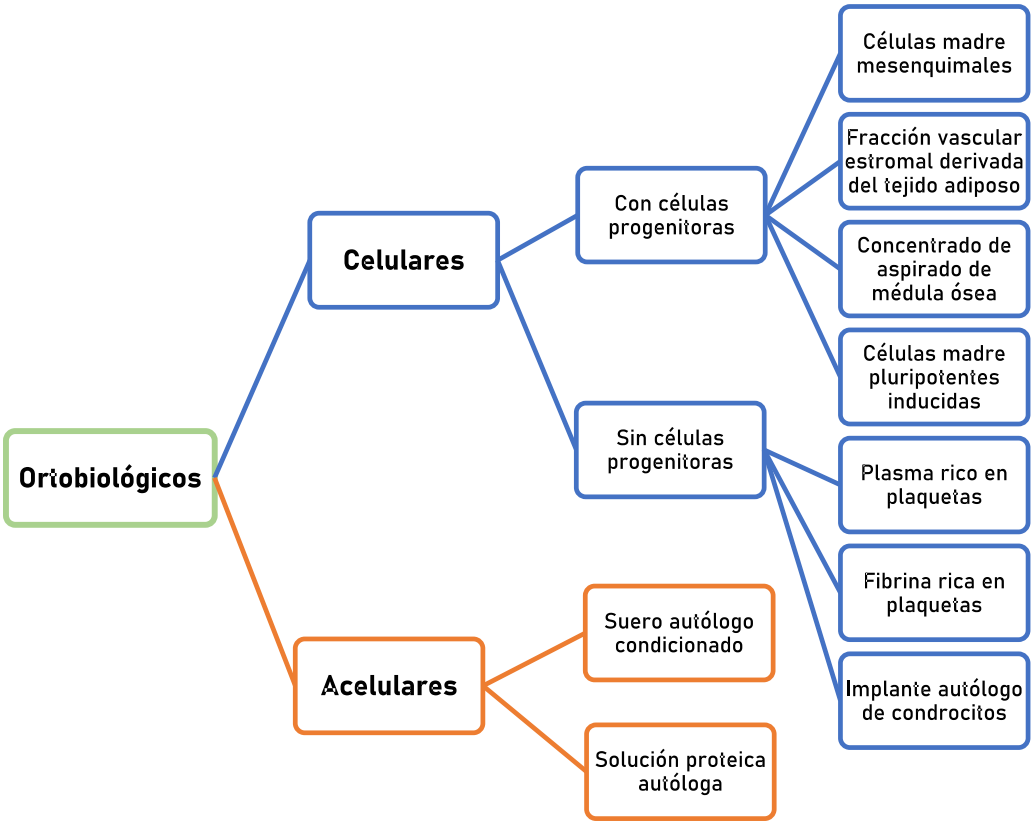


Figura 1. Clasificación productos ortobiológicos más empleados en la clínica equina (Mateu 2021).

Entre los productos ortobiológicos más empleados en la clínica equina para el tratamiento de patologías del aparato locomotor están las MSCs, el suero autólogo condicionado, la solución proteica autóloga y el plasma rico en plaquetas (Knott et al. 2022). Estos tres últimos se componen de derivados sanguíneos autólogos mínimamente manipulados (Ribitsch et al. 2021). La administración de MSCs en la clínica equina es más compleja y puede realizarse de forma autóloga pero también alogénica, obteniendo las células de otro individuo de la misma especie que actúe como donante (Colbath et al. 2020b).

A continuación, se explicarán algunos conceptos básicos sobre las células madre para posteriormente centrarnos en las MSCs, que son el objeto principal de la presente Tesis Doctoral.

3.1.1 Definición y clasificación de las células madre

Las células madre son células indiferenciadas, presentes en distintos tejidos y fases de desarrollo, que poseen gran capacidad de autorrenovación y proliferación, además de un potencial de diferenciación en un amplio rango de tipos celulares especializados (Ullah et al. 2015; Rajabzadeh et al. 2019). Existen distintas formas de clasificarlas, siendo las más comunes las que se refieren a su potencial de diferenciación y a su origen (**Figura 2**) (Hayes et al. 2012; Kalra y Tomar 2014).

3.1.1.1 Clasificación según capacidad de diferenciación

Atendiendo a su capacidad de diferenciación, de mayor a menor potencial, las células madre se pueden dividir en totipotentes, pluripotentes, multipotentes, oligopotentes y unipotentes (Kalra y Tomar 2014; Gugjoo et al. 2019). Las células totipotentes son aquellas que poseen el potencial para diferenciarse en un organismo vivo completo, dando lugar a células de cualquiera de las tres capas germinales (endodermo, mesodermo y ectodermo) y también a las estructuras extraembrionarias como la placenta. Las células madre pluripotentes, como las células madre embrionarias, tienen la capacidad de poder diferenciarse a cualquiera de los tipos celulares de las tres capas germinales. En cambio, la capacidad de diferenciación de las células madre multipotentes, como por ejemplo la de las MSCs, está restringida a los distintos tipos celulares dentro de una misma capa embrionaria. Las células oligopotentes y las unipotentes, cuyo potencial de diferenciación es mucho más reducido, solo pueden dar lugar a unos pocos o a un único tipo celular, respectivamente (Kalra y Tomar 2014).

3.1.1.2 Clasificación según origen

Por otro lado, según la clasificación centrada en su origen o fase de desarrollo del organismo, las células madre se pueden agrupar en células embrionarias, células adultas o iPSCs (Jiang et al. 2021).

Las células madre embrionarias, presentes en la masa celular de la mórula y del blastocisto, pueden diferenciarse en cualquier tipo de célula adulta, siendo totipotentes o pluripotentes según el estadio de desarrollo embrionario. En cambio, las células madre adultas, presentes en los órganos y tejidos del individuo nato, poseen diferente potencial de diferenciación pudiendo ser multipotentes, oligopotentes o unipotentes (Rajabzadeh et al. 2019). La función de las células madre adultas es el correcto mantenimiento del tejido u órgano donde se encuentran, para lo cual existen diferentes tipos celulares: células madre mesenquimales, hematopoyéticas, intestinales, neurales, hepáticas, del hueso o del cartílago, pancreáticas, oculares y epidérmicas (Prochazkova et al. 2015; Mishra et al. 2020).

Por último, las iPSCs no están presentes de forma natural en el organismo, sino que son fruto de la intensa investigación en el campo de las células madre. Las iPSCs se consiguen reprogramando células somáticas adultas, como, por ejemplo, fibroblastos mediante la introducción de factores de transcripción relacionados con la pluripotencia (Oct4, Sox2, c-Myc y Klf4). Estos factores reprograman los fibroblastos y los conducen a un estado indiferenciado en el cual tienen capacidad ilimitada de autorrenovación y de diferenciación a cualquier célula del organismo (Takahashi y Yamanaka 2006).

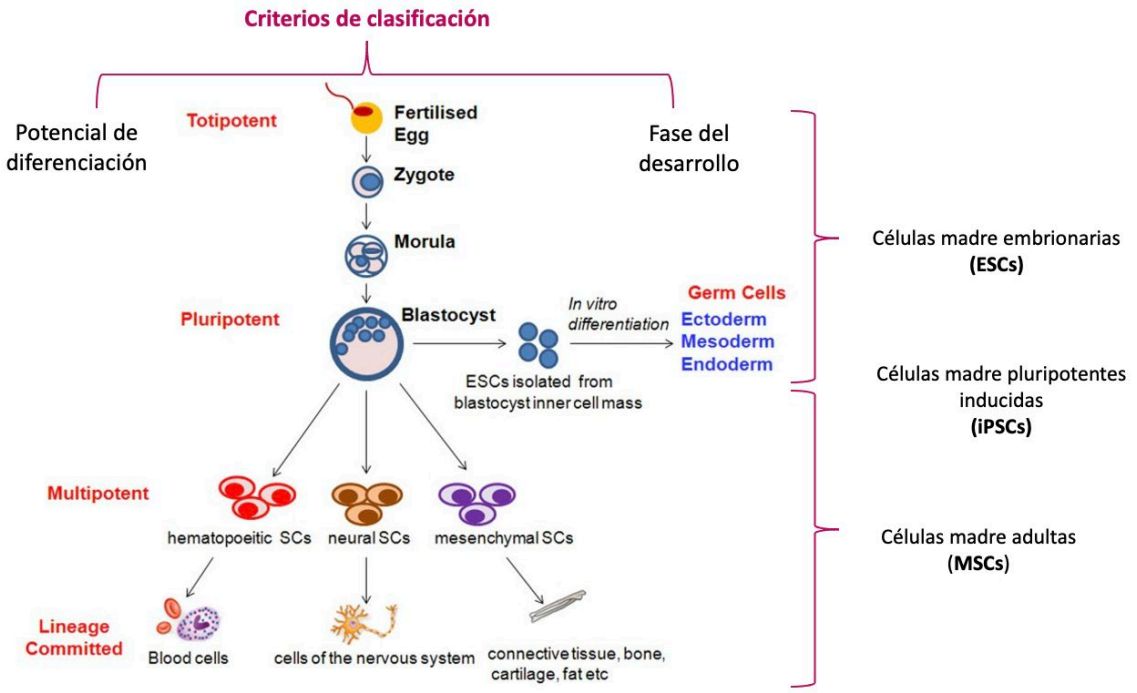


Figura 2. Representación esquemática de la clasificación de células madre según su capacidad de diferenciación y su origen (Hayes et al. 2012).

4. CÉLULAS MADRE MESENQUIMALES

Las MSCs son células madre multipotentes adultas procedentes del mesodermo que se caracterizan principalmente por la capacidad para diferenciarse en diversos tipos celulares de su mismo linaje como por ejemplo osteoblastos, condrocitos, adipocitos (Ullah et al. 2015). Las MSCs fueron descritas por primera vez en 1970 en células del bazo y de médula ósea de conejillos de Indias, y se las denominó ‘’unidades formadoras de colonias con morfología fibroblástica’’ (CFU-F) por sus propiedades observadas *in vitro* (Friedenstein et al. 1970).

Una de las principales ventajas de las MSCs en comparación con otros tipos de células madre, como las células embrionarias o fetales, es que pueden obtenerse de pacientes adultos de manera relativamente sencilla y no acarrean los dilemas éticos relacionados con su utilización (Prządka et al. 2021).

Las MSCs se encuentran ampliamente distribuidas por todo el organismo, pero su fuente originaria *in vivo* sigue siendo objeto de estudio y todavía no ha sido completamente dilucidada. Estudios previos señalan que las MSCs podrían provenir de los pericitos o tejido perivascular, debido a los marcadores fenotípicos de superficie que comparten con estos tipos celulares como son la molécula de adhesión celular CD146 o el receptor del factor de crecimiento derivado de las plaquetas (Murphy et al. 2013).

4.1 Fuentes y cultivo de las células madre mesenquimales

Las MSCs de las diferentes especies animales, incluida la especie equina, están presentes en gran variedad de órganos, tejidos y fluidos del organismo (Kornicka et al. 2019; Prządka et al. 2021). Las MSCs equinas han sido aisladas de médula ósea (BM-MSCs), tejido adiposo (AT-MSCs), músculo y periostio, sangre periférica (PB-MSCs), tejidos dentales (encía, pulpa dentaria y ligamento periodontal), tendones, membrana y líquido sinoviales, endometrio y folículos capilares, entre otros (Ranera et al. 2011; Iacono et al. 2015; Gugjoo et al. 2019). Además, también pueden encontrarse en tejidos perinatales, como el cordón umbilical (CT-MSCs), en sangre del cordón umbilical (CB-MSCs), gelatina de Wharton así como en la membrana amniótica (Iacono et al. 2012; Broeckx et al. 2018; Lanci et al. 2019) (**Figura 3**).

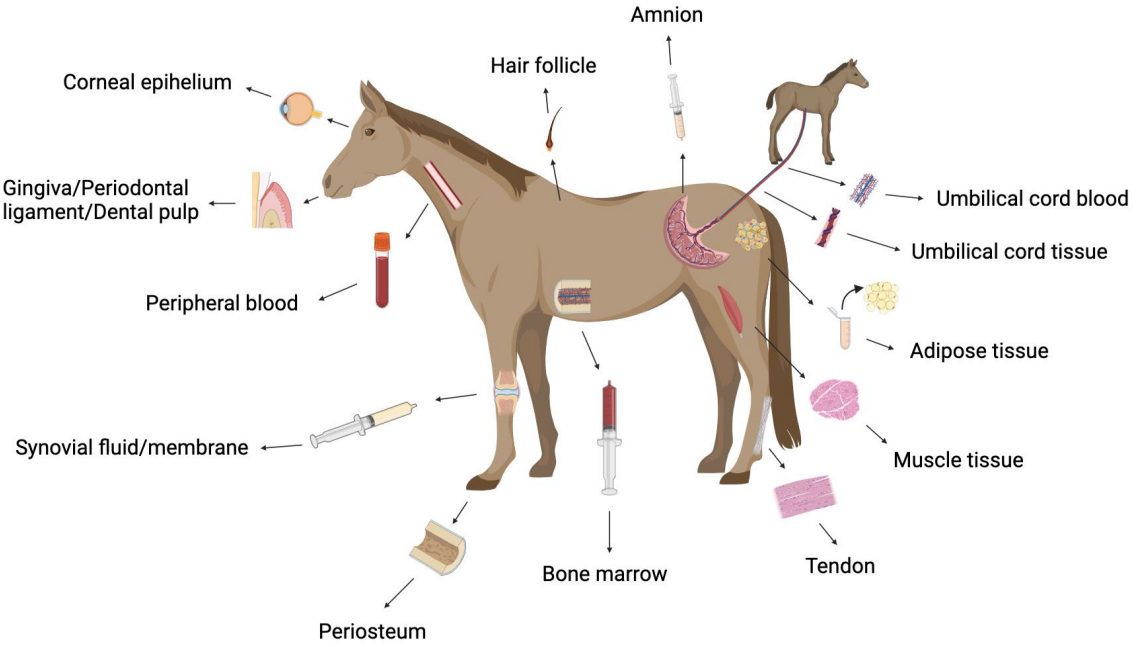


Figura 3. Representación de las diferentes fuentes de origen de las MSCs equinas.

A pesar de la amplia variedad de fuentes, no todas son apropiadas para su aislamiento con fines terapéuticos, por lo que es conveniente priorizar la selección de aquellas fuentes menos invasivas para su posterior aplicación clínica. Las fuentes más comúnmente utilizadas en la especie equina son la médula ósea, el tejido adiposo, la sangre periférica y los tejidos perinatales como la sangre o el tejido del cordón umbilical (Bastos et al. 2017; Kornicka et al. 2019).

Asimismo, resultan de especial interés las MSCs derivadas de sangre periférica y de tejidos perinatales por poder obtenerse a partir de procedimientos no invasivos. Sin embargo, en el caso de los tejidos perinatales, se requiere disponer de yeguas a término y potros recién nacidos para obtener MSCs, siendo este su principal inconveniente. Cabe destacar que estos tipos de MSCs presentan una baja expresión de marcadores inmunogénicos lo que las hace muy apropiadas para su aplicación alogénica (Tessier et al. 2015; Cassano et al. 2018a).

En la actualidad, debido a su abundancia y también a la facilidad de acceso a ellas, el tejido adiposo y la médula ósea son los tejidos más utilizados para el aislamiento de MSCs y su posterior administración para el tratamiento de diferentes patologías en la especie equina (Barrachina et al. 2018c; Voga et al. 2020).

La concentración de MSCs en estos tejidos es de aproximadamente un 2% de las células nucleadas en el tejido adiposo y entre el 0,01-0,001% en la médula ósea (Strem et al. 2005). Estudios posteriores describen que el número de células nucleadas derivadas del tejido adiposo es de aproximadamente $2,3 \times 10^6$ células/g (Nixon et al. 2008; Tuemmers et al. 2012). En el caso de la médula ósea adulta, se suelen obtener $6,4 \pm 3,4 \times 10^6$ células nucleadas/mL de aspirado (Hildebrand et al. 2002; Baghaban Eslaminejad et al. 2009). En cambio, de entre todas las fuentes, la sangre periférica equina contiene la menor concentración de MSCs, con una tasa de éxito en la recolección que oscila entre el 33% (Koerner et al. 2006), el 44% (Martinello et al. 2010) y el 66% (Carvalho et al. 2013), bastante baja en comparación con la del tejido adiposo y la médula ósea.

Debido a esta baja concentración en los diferentes tejidos de origen, es necesaria la expansión *in vitro* de estas células para obtener un número suficiente que permita potenciar su efecto terapéutico (Gugjoo et al. 2019). En el caso de las BM-MSCs se ha observado que tienen la capacidad de replicarse más de mil millones de veces a partir de un único cultivo celular (Gugjoo et al. 2019).

En la especie equina, las MSCs de la médula ósea se extraen del esternón o del ilion (Lombana et al. 2015; Bastos et al. 2017), mientras que las MSCs del tejido adiposo se obtienen de la región supraglútea (Schnabel et al. 2013; Barrachina et al. 2018c; Bogers 2018). Tras la obtención de estos tejidos, en ambos casos las células se aíslan y se expanden en medios de cultivo celulares, como el *Dulbecos´s modified Eagle´s médium* (DMEM), con la adición de varios suplementos, incluyendo suero fetal bovino (FBS, de sus siglas en inglés *fetal bovine serum*), factores de crecimiento, antibióticos y aminoácidos (Barrachina et al. 2018c; Bogers 2018).

Las MSCs son generalmente cultivadas en un medio con concentraciones variables de FBS, que contiene hormonas, vitaminas, proteínas de adhesión al plasma y factores de crecimiento (Naskou et al. 2018). A pesar de su utilidad, el uso de FBS plantea una serie de limitaciones como son la inducción de cambios de comportamiento en las células debido a las proteínas xenogénicas, la variabilidad ente diferentes lotes y problemas de inmunogenicidad (Joswig et al. 2017; Gugjoo et al. 2019) que se abordarán en las siguientes secciones. Estos inconvenientes han hecho que se estén estudiando nuevas opciones como el lisado plaquetario, el suero autólogo o alogénico o diferentes sustitutos comerciales (Even et al. 2022). Sin embargo, estas nuevas alternativas se han asociado a cambios en la adhesión celular y morfología (Clark et al. 2016; Schubert et al. 2018), así como reducción de la capacidad inmunomoduladora de las MSCs (Clark et al. 2016). Además, se ha observado que estas alternativas afectan a los patrones de crecimiento celulares disminuyendo la proliferación celular de las MSCs (Russell y Koch

2016; Even et al. 2022). En los cultivos celulares suplementados con FBS se requieren aproximadamente de 3 a 4 semanas para obtener una cantidad suficiente de MSCs para su uso terapéutico (Schnabel et al. 2013; Barrachina et al. 2018c), por lo que este proceso requiere unas condiciones y un tiempo de los que no siempre se puede disponer dependiendo de la patología que tenga el paciente.

La gran variedad de distribuciones de las MSCs junto a la variabilidad en la velocidad de crecimiento y expansión de los cultivos de estas células refleja que no son un solo tipo celular, sino que son una población heterogénea, lo que ha convertido en un desafío el caracterizarlas completamente.

4.2 Caracterización de las células madre mesenquimales

En 2006, la *International Society for Cellular Therapy* (ISCT) con el objetivo de estandarizar y facilitar la comparación entre los estudios realizados con MSCs, propuso los criterios que las MSCs humanas debían cumplir para ser consideradas como tales. Deben ser adherentes al plástico del sistema de cultivo estándar, expresar o carecer de determinados marcadores de superficie (inmunofenotipo) y ser capaces de diferenciarse en osteoblastos, adipocitos y condrocitos *in vitro* (Dominici et al. 2006).

Aunque estos criterios se establecieron en un intento de estandarizar la utilización de las MSCs humanas, en los últimos años las terapias con estas células han ido cobrando mucha importancia en modelos animales de investigación y en la clínica veterinaria, siendo necesario caracterizar las MSCs en estas especies (Gugjoo et al. 2019; Guest et al. 2022).

Diferentes estudios han demostrado que las MSCs son una población celular muy heterogénea, presentando variaciones entre especies e incluso entre los distintos tejidos de una misma especie (Radcliffe et al. 2010; Ranera et al. 2011). Por ello, es importante caracterizar correctamente las MSCs de las diferentes especies y fuentes de interés, con el fin de identificarlas correctamente antes de su administración terapéutica. Con el objetivo de estandarizar su utilización en otras especies, la caracterización de las MSCs se ha basado en los mismos criterios propuestos por la ISCT para las MSCs humanas, pero destacando algunas diferencias especie específicas (Gugjoo et al. 2019; Guest et al. 2022).

4.2.1 Inmunofenotipo

El inmunofenotipo es el conjunto de marcadores o antígenos expresados en la superficie de las células, identificados normalmente por medio de anticuerpos mediante citometría de flujo (Wang y Lebrec 2017). Teniendo en cuenta que las MSCs son una población heterogénea, la ISCT propuso que más del 95% de la población de MSCs en la especie humana debían expresar las moléculas CD105 (endoglina), CD73 (ecto-5' nucleotidasa) y CD90 (Thy-1). Además, menos de un 2% de las células debían expresar CD45 (marcador de leucocitos), CD34 (marcador hematopoyético), CD14 o CD11b

(marcadores de macrófagos), CD79α o CD19 (marcadores de linfocitos B) y el antígeno leucocitario humano (HLA, de sus siglas en inglés *human leukocyte antigen*) tipo II (Dominici et al. 2006).

En la última década, la disponibilidad limitada de anticuerpos monoclonales comerciales específicos en medicina veterinaria ha dificultado la posibilidad de establecer el inmunofenotipo de las MSCs por citometría de flujo en varias especies (Gugjoo et al. 2019; Guest et al. 2022); aunque se ha observado que las MSCs equinas suelen expresar la mayoría de los marcadores establecidos por la ISCT (Rink et al. 2017; Gugjoo et al. 2019; Hunka et al. 2020).

La falta de anticuerpos comerciales específicos disponibles para poder caracterizar las MSCs equinas se intentó resolver mediante la combinación de citometría de flujo con anticuerpos de otras especies, pero con reactividad cruzada, junto con la detección semicuantitativa de la expresión génica de los marcadores específicos mediante RT-qPCR (Radcliffe et al. 2010). A pesar de que la RT-qPCR puede no reflejar directamente la expresión de superficie, esta técnica no presenta problemas de especificidad de anticuerpos. Por ello, la combinación de ambas técnicas ha facilitado el estudio del inmunofenotipo de las MSCs equinas, a pesar de que se trata de una medición indirecta (Ranera et al. 2011; Schauwer et al. 2011).

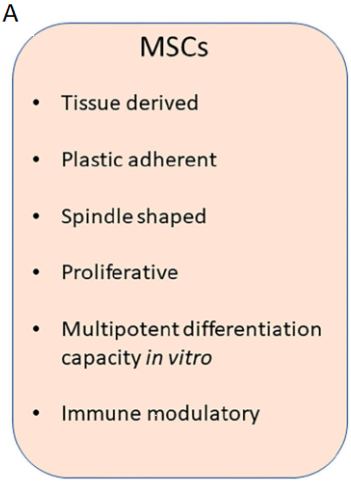
En un intento de estandarizar los resultados obtenidos en las diferentes investigaciones realizadas hasta el momento, en 2011, De Schauwer y colaboradores propusieron que las MSCs equinas debían ser positivas para CD29 (integrina beta 1), CD44 (H-CAM), y CD90 y negativas para CD14, CD79α y MHC-II. Sin embargo, a lo largo de estos últimos años diferentes autores han publicado resultados discrepantes que muestran la dificultad de establecer una caracterización única en una población celular heterogénea.

En el año 2014, se llevó a cabo un estudio con el propósito de estandarizar la caracterización de las MSCs equinas en el que se observó que las MSCs obtenidas de diversas fuentes (tejido adiposo, médula ósea, sangre, tejido de cordón umbilical, y tejido tendinoso) y de distintos donantes, presentaban variaciones en su patrón de expresión (Paebst et al. 2014). Todos los tipos de células independientemente del origen fueron positivas para CD29 y CD44, aunque la expresión de CD90 y CD105 fue muy heterogénea y la mayoría fueron negativas para CD73. Todos los anticuerpos anti-humanos utilizados para los marcadores de exclusión CD14, CD34, CD45, CD79α y MHC-II fueron positivos en las células mononucleares de sangre periférica equina y negativos para la mayoría de las MSCs. Anteriormente, se habían descrito diferencias en los patrones de expresión génica de *CD29*, *CD44* y *CD90*, los cuales fueron significativamente mayores en AT-MSCs en comparación con las BM-MSCs equinas. Incluso se había descrito la expresión del marcador hematopoyético *CD34* en AT-MSCs, pese a ser un marcador considerado negativo por la ISCT (Ranera et al. 2011).

Estas diferencias se han atribuido a los distintos métodos empleados en la obtención, aislamiento y cultivo, así como a las enzimas empleadas para la digestión de los tejidos en el aislamiento celular (Bastos et al. 2017; Gugjoo et al. 2019). Además, el inmunofenotipo de las MSCs no solo puede ser variable entre los distintos tejidos de una misma especie, sino que también puede ser variable entre distintos tejidos de un mismo animal (Cassano et al. 2018a).

Aunque a día de hoy aún no se dispone de un consenso para caracterizar las MSCs de las distintas especies animales, recientemente se han propuesto unos nuevos criterios mínimos que deben cumplir las MSCs equinas y caninas (Guest et al. 2022).

A



B

Defining Characteristics	Minimum	Optimal
Plastic adherence	✓	✓
Tri-lineage differentiation	✓	✓
Surface antigen expression: **Markers commonly positive:** CD90 CD73 CD105 CD29 CD44	Two positive markers.	All markers.
Markers commonly negative: CD79α/CD19 CD14/CD11b CD34 CD45	Two negative markers.	All markers.
MHC II expression (where used allogeneically)	✓	✓
***In vitro* immune suppression**		✓
Other analytical methods of potency		✓

Figura 4. Características (A) y marcadores propuestos (B) para la caracterización de las MSCs en la especie equina y canina (Guest et al. 2022).

En el "*position statement*" publicado por Guest y colaboradores en 2022 indican que en las investigaciones llevadas a cabo en las MSCs de las distintas especies veterinarias sería necesario adoptar un enfoque homogéneo en cuanto a los criterios de evaluación estandarizados, así como en la aplicación de estos criterios. También señalan que son necesarios más estudios para estandarizar los criterios de caracterización inmunofenotípica de las MSCs equinas, lo que ayudará a garantizar la consistencia en su identificación y uso clínico (Guest et al. 2022).

4.2.2 Multipotencia

Según los criterios de las ISCT, las MSCs deben ser capaces de diferenciarse a células del linaje mesodérmico, considerándose como criterio mínimo la diferenciación trilinaje a osteoblastos, condrocitos y adipocitos cuando son cultivadas con medios específicos para ello. Las MSCs equinas son capaces de diferenciarse a otras células del mesodermo,

como miocitos o hepatocitos, e incluso se ha observado *in vitro* la diferenciación a tipos celulares de otros linajes como son las neuronas (Al Naem et al. 2019).

La capacidad de diferenciación trilinaje se ha descrito en MSCs equinas de distintos orígenes como médula ósea, tejido adiposo o cordón umbilical, aunque se ha observado que dicha capacidad es heterogénea según las fuentes (Barberini et al. 2014). Por ejemplo, se ha observado que el potencial de diferenciación osteogénico de las MSCs equinas es mayor en las BM-MSCs que en otras fuentes como son las AT-MSCs (Arévalo-Turrubiarte et al. 2019), coincidiendo con lo observado en las MSCs humanas (Fideles et al. 2019). Sin embargo, mientras que algunos autores consideran que el potencial condrogénico es superior en las CB-MSCs equinas (Burk et al. 2013), otros consideran que es en las BM-MSCs (Branly et al. 2017). También se ha descrito que las BM-MSCs y las AT-MSCs son las fuentes que presentan una capacidad de diferenciación más rápida en la especie equina (Barberini et al. 2014).

5. CÉLULAS MADRE MESENQUIMALES EQUINAS: AUTÓLOGAS VS. ALOGÉNICAS

La utilización de terapias celulares alogénicas siempre ha despertado un gran interés en medicina regenerativa por todas las ventajas que ofrece. La utilización de MSCs autólogas presenta varios inconvenientes relacionados con el tiempo necesario para su aislamiento y cultivo, así como también con la edad avanzada, las enfermedades genéticas o patologías concomitantes del paciente (Carter-Arnold et al. 2014; Colbath et al. 2020a). Las MSCs procedentes de pacientes de edad avanzada suelen estar en menor cantidad además de tener menor viabilidad y capacidad de expansión, por lo que los cultivos se prologan en el tiempo hasta conseguir una dosis de MSCs apropiada para la terapia (Marycz et al. 2019). Además, las MSCs de pacientes con enfermedades genéticas, presentan el mismo defecto genético y por tanto sus MSCs tampoco son apropiadas para la terapia celular (Zhang et al. 2015a; Kornicka et al. 2017).

La creación de bancos celulares a partir de MSCs alogénicas de donantes sanos permite disponer de MSCs bien caracterizadas para la administración en fases tempranas de la patología. Además, pueden seleccionarse los donantes de MSCs con mayor capacidad de expansión y con mejores propiedades terapéuticas. Asimismo, se pueden conservar lotes específicos de células caracterizadas, evitando la variabilidad ligada al uso de células autólogas, y permitiendo una terapia homogénea y más rápida, ya que su tiempo de expansión *ex vivo* es más corto (Barrachina et al. 2023).

De esta forma, se evita el retraso asociado a la obtención y expansión de MSCs autólogas durante semanas, que es lo que más limita su uso en las fases tempranas de la lesión (Colbath et al. 2020a). Además, el hecho de que el paciente no se encuentre en condiciones óptimas de salud, lo que repercute en la cantidad y calidad de sus MSCs, deja de suponer un inconveniente (Berglund et al. 2017b).

Por todo ello, el uso de MSCs alogénicas en la especie equina y humana ha cobrado una enorme importancia, tal y como refleja el número creciente de ensayos clínicos utilizando células alogénicas y de publicaciones científicas sobre el tema (Colbath et al. 2020b; Jiang et al. 2021). Según la Alliance for Regenerative Medicine (ARM), existen más de 1.000 compañías en todo el mundo produciendo y comercializando productos de medicina regenerativa para distintas aplicaciones. Asimismo, un número creciente de compañías farmacéuticas y biotecnológicas están interesadas en ofrecer productos celulares para pacientes veterinarios (ej. Global Stem Cell Technology, AniCell Biotech, Centauri Bioech S.L., EquiCord YMAS S.L.). De hecho, la Agencia Europea del Medicamento aprobó en 2018 la comercialización del primer producto basado en células madre para el tratamiento de la osteoartritis (OA) en caballos: Arti-Cell Forte, desarrollado por la compañía Global Stem Cell Technologies y comercializado en Europa por la farmacéutica Boehringer-Ingelheim. En 2019 se aprobó también para el tratamiento de OA el producto Horstem, desarrollado por la compañía EquiCord. En 2021 la Agencia Española de Medicamentos y Productos Sanitarios autorizó la comercialización del primer producto veterinario con células madre en España para tratar la OA en caballos, BestaStem, desarrollado por la compañía gallega Centauri Biotech. El pasado año 2022, el Comité de Medicamentos Veterinarios adoptó un dictamen positivo para el medicamento veterinario RenuTend, también desarrollado por Global Stem Cell Technologies, que promueve la restauración tisular de tendones y ligamentos. Sin embargo, tres de estos productos basados en células madre alogénicas solo están aprobados para una sola administración, aunque en el caso de BestaStem indican que la administración puede repetirse a los 45 días.

En diferentes especies animales se ha observado que la administración de MSCs alogénicas puede producir cierto nivel de respuesta inmune, tanto a nivel celular como humoral (mediada por la producción de anticuerpos aloespecíficos) (Pezzanite et al. 2015; Rowland et al. 2021; Rosa et al. 2022). Asimismo, la administración repetida de MSCs alogénicas podría conllevar reacciones adversas asociadas a la respuesta del sistema inmunitario del paciente al reconocer dichas células como extrañas, además de comprometer el efecto terapéutico de las MSCs (Owens et al. 2016).

En consecuencia, en los últimos años, numerosos estudios han demostrado que el concepto de “inmunoprivilegio” por el que se caracterizaban las MSCs no era correcto y actualmente las MSCs se consideran inmunoevasivas (Ankrum et al. 2014). Así pues, la terapia con MSCs alogénicas presenta ciertas consideraciones referentes tanto a su capacidad inmunomoduladora como inmunogénica que deben tenerse en cuenta y que se desarrollarán en las siguientes secciones por ser el principal objetivo de esta Tesis Doctoral, ya que podrían afectar negativamente a la efectividad y seguridad de las MSCs (Berglund et al. 2017b).

6. MECANISMOS DE ACCIÓN TERAPÉUTICOS DE LAS CÉLULAS MADRE MESENQUIMALES

En los últimos años, las MSCs se han convertido en las células de elección en las terapias avanzadas, no solo por su facilidad de cultivo, su carácter multipotencial y su habilidad para migrar, integrarse y diferenciarse en el tejido dañado (Voga et al. 2020), sino fundamentalmente por su capacidad de promover la reparación de tejidos, de evitar la formación de fibrosis cicatricial y de modular la respuesta inmunitaria y la inflamación mediante su acción paracrina, lo que incrementa su potencial (Gugjoo et al. 2019).

Inicialmente se atribuyó a la capacidad de diferenciación de las MSCs la base de su acción terapéutica para la reparación y la regeneración de los tejidos afectados. Sin embargo, al contrario de lo que se planteó inicialmente, las MSCs no necesitan integrarse o diferenciarse en células de un tejido concreto para ejercer sus acciones terapéuticas, aunque se sigue considerando que los mecanismos de integración y diferenciación participan en la acción terapéutica de las MSCs (Voga et al. 2020; Prządka et al. 2021).

6.1 Capacidad de migración, integración y diferenciación

Las MSCs administradas de forma sistémica son capaces de migrar hacia los tejidos dañados, propiedad que se atribuye a diversos factores de crecimiento, quimiocinas y receptores de la matriz extracelular localizados en su superficie celular (Gugjoo et al. 2019; MacDonald y Barrett 2020). Además, se ha descrito que la capacidad de migración e integración de las MSCs depende en gran medida de la reacción inflamatoria del lugar de la lesión (Mund et al. 2020). Por ello, la exposición de las MSCs a moléculas proinflamatorias como el interferón ɣ (IFNɣ), el factor de necrosis tumoral α (TNFα) y los lipopolisacáridos (LPS) les permite dirigirse específicamente hacia los lugares donde se ha producido la inflamación (Shi et al. 2012; MacDonald y Barrett 2020). En las siguientes secciones, se desarrollará en detalle la relevancia de estas citoquinas en el contexto mencionado.

Cuando se produce daño tisular, las células endoteliales expresan diferentes selectinas (E y P) y moléculas de adhesión celular vascular 1 (VCAM1) que facilitan las diferentes etapas del proceso de anidamiento de las MSCs, incluyendo el anclaje y rodamiento, activación, detención, transmigración o diapédesis y migración (Ullah et al. 2019; MacDonald y Barrett 2020) (**Figura 5**). Los ligandos como CD44 y CD29/integrina-β1, expresados en la superficie de las MSCs, son cruciales en el proceso de migración y anidamiento al permitir que las MSCs se unan a las células endoteliales y pasen del torrente sanguíneo al tejido dañado (Stewart y Stewart 2011; Gugjoo et al. 2019).

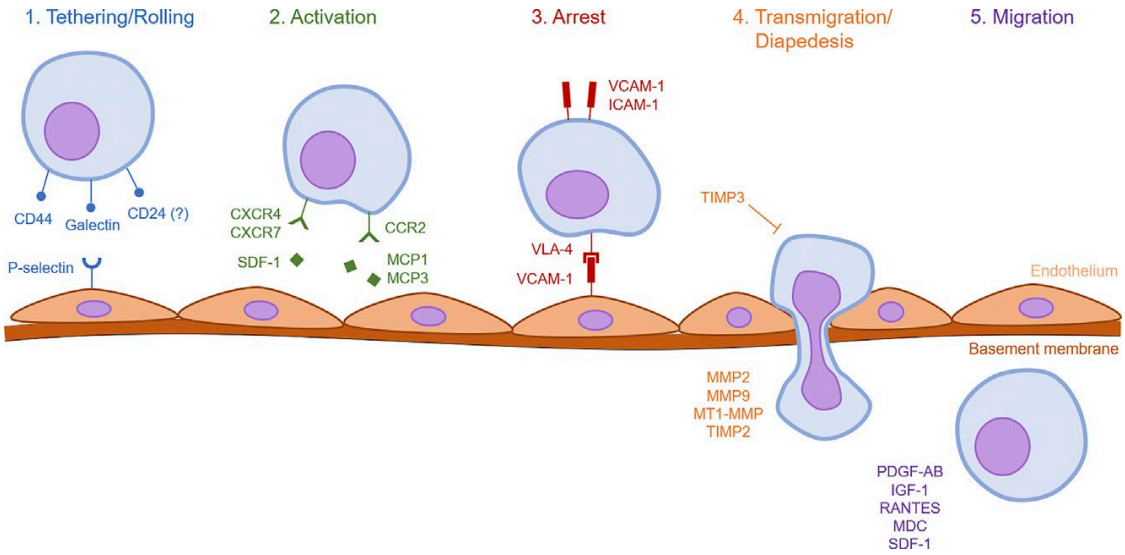


Figura 5. Descripción general de los mecanismos moleculares que facilitan cada paso del anidamiento de las MSCs (Ullah et al. 2019).

Sin embargo, en la especie humana (White et al. 2016) y equina (Mund et al. 2020) se ha descrito que la administración intravenosa de MSCs puede resultar en el atrapamiento de las mismas en el pulmón en las horas siguientes a la administración, sin que llegue un número significativo de MSCs al tejido lesionado. En el estudio de Mund y colaboradores (2020) señalaron que, aun así, se observaron MSCs en las biopsias de los tejidos lesionados, por lo que las MSCs equinas habrían podido abandonar el torrente sanguíneo y migrar hacia el lugar de la lesión gracias a los mecanismos moleculares explicados previamente. El atrapamiento de las MSCs en los pulmones podría ser debido al tamaño de las MSCs, que es de aproximadamente 30 µm cuando están en suspensión. Debido a su tamaño relativamente grande en comparación con los capilares pulmonares, que tienen un diámetro de 14 µm, existe la posibilidad de que las MSCs queden atrapadas en los pulmones (Voga et al. 2020).

Cuando las MSCs equinas son administradas de forma local, algunos estudios señalan que, transcurridas 24 horas, menos del 30% de las MSCs pueden encontrarse en el lugar de la lesión (Becerra et al. 2013), debido a la movilización de MSCs hacia la sangre después de su administración (Burk et al. 2016). Sin embargo, otros estudios indican que las MSCs equinas pueden permanecer en el sitio de inyección de 2 a 4 semanas (Consentius et al. 2015; Mund et al. 2020), incluso algunas células siguen dentro de las lesiones transcurridas 9 semanas de la inyección local (Geburek et al. 2016).

Es importante destacar que no se pueden extrapolar los resultados de seguimiento observados tras las inyecciones tendinosas de MSCs a lo que sucede después de una administración intraarticular (Colbath et al. 2020a). El entorno articular es único, ya que cuenta con una importante barrera de revestimiento sinovial y pequeños capilares dentro de la membrana sinovial. Además, debido a la escasa vascularización y celularidad del cartílago, su capacidad de autorreparación es limitada (Gugjoo et al. 2019; Voga et al. 2020). Es probable que la mayoría de las MSCs queden retenidas en la

articulación tras su administración intraarticular, aunque este aspecto aún se encuentra en fase de investigación (Colbath et al. 2020a).

Aunque se ha documentado ampliamente la capacidad de diferenciación de las MSCs *in vitro*, sugiriendo un mayor potencial terapéutico de estas células, es difícil evaluar su capacidad de diferenciación *in vivo* después de su administración. Además, la tendencia clásica a considerar que las MSCs inyectadas colonizan el tejido dañado, diferenciándose en los tipos celulares y actuando directamente en el lugar de la lesión, no reflejaría completamente sus capacidades terapéuticas a largo plazo (Gugjoo et al. 2019; Voga et al. 2020).

En la especie equina están cobrando mucho interés los tratamientos con MSCs pre-diferenciadas con el objetivo de mejorar su integración en el lugar de la lesión (Broeckx et al. 2019a; Depuydt et al. 2021; Van Hecke et al. 2021). Además, también se están investigando diferentes estrategias para incorporar las células en diferentes soportes con el fin de facilitar su integración en la lesión (Ho et al. 2016). Sin embargo, sigue en estudio cómo estas estrategias pueden afectar a la viabilidad y propiedades inmunomoduladoras de las MSCs (Colbath et al. 2017b). Aunque en este sentido, cabe destacar que el efecto terapéutico observado no parece estar correlacionado con la vida media de las MSCs tras su administración, sino más bien con su acción paracrina, según estudios recientes en la especie equina (Mund et al. 2020) y canina (Abe et al. 2020). Es necesario seguir investigando para comprender en profundidad las señales de reclutamiento, migración y retención de las MSCs equinas, así como para determinar la importancia de la diferenciación de las MSCs en el tejido lesionado (MacDonald y Barrett 2020).

6.2 Acción paracrina

Las investigaciones llevadas a cabo durante los últimos años en la especie humana y en las especies animales han demostrado que la capacidad terapéutica de las MSCs se debe principalmente a sus mecanismos paracrinos (Alvites et al. 2022; Iacono y Merlo 2022). Estos mecanismos se basan en la secreción de diversas moléculas por parte de las MSCs, con distintas funciones reguladoras, lo que amplía considerablemente sus aplicaciones terapéuticas (Gugjoo et al. 2019).

En diferentes especies animales se ha observado la capacidad de las MSCs de secretar diversas moléculas bioactivas como factores de crecimiento, citoquinas y quimiocinas. Estas moléculas desempeñan un papel crucial en la reparación y cicatrización del tejido al regular la respuesta inflamatoria del organismo, aunque estos mecanismos varían considerablemente según la especie y el tejido de origen de las MSCs (Voga et al. 2020; Prządka et al. 2021).

Los efectos de los mediadores liberados por parte de las MSCs pueden dividirse en tróficos (antiapoptóticos, de soporte y angiogénicos), antifibróticos o anticicatriciales, quimioatrayentes e inmunomoduladores (**Figura 6**).

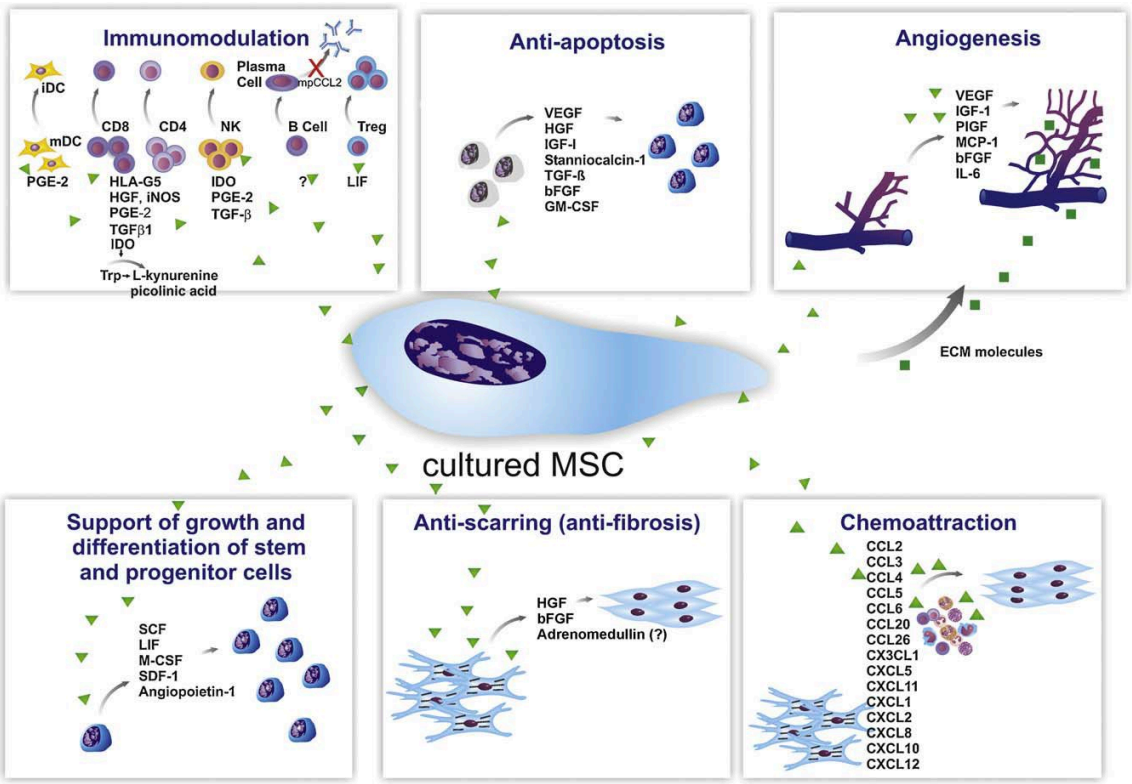


Figura 6. Efectos paracrinos que pueden ejercer las MSCs a través de la secreción de diversas moléculas (da Silva Meirelles et al. 2009).

6.2.1 Efectos tróficos

Los factores solubles responsables de los efectos tróficos ejercidos por las MSCs pueden ser clasificados en tres categorías: antiapoptóticos, de soporte (estimulando la mitosis, proliferación y diferenciación de las células progenitoras propias del órgano o tejido) y angiogénicos (Liang et al. 2014; Fan et al. 2020).

6.2.1.1 Antiapoptóticos

Mediante la secreción de diversas moléculas, las MSCs son capaces de disminuir y limitar la apoptosis de otros tipos celulares cuando se produce una lesión tisular (Chen et al. 2018; Chang et al. 2020). Algunos de los factores implicados en reducir la muerte celular en el tejido dañado son el factor de crecimiento endotelial (VEGF), el factor de crecimiento insulínico tipo 1 (IGF1) y el factor de crecimiento hepatocítico (HGF), los cuales mejoran la supervivencia de las células endoteliales (Bagge et al. 2020; Fan et al. 2020; Voga et al. 2020).

Además, se ha observado que la expresión de estas moléculas por parte de las MSCs aumenta bajo condiciones de hipoxia, que es la situación que se pueden encontrar las MSCs en un primer estadio de daño tisular, por lo que ayudarían a controlar y limitar las lesiones en sus fases iniciales (Textor et al. 2018; Pfeiffenberger et al. 2019).

6.2.1.2 De soporte

En cuanto a los efectos de estimulación del crecimiento y proliferación celular, las MSCs son capaces de secretar moléculas que se encargan de reclutar y mantener tanto a otras células progenitoras como a los precursores de las células del tejido dañado (Kocan et al. 2017; Alvites et al. 2022). Algunas de las moléculas secretadas son el factor 1 derivado de células estromales (SDF-1), interleuquina 6 (IL6), indolamina 2,3-dioxigenasa (IDO1) y prostaglandina E2 (PGE2), entre otras (Wang et al. 2014). Como se desarrollará más adelante, estas moléculas se encuentran a la vez implicadas en otros mecanismos terapéuticos de las MSCs.

6.2.1.3 Angiogénicos

El restablecimiento del riego sanguíneo es fundamental para la recuperación de los tejidos dañados, por lo que los mecanismos proangiogénicos se consideran de gran importancia (Bussche y Van De Walle 2014). Las MSCs desarrollan efectos proangiogénicos mediante la implicación de moléculas como el factor de crecimiento fibroblástico (FGF), VEGF, SDF-1, IL6, el factor de crecimiento placentario (PGF) o la proteína quimioatrayente de monocitos 1 (MCP-1) (Fan et al. 2020). De forma simultánea, las moléculas antiapoptóticas mencionadas, a la vez que limitarían la muerte de las células endoteliales, estimularían la angiogénesis de las células endoteliales para la formación de nuevos vasos sanguíneos (Fan et al. 2020).

En conjunto, la secreción de todos estos factores puede inhibir las respuestas inflamatorias, desencadenar procesos angiogénicos, promover las actividades endoteliales y fibroblásticas, y facilitar la proliferación y diferenciación de las células progenitoras en los tejidos *in situ* (Wang et al. 2014).

6.2.2 Efectos antifibróticos

El proceso de reparación que se inicia tras un daño tisular, sobre todo en tejidos del sistema locomotor debido a su limitada capacidad de reparación, promueve la formación de tejidos cicatriciales con características funcionales y biomecánicas inferiores al original, formándose tejidos más débiles y menos elásticos (Smith et al. 2013). Las MSCs poseen propiedades antifibróticas que favorecen la formación de un tejido más similar al original en lugar del tejido fibroso comúnmente generado durante el proceso natural de cicatrización (Voga et al. 2020).

El efecto antifibrótico de las MSCs se atribuye a la liberación de moléculas inmunosupresoras como son PGE2, IL10, HGF y óxido nítrico (NO) entre otras, que regulan la actividad de las células inflamatorias y disminuyen el proceso de fibrosis (Seo y Jung 2016). Es por ello que se han obtenido mejores resultados terapéuticos cuando las MSCs se administran de forma temprana, antes de que la fibrosis del tejido dañado sea excesiva (Prządka et al. 2021).

6.2.3 Quimioatracción

Las MSCs secretan una amplia variedad de moléculas quimioatrayentes, como los ligandos de quimiocinas CCL y CXCL, que favorecen el reclutamiento y regulación de las células que se encuentran involucradas en el proceso de inflamación (Cassano et al. 2018b). Asimismo, las MSCs también son capaces de influir en las funciones de reclutamiento, proliferación, maduración, activación y producción de citoquinas de diferentes células inmunitarias (Alvites et al. 2022). Entre las células diana atraídas al lugar de la lesión para ejercer sus correspondientes funciones se encuentran los monocitos, eosinófilos, neutrófilos, basófilos, linfocitos T y B, células natural killer (NK), células dendríticas, progenitores hematopoyéticos y endoteliales (Cassano et al. 2018b; Voga et al. 2020).

7. PROPIEDADES INMUNOMODULADORAS DE LAS CÉLULAS MADRE MESENQUIMALES

Durante los últimos años, las investigaciones en terapia celular han demostrado que el potencial terapéutico de las MSCs se basa sobre todo en sus propiedades para modular el sistema inmunitario (Voga et al. 2020; Jiang et al. 2021).

Además de la gran cantidad de mediadores relacionados con la actividad paracrina de las MSCs, se ha observado que los mecanismos de contacto directo con las células inmunitarias también están implicados en su mecanismo terapéutico (Berglund et al. 2017b; Gugjoo et al. 2019; Foo et al. 2021). Tanto la capacidad inmunomoduladora como la antiinflamatoria de las MSCs están mediadas por señales dependientes e independientes del contacto entre células (Fan et al. 2020) y todo ello constituye un proceso multifactorial en el que participan diversos mecanismos y moléculas (**Figura 7**).

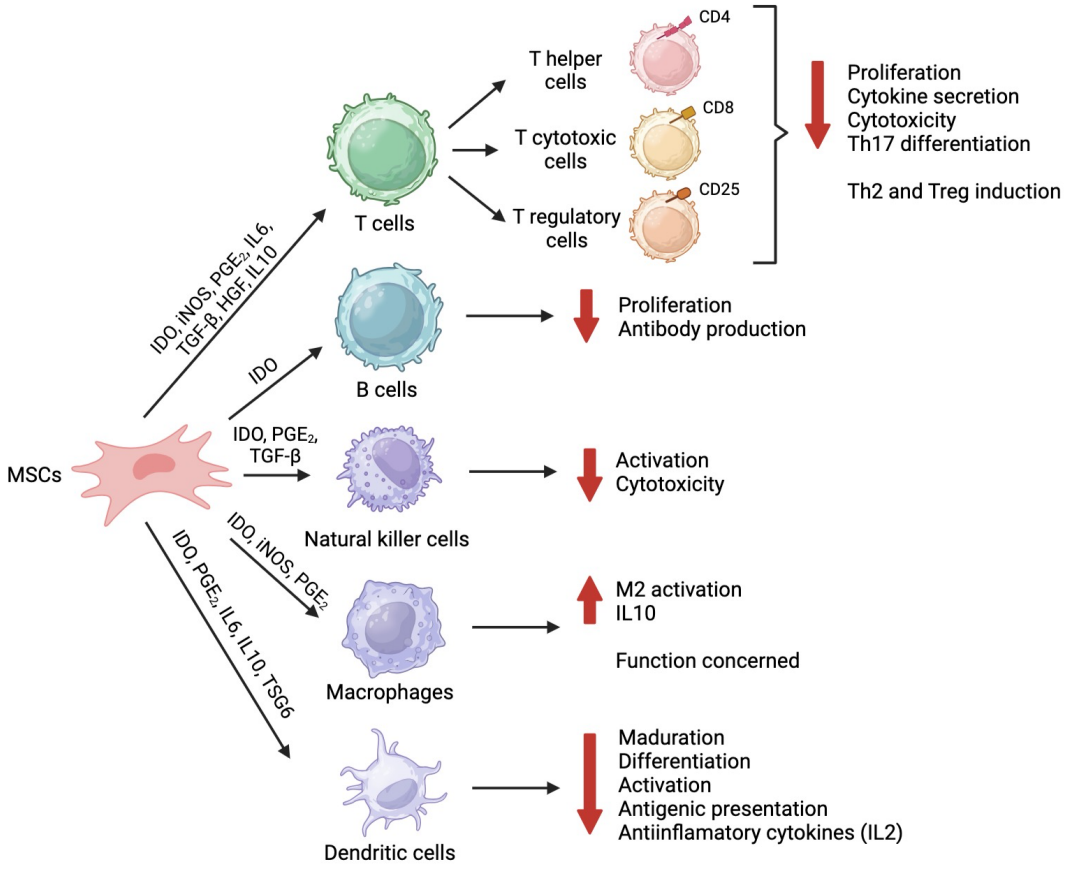


Figura 7. Principales interacciones inmunomoduladoras entre las MSCs y células inmunitarias.

Debido a la complejidad de las interacciones entre las MSCs y las diferentes células inmunes, se presenta a continuación una revisión de las principales células inmunitarias con las que las MSCs interactúan con el fin de detallar las acciones que se conocen hasta el momento. Esta revisión también busca esclarecer aspectos relacionados con la inmunogenicidad y su relevancia en la utilización alogénica de las MSCs, temas que serán abordados más adelante.

7.1 Poblaciones celulares esenciales en la respuesta inmunitaria

La capacidad inmunomoduladora se produce generalmente a través de la interacción de las MSCs con otras células inmunitarias como linfocitos T, linfocitos B, células dendríticas, células NK, neutrófilos o macrófagos (Voga et al. 2020). El mecanismo de acción de las MSCs se centra en modular la proliferación, diferenciación, migración y apoptosis de estas células inmunitarias (Ma et al. 2014; Aldrich et al. 2021; Wang et al. 2022).

7.1.1 Células T

Las células T son unas de las principales células efectoras de la respuesta inmunitaria adaptativa y controlan el tipo de respuesta contra patógenos específicos (Caffi et al. 2020). Estas células solo expresan un tipo de receptor de antígeno, conocido como T-cell receptor (TCR), y necesitan la activación por parte de las células presentadoras de antígenos (CPAs) para reconocer de manera específica un antígeno en particular (Marshall et al. 2018).

El complejo MHC-antígeno de la CPA activa el TCR de las células T y, en respuesta, estas secretan citoquinas que controlarán la respuesta inmunitaria. Una vez activadas, las células T pueden diferenciarse a células T citotóxicas (CD8+) o colaboradoras (CD4+) (Kamm et al. 2021a).

Los linfocitos T citotóxicos (CD8+) desempeñan un papel principal en la eliminación de células infectadas por agentes extraños. Reconocen a las células infectadas al reconocer el antígeno presentado por las CPAs a través de su MHC de clase I. Una vez reconocidas, los linfocitos T citotóxicos pueden destruir las células infectadas mediante un proceso conocido como lisis (Marshall et al. 2018). Tras la destrucción del antígeno, algunas de estas células permanecen como células de memoria para proporcionar una respuesta más rápida en caso de reexposición al antígeno, contribuyendo así a la respuesta adaptativa (Lohan et al. 2017).

Los linfocitos T colaboradores (CD4+) desempeñan un papel fundamental en el establecimiento y la maximización de la respuesta inmunitaria (Marshall et al. 2018). Estas células no tienen acción citotóxica ni fagocítica, y tampoco son capaces de destruir las células infectadas ni eliminar patógenos. Sin embargo, su función es la de mediar la respuesta inmunitaria dirigiendo a otras células para que realicen estas tareas mediante la secreción de distintas citoquinas (Marshall et al. 2018).

La respuesta de los linfocitos T colaboradores se caracteriza por la producción de la citoquina proinflamatoria IFNɣ, cuya función principal es estimular las respuestas inmunitarias innatas y adaptativas (Cassano et al. 2018a) mediante la activación de citoquinas que inducen a los linfocitos B a producir anticuerpos opsonizantes (de revestimiento) y neutralizantes (Marshall et al. 2018). En el contexto de las terapias celulares, la secreción de IFNɣ podría indicar la expansión de las células efectoras y de

memoria en respuesta a las MSCs recibidas (Berglund et al. 2017b). Sin embargo, como se desarrollará más adelante, se reconoce que IFNɣ desempeña un papel muy importante en la capacidad inmunosupresora de las MSCs.

Los efectos inmunomoduladores de las MSCs se describieron al observar su capacidad de inhibir la proliferación de los linfocitos T CD4+ y CD8+ (Briones et al. 2011) y de evitar la lisis producida por los linfocitos T citotóxicos (Haddad y Saldanha-Araujo 2014) en la especie humana. En la especie equina, se ha descrito que las MSCs también tienen la capacidad de inhibir la proliferación de las células T y suprimir la respuesta de las células T alogénicas (Carrade et al. 2012; Carrade Holt et al. 2014; Paterson et al. 2014; Remacha et al. 2015).

Las células T reguladoras (Treg), un tipo de células T colaboradoras, desempeñan un papel crucial al limitar y suprimir la actividad del sistema inmunológico. Su función principal es controlar las respuestas inmunitarias aberrantes o dirigidas contra antígenos propios, lo cual puede conducir al desarrollo de enfermedades autoinmunes (Marshall et al. 2018). Estudios recientes han demostrado que el aumento de las Treg estaría implicado en la capacidad de inmunosupresión de las MSCs equinas administradas de forma alogénica. Este aumento serviría para amortiguar la respuesta inmunitaria adaptativa y podría evitar el rechazo de células extrañas por parte del receptor (Kamm et al. 2021a).

7.1.2 Células B

La función principal de las células B es la producción de anticuerpos específicos contra antígenos extraños. A diferencia de las células T, los linfocitos B son capaces de reconocer un antígeno libre de forma directa, sin que las CPAs medien en la presentación. Cuando las células B son activadas por el antígeno extraño, proliferan y se diferencian en células B de memoria o en células plasmáticas (Marshall et al. 2018).

Las células B de memoria expresan receptores de unión a antígenos y permanecen presentes una vez que la infección ha sido superada. Su función es proporcionar una respuesta rápida y efectiva en caso de una reexposición a los mismos antígenos, contribuyendo así a la inmunidad adaptativa humoral (Lohan et al. 2017; Marshall et al. 2018). Las células plasmáticas, en cambio, no expresan receptores de unión a antígenos y morirán cuando se haya eliminado el agente que indujo la respuesta inmunitaria (Marshall et al. 2018).

Existe cierta controversia en relación a los mecanismos por los cuales las MSCs ejercen un efecto supresor sobre los linfocitos B. Varios autores coinciden en que las MSCs son capaces de inhibir la proliferación de células B (Voga et al. 2020; Foo et al. 2021), así como de reducir la producción de inmunoglobulinas (Fan et al. 2020). Sin embargo, otros estudios han mostrado un papel estimulador de la proliferación y diferenciación de las células B por parte tanto de las MSCs humanas (Rosado et al. 2015; García-Sancho et al. 2017) como murinas (Ryan et al. 2014).

En la especie equina hay muy pocos estudios en los que se haya valorado cómo las MSCs afectan a la proliferación y maduración de las células B. Un estudio reciente en caballos de edad avanzada describió que la proliferación de células B no se veía aumentada cuando se enfrentaban *in vitro* a MSC alogénicas (Kamm et al. 2021a). Pero cabe destacar que en caballos de edad avanzada se produce una disminución de la cantidad linfocitos T y B, lo que acarrea un deterioro de la respuesta inmunitaria (Paillot 2014).

7.1.3 Células natural killer

Los linfocitos natural killer (NK) son un tipo de células inmunológicas que desempeñan un papel muy importante en la inmunidad innata. Estas células tienen la capacidad de reconocer y destruir células infectadas o tumorales sin necesidad de una señal previa, lo que les permite actuar rápidamente en caso de una infección o crecimiento anormal (Marshall et al. 2018).

Para ejercer su citotoxicidad, las células NK utilizan receptores tanto activadores como inhibidores. Los receptores activadores toll like receptors (TLRs) se unen a ligandos que son expresados por células en distrés, como aquellas infectadas por virus. Esta unión a su vez induce la producción de IFNɣ por parte de las células NK, lo que aumenta su poder citotóxico (Barrachina 2017).

Por otro lado, los receptores inhibidores reconocen los ligandos propios de las células del organismo como el MHC-I y, al unirse a ellos, inducen una inhibición del mecanismo citotóxico. Sin embargo, la ausencia de expresión de MHC-I es un indicativo de que la célula está en distrés y, por tanto, la citotoxicidad de las células NK puede ser desencadenada (Kamm et al. 2021a).

Además de su función citotóxica, las células NK también desempeñan un papel importante en la regulación de la respuesta inmunitaria y pueden influir en la función de otras células inmunitarias, como las células dendríticas, células T y células B. Las células NK también son capaces de producir diversas citoquinas como IFNɣ cuando son activadas por otras moléculas como IL2, IL12, IL15 o IL18, por lo que estas células son un componente crucial de la inmunidad innata y tienen un papel muy importante en la protección contra infecciones y tumores (Barrachina 2017).

7.1.4 Macrófagos

Los macrófagos son células inmunitarias que desempeñan un papel fundamental en la inmunidad innata. Además, también establecen una conexión crucial entre la inmunidad innata y la adaptativa al actuar como CPAs (Marshall et al. 2018). En todos los tejidos, se encuentran macrófagos residentes que constituyen la primera línea de defensa contra invasores. Sin embargo, durante la inflamación, también pueden reclutarse macrófagos inmaduros, conocidos como monocitos, desde la sangre.

Estos monocitos convertidos en macrófagos llegan más tarde al lugar de la inflamación y tienen una actividad principalmente proinflamatoria, encargándose de eliminar los

microorganismos y los restos de los neutrófilos apoptóticos, que son las primeras células fagocíticas en llegar (Kamm et al. 2021a). Estos macrófagos se conocen como macrófagos M1 activados de forma clásica. Después de la fase de destrucción tisular, sigue una fase de reparación en la que prevalece la actividad antiinflamatoria. Durante esta fase, los macrófagos producen poliaminas y prolina a partir de arginina, moléculas necesarias para la síntesis de fibrinógeno y otras fibras de la matriz extracelular. En esta forma de activación alternativa a los macrófagos se les denomina M2 (Barrachina 2017). El equilibrio entre los macrófagos proinflamatorios (M1) y antiinflamatorios (M2), es crítico (Cassano et al. 2018b) debido a que un exceso de M1 puede resultar en una inflamación crónica, mientras que un exceso de M2 puede causar una fibrosis excesiva del tejido (Tanaka et al. 2014; Braune et al. 2017).

Actualmente, solo hay dos estudios en la especie equina en los que se haya valorado el efecto de las MSCs en los macrófagos. En uno de ellos se observó un efecto mínimo de las MSCs sobre los macrófagos activados *in vitro*, lo que podría indicar que las MSCs no tendrían una gran capacidad inmunorreguladora para desactivar los macrófagos equinos (Cassano et al. 2018c). Sin embargo, en el otro estudio del mismo grupo, las MSCs expuestas a macrófagos activados demostraron tener propiedades inmunosupresoras en los ensayos de proliferación de linfocitos T activados (Cassano et al. 2018b). Aunque los datos en este campo son escasos, los autores plantean la posibilidad de que las MSCs alogénicas serían menos efectivas en la modulación de la actividad de los macrófagos activados (Cassano et al. 2018c).

7.1.5 Neutrófilos

Los neutrófilos son leucocitos polimorfonucleares (PMN) que representan la población celular más abundante en la respuesta innata y suelen ser los primeros leucocitos en infiltrarse en un tejido alogénico (Scozzi et al. 2017; Kamm et al. 2021a). No obstante, los mecanismos que promueven la proliferación de PMN, como uno de los subconjuntos celulares principales en la respuesta defensiva contra los patógenos, también podrían desempeñar un papel en la fisiopatología de los estados inflamatorios desregulados (Espinosa et al. 2020). Los neutrófilos son reclutados a las áreas inflamadas por el endotelio vascular y se ha observado que también son atraídos hacia las MSCs a través de quimiocinas como la CXCL8 (IL-8) (Scozzi et al. 2017; Mardpour et al. 2019).

La interacción entre las MSCs y los neutrófilos ha sido objeto de estudio en diversas especies, y se ha observado que las MSCs tienen la capacidad de modular la respuesta inflamatoria neutrofílica en ciertos modelos de enfermedades en los que los neutrófilos desempeñan un papel fundamental como células efectoras (Mittal et al. 2018). Asimismo, estudios han demostrado que las MSCs alogénicas son capaces de suprimir la activación de los neutrófilos y reducir significativamente la producción de especies reactivas de oxígeno (ROS) *in vitro*, especialmente cuando los neutrófilos han sido previamente activados (Mumaw et al. 2015; Salami et al. 2018).

Sin embargo, uno de los fenómenos más preocupantes del sistema inmunitario innato en el contexto equino es la rápida afluencia de neutrófilos tras la administración intraarticular de MSCs, ya sea de forma autólogas o alogénicas (Ardanaz et al. 2016; Joswig et al. 2017)

En varios estudios se ha observado un aumento del recuento de neutrófilos en el líquido sinovial durante 48-72 horas después de la administración de MSCs autólogas y alogénicas (Ardanaz et al. 2016; Joswig et al. 2017; Colbath et al. 2020a). En uno de los estudios (Joswig et al. 2017) determinaron que las MSCs no eran la causa de la infiltración neutrofílica y que esta invasión podría ser debida al FBS utilizado en el medio de cultivo de las MSCs.

En otro estudio en la especie equina se evaluó el efecto de las MSCs en la respuesta inflamatoria de los neutrófilos en una articulación inflamada mediante LPS (Williams et al. 2016). Los resultados mostraron una disminución significativa de la infiltración de neutrófilos en la articulación tratada con MSCs indicando que la presencia de MSCs podría suprimir la activación del sistema inmunitario innato y limitar la respuesta inflamatoria de los neutrófilos en un entorno de inflamación.

7.2 Principales moléculas involucradas en la capacidad inmunomoduladora de las células madre mesenquimales

Con el avance de las investigaciones, se va descubriendo un número creciente de mediadores secretados por las MSCs que participan en su función inmunomoduladora, aunque varían entre especies e incluso entre tejidos de origen (Gugjoo et al. 2019). Actualmente se están llevando a cabo diferentes estudios en los que se están investigando cuáles son las principales moléculas implicadas en la capacidad inmunomoduladora de las MSCs equinas. Además, aunque las MSCs pueden ejercer sus efectos inmunorreguladores en condiciones basales, se ha observado que su función inmunomoduladora se ve potenciada cuando se encuentran en un entorno inflamatorio, como el que se presenta durante una lesión. En este contexto, la activación de las MSCs mediante señales inflamatorias favorece su capacidad para modular la respuesta inmunológica (Cuerquis et al. 2014; Barrachina et al. 2017).

A continuación, se presentan y describen las principales moléculas implicadas en los mecanismos inmunomoduladores de las MSCs de forma que se pueda comprender la relación entre su secreción y los efectos que ejercen las MSCs en las lesiones cuando son administradas.

7.2.1 Interleuquina 6 (IL6)

IL6 es una citoquina proinflamatoria con actividad pleiotrópica que actúa como mediador en una situación patológica, ya que se produce ante una inflamación y daño tisular. Sus efectos se centran principalmente en la modulación de la inflamación, la inmunidad y la hematopoyesis (Tanaka et al. 2014; Braune et al. 2017). Entre los

diferentes efectos se encuentra la inducción de la síntesis de proteínas de fase aguda como el fibrinógeno y el serum amiloide A y la reducción de la producción de fibronectina, albúmina y transferrina. En relación a su papel inmunológico, se ha observado que desempeña un papel importante en la generación de la respuesta inmunitaria adquirida. Esto se debe a su capacidad para aumentar la producción de anticuerpos, induciendo la diferenciación de los linfocitos B en células plasmáticas, así como promoviendo el desarrollo de linfocitos T efectores (Tanaka et al. 2014; Foo et al. 2021; Alvites et al. 2022).

Sin embargo, en la especie humana se ha descrito que la secreción de IL6 por parte de las MSCs está relacionada con una inmunosupresión más eficiente de los linfocitos B (Foo et al. 2021; Alvites et al. 2022). Además, cabe destacar que tanto la producción de PGE2 como la de NO están retroalimentadas de forma positiva por IL6 y su secreción se reduce fuertemente en ausencia de la misma (Philipp et al. 2018).

En la especie equina, la molécula IL6 ha sido descrita como una de las citoquinas más importantes en la regulación de la proliferación de los linfocitos por parte de las MSCs debido a su actividad inmunorreguladora en los procesos inflamatorios (Remacha et al. 2015). Aunque otros autores indican que no es la principal molécula responsable de la inhibición de la proliferación de células T por parte de las MSCs equinas, ya que cuando se bloquea la secreción de IL6, las MSCs siguen siendo capaces de inhibir la proliferación de células T (Carrade Holt et al. 2014).

7.2.2 Ciclooxigenasa 2 (COX2)

COX2 es la enzima responsable del aumento de la formación de prostaglandinas en estados inflamatorios (Caffi et al. 2020) debido a que cataliza la formación de diversos prostanoides a partir del ácido araquidónico (Saul et al. 2019).

Su síntesis es inducida rápidamente por diferentes citoquinas, factores de crecimiento, mediadores inflamatorios, mitogénicos y hemodinámicos (Caffi et al. 2020; Sareen et al. 2020). En el modelo murino se ha descrito que la molécula PGE2, producida en las MSCs mediante la oxidación secuencial del ácido araquidónico por COX2, preservaría el privilegio inmunitario de las MSCs alogénicas ya que promovería la inhibición de la proliferación de células T citotóxicas e impediría la activación de células B (Sareen et al. 2020).

Diferentes estudios en la especie equina han demostrado que PGE2 parece ser la principal responsable de la inhibición de la proliferación de linfocitos T equinos mediada por las MSCs equinas (Carrade Holt et al. 2014; Remacha et al. 2015; Colbath et al. 2017a; Caffi et al. 2020). Cabe destacar que mediante el bloqueo de PGE2, se observó que la capacidad inhibitoria de las MSCs equinas sobre la proliferación de los linfocitos quedaba totalmente anulada. En cambio, mediante el bloqueo de otras moléculas como son IL6 y NO, no se inhibió por completo el efecto supresor sobre los linfocitos (Carrade

et al. 2012; Carrade Holt et al. 2014), sugiriendo que es PGE2 quien tiene un papel central en la inmunorregulación de las MSCs equinas.

7.2.3 Indolamina 2,3-dioxigenasa (IDO1)

IDO1 es una enzima que regula la proliferación celular mediante el catabolismo del triptófano (Remacha et al. 2015). Sus funciones radican en la regulación de la inflamación y en la creación de tolerancia inmunológica (Munn y Mellor 2016). Un estado inflamatorio mejora sus efectos en lo relativo a la reparación tisular, limita sus efectos destructivos sobre los tejidos y promueve la diferenciación de los linfocitos T reguladores. En consecuencia, se altera la presentación natural de antígenos e indirectamente IDO1 ejerce un efecto inmunosupresor y de tolerancia inmunológica (Munn y Mellor 2016) debido a que los linfocitos T reguladores actúan suprimiendo la respuesta inmunitaria y manteniendo la homeostasis del organismo (Wu et al. 2018; Caffi et al. 2020).

En MSCs humanas, IDO1 se ha descrito como una molécula clave en la capacidad inmunomoduladora de las MSC ya que la catabolización de IDO1 producida por las MSCs da lugar a catabolitos capaces de inhibir los linfocitos T y provocar su apoptosis (Wu et al. 2018; Alvites et al. 2022).

En cambio, estudios en la especie equina indican que IDO1 no parece estar implicada en el proceso inmunomodulador ni en la capacidad de las MSCs alogénicas para inhibir la proliferación de linfocitos (Colbath et al. 2017a). En varios estudios en los que se ha evaluado la capacidad inmunomoduladora e inmunogénica de las MSCs equinas, no se ha detectado secreción de IDO1 por parte de las MSCs (Carrade et al. 2012; Colbath et al. 2017a). Todo ello sugiere que esta vía no está funcionalmente activa y que en presencia de células T activadas, las MSCs equinas no producen IDO1.

Sin embargo, en lo relativo a su expresión génica, en la especie equina existe cierta controversia ya que algunos autores tampoco han observado expresión de *IDO1* en las MSCs equinas por RT-qPCR (De Schauwer et al. 2014), mientras que otros indican que las MSCs equinas sí expresan *IDO1*, especialmente cuando las MSCs son estimuladas (Cassano et al. 2018a; Caffi et al. 2020).

7.2.4 Sintasa de óxido nítrico inducible (iNOS2)

iNOS2 es una enzima que cataliza la formación de óxido nítrico a partir de L-arginina y forma parte de la familia de las sintasas de óxido nítrico. En condiciones basales no está presente en la mayoría de las células pero su expresión es inducible y se asocia a procesos inflamatorios y patológicos (Kielbik et al. 2019). Los estímulos y las condiciones que influyen en esta enzima son específicos para cada especie y para cada tipo celular (Su et al. 2014; Han et al. 2022).

Mientras que iNOS2 es reconocida como la principal enzima que media los efectos inmunorreguladores de las MSCs en roedores (Su et al. 2014; Han et al. 2022), el papel

de iNOS2 en la capacidad inmunomoduladora de las MSCs equinas se sigue investigando. Aunque varios autores coinciden en que no es una de las principales moléculas responsables de la inhibición de la proliferación de células T por parte de las MSCs equinas (Carrade Holt et al. 2014; Colbath et al. 2017a; Caffi et al. 2020).

Algunos trabajos han observado que las MSCs equinas de origen hemático (BM-MSCs y CB-MSCs) producen NO, mientras que las derivadas de tejidos sólidos (AT-MSCs y CT-MSCs) (Carrade et al. 2012) no secretan dicha molécula. Cabe destacar que también varía el mecanismo por el cual las MSCs equinas de distintos orígenes inhiben la proliferación de los linfocitos T, ya que mientras las BM-MSCs y CB-MSCs detienen el ciclo celular, las AT-MSCs y CT-MSCs inducen su apoptosis (Carrade et al. 2012). Sin embargo, a pesar de presentar mecanismos diferentes, la capacidad inmunomoduladora *in vitro* es similar entre las MSCs equinas de distintos orígenes cuando son expuestas a estímulos inflamatorios (Cassano et al. 2018a).

7.2.5 Molécula de adhesión celular vascular 1 (VCAM1)

Además de mediante su acción paracrina, las MSCs también pueden ejercer su efecto a través del contacto directo con células inmunitarias, interviniendo en el proceso diferentes moléculas de adhesión como por ejemplo VCAM1 (Fan et al. 2020).

VCAM1 es una proteína de la familia de las inmunoglobulinas que se expresa en las células endoteliales y participa en el reclutamiento de los leucocitos de la sangre al tejido dañado donde se ha producido una reacción inflamatoria. La unión de los leucocitos a las células endoteliales desencadena la activación de señales específicas, lo que conduce a la formación de pasajes intracelulares a través de los cuales los leucocitos pueden migrar del endotelio hacia los tejidos (Cook-Mills et al. 2011).

Las MSCs pueden migrar hasta los tejidos dañados gracias a la molécula de superficie CD29, que es ligando de VCAM1, cuya expresión aumenta en las células endoteliales dañadas. Por ello, en caso de daño tisular, las MSCs tienen la capacidad de migrar al endotelio y unirse a él (Foo et al. 2021). Este mecanismo de migración resulta muy importante para el reclutamiento de las MSCs hacía las regiones lesionadas para facilitar la reparación del tejido dañado (Barrachina et al. 2016).

Trabajos realizados por diferentes autores han destacado la importancia del contacto celular en la capacidad inmunomoduladora de las MSCs humanas y equinas (Ren et al. 2010; Barrachina et al. 2016). Se ha observado que tras el cocultivo de MSCs con linfocitos T la expresión de VCAM1 en las MSCs aumentaba y en consecuencia su capacidad de migración e inmunomodulación se potenciaba (Ma et al. 2014). Además, cabe destacar que cuando no había contacto directo entre las MSCs y los linfocitos, no se observaba la expresión de moléculas de adhesión como VCAM1 (Pourgholaminejad et al. 2016).

En resumen, conocer las moléculas presentes en un entorno inflamatorio y los mediadores secretados por las MSCs en respuesta a dicho entorno es fundamental para optimizar sus aplicaciones terapéuticas. Sin embargo, identificar las moléculas responsables se ve dificultado por la complejidad de las interacciones entre las MSCs y las células inmunitarias (Barrachina et al. 2016). Debido a que estas moléculas tienen efectos sobre el sistema inmunitario y por ello, dependiendo de su nivel de expresión y/o secreción, las propiedades inmunomoduladoras de las MSCs serán mayores o menores, su investigación es crucial para potenciar la efectividad terapéutica de las MSCs.

8. INMUNOGENICIDAD DE LAS CÉLULAS MADRE MESENQUIMALES

Las MSCs se habían considerado tradicionalmente como inmunoprivilegiadas, lo que significaba que eran capaces de eludir el reconocimiento por el sistema inmunitario cuando se administraban de forma alogénica y por consiguiente, tanto las terapias autólogas como las alogénicas se consideraban seguras y sin efectos adversos (Ankrum et al. 2014; Berglund et al. 2017b).

En los últimos años, numerosos estudios han demostrado que el concepto de "inmunoprivilegio" no era correcto y actualmente las MSCs se consideran inmunoevasivas. Este cambio de paradigma se basa en la capacidad de las MSCs para regular la respuesta inmunitaria, desarrollada anteriormente, y también en que serían menos inmunogénicas que otros tipos celulares (Ankrum et al. 2014).

Sin embargo, se han llevado a cabo estudios *in vitro* (Schnabel et al. 2014; Berglund y Schnabel 2017) e *in vivo* en diferentes especies, como roedores (Ryan et al. 2014; Oliveira et al. 2017), caballos (Pezzanite et al. 2015; Joswig et al. 2017; Ursini et al. 2019) y perros (Cabon et al. 2019), que indican que las MSCs alogénicas pueden provocar una respuesta inmunitaria en el receptor. Por tanto, la generación de una respuesta inmunitaria tras la administración repetida de MSCs alogénicas podría constituir un obstáculo en el desarrollo de este tipo de tratamientos (Kamm et al. 2021b).

Varios autores han señalado que tras la administración repetida de MSCs alogénicas equinas solo se generaba una leve reacción inflamatoria (Joswig et al. 2017; Bertoni et al. 2019) que podría deberse al reconocimiento inmunitario de las MSCs por parte del receptor tras la reexposición. No obstante, dicha reacción inmunitaria no siempre se ha observado ni ha sucedido de la misma forma o en el mismo grado (Ardanaz et al. 2016; Magri et al. 2019; Colbath et al. 2020a), sugiriendo que existen diversos factores que pueden influir en el proceso de reconocimiento de las MSCs alogénicas.

Entre dichos factores, uno de los más importantes es el nivel de expresión de las moléculas inmunogénicas en las MSCs y la compatibilidad para el MHC entre donante y receptor.

8.1 Moléculas del complejo mayor de histocompatibilidad

Los genes del MHC codifican proteínas de la superficie celular que tienen la función de reconocer y presentar diferentes péptidos a las células inmunitarias (Colbath et al. 2020b). Las moléculas del MHC son de dos clases: MHC-I y MHC-II.

El MHC-I está expresado en todas las células nucleadas del organismo y su función es presentar péptidos a los linfocitos T citotóxicos. Estos péptidos pueden ser componentes propios de la célula o bien péptidos extraños al organismo. Cuando el MHC-I es reconocido por las células T citotóxicas, portadoras de la molécula CD8 en su membrana plasmática, son capaces de detectar si lo mostrado es propio o extraño. Si el péptido presentado no es propio del organismo, las células T citotóxicas desencadenarán la destrucción de la célula portadora (Marshall et al. 2018; Kamm et al. 2021a).

El MHC-II sólo se expresa en las CPA, cuya función es transportar péptidos de antígenos previamente fagocitados. Cuando las células T reconocen a los péptidos unidos a las moléculas del MHC-II de las CPA, se activan e inician la respuesta inmunitaria. Este proceso de presentación del antígeno activa a las células T para que se diferencien en células T citotóxicas (células CD8+) o células T colaboradoras (células CD4+). Como se ha desarrollado anteriormente, una vez activadas, las células T colaboradoras liberan citoquinas que influyen en la actividad de muchos tipos de células, incluidas las mismas CPA que las activan (Marshall et al. 2018).

Tras varios estudios contradictorios sobre la presencia de marcadores MHC en la superficie de las MSCs equinas, se ha concluido que tanto MHC-I como MHC-II pueden expresarse aunque su expresión puede ser variable entre animales y entre tejidos de origen (De Schauwer et al. 2014; Guest et al. 2022), y además dicha expresión puede variar dependiendo del ambiente en que en encuentren las MSCs (Schnabel et al. 2014).

Estudios *in vitro* han demostrado que cuando las MSCs equinas expresan altos niveles de MHC-II estimulan la generación de la respuesta inmunitaria (Kamm et al. 2021b). En cambio, cuando la expresión es baja, las MSCs pueden pasar desapercibidas y no inducir respuesta inmunitaria (Schnabel et al. 2014; Broeckx et al. 2019a). Además, existen diferentes estudios que se han centrado en cómo reducir la expresión del MHC en las MSCs equinas (Berglund et al. 2017a; Hill et al. 2017).

Como se ha mencionado anteriormente, las MSCs pueden alterar su expresión del MHC-II en función de la exposición ambiental. En el estudio de Schnabel et al. (2014) analizaron MSCs de distintos caballos, tanto jóvenes como adultos, y encontraron resultados muy heterogéneos en cuanto a la expresión del MHC-II. En su estudio concluyeron que el porcentaje de MSCs que expresan MHC-II es variable dependiendo del donante y, además, puede verse modificado por un considerable número de factores tales como la genética, la calidad del aspirado de médula o incluso el estado inmune del animal en ese momento. Los autores proponían que todos los animales donantes de

MSCs fueran previamente inmunofenotipados, ya que aquellos con altos niveles de MHC-II producían mayor respuesta inmunitaria *in vitro* (Schnabel et al. 2014).

En consonancia, estudios recientes en caballos (Kamm et al. 2019, 2021b) han descrito que el nivel de expresión del MHC en las MSCs depende de cada individuo y que las MSCs podrían clasificarse según el nivel de expresión del MHC-II; alta o baja expresión. En este estudio concluyeron que cuando los antígenos de superficie celular son tenidos en cuenta, no se observaba proliferación linfocitaria tras el cocultivo con MSCs alogénicas, pero que son necesarios más estudios que esclarezcan la relación entre las propiedades *in vitro* de las MSCs equinas y su actividad *in vivo*.

8.2 Moléculas CD40

Además del MHC, hay otras moléculas que intervienen en la inmunogenicidad de las MSCs como son CD40 y CD80, que están muy relacionadas con el perfil inmunogénico de las MSCs y pueden desarrollar un papel muy importante en el rechazo inmunológico. Ambas moléculas actúan como activadoras del sistema inmunitario promoviendo la estimulación de los linfocitos T, y la aparición y proliferación de linfocitos B de memoria (Lee et al. 2021).

CD40 es una proteína de membrana presente en las CPA, linfocitos B, macrófagos y células dendríticas (Buyl et al. 2020; Lee et al. 2021). Para que se produzca la activación de los linfocitos B por antígenos solubles, se requiere la colaboración de los linfocitos T colaboradores (CD4+). De este modo, cuando la molécula CD40 de la superficie de los linfocitos B se acopla al ligando CD40L presente en los linfocitos T activados, se induce la proliferación de los linfocitos B. Además, el acoplamiento de CD40-CD40L también estimula la expresión de moléculas coestimuladoras como CD80, contribuyendo a una activación más eficaz de las células T (Briones et al. 2011).

Aunque algunos autores señalan que aproximadamente el 50% de las AT-MSC humanas expresan *CD40* en condiciones basales (Buyl et al. 2020), un estudio reciente en la especie humana indicó que las MSCs derivadas de amígdalas estimuladas con citoquinas proinflamatorias incrementaban su función inmunomoduladora a medida que aumentaba la expresión de *IDO1* y *CD40* (Lee et al. 2021).

Estudios anteriores también habían descrito que la expresión de la molécula coestimuladora *CD40* aumentaba en las MSCs cuando se encontraban en condiciones inflamatorias tanto en la especie humana como en la equina (Haddad y Saldanha-Araujo 2014; Barrachina et al. 2017). A pesar de este aumento, esto no necesariamente se traduce en un aumento de la inmunogenicidad, ya que es necesaria la colaboración de otras moléculas para que se produzca el reconocimiento antigénico (Barrachina et al. 2017) y la activación de las células T (Briones et al. 2011). Sin embargo, existen muy pocos estudios *in vitro* en la especie equina en los que se haya valorado la importancia e influencia de CD40 en la capacidad inmunomoduladora o inmunogénica de las MSCs equinas.

8.3 Moléculas CD80

CD80 es una molécula coestimuladora localizada en la membrana de las CPA que hace de ligando de dos receptores, CD152 y CD28, presentes en la superficie de los linfocitos T (Briones et al. 2011). Para que las células T puedan activarse es necesario que sus receptores se acoplen a las moléculas coestimuladoras B7 (CD80 y CD86) presentes en la superficie de las CPAs. Este acoplamiento induce la proliferación de células T y la secreción de diferentes citoquinas (Kindt et al. 2020).

En ambientes no inflamatorios, la molécula CD80 contribuye al mantenimiento de la homeostasis y la tolerancia hacia los autoantígenos. Además, desempeña un papel en la regulación de la activación de los linfocitos T, limitando su respuesta y promoviendo un estado de equilibrio inmunológico (Kindt et al. 2020). Por contraposición, ante estímulos inflamatorios, promueve la maduración de las células dendríticas y además aumenta los niveles de CD86, que al unirse al CD28 estimula la proliferación de los linfocitos T (Briones et al. 2011).

Estudios en MSCs humanas han señalado que tras la estimulación con citoquinas, las MSCs no aumentan la expresión de *CD80*, incluso disminuye, a diferencia de lo que ocurre con otras moléculas inmunitarias (Van Megen et al. 2019; Ozdemir et al. 2021). Un estudio reciente en la especie murina ha descrito que las MSCs inducen la expresión de FoxP3 en las Tregs, sin embargo, al bloquear la expresión de *CD80* se dejaba de observar inhibición de la proliferación de células T mediada por los linfocitos T reguladores. En cambio, la adición de CD80 soluble aumentaba significativamente la expresión de FoxP3 (Mittal et al. 2022).

En las MSCs equinas se ha observado una expresión de moderada a fuerte de las moléculas coestimuladoras *CD40* y *CD80* en las UC-MSCs y PB-MSCs (De Schauwer et al. 2014). Sin embargo, tras la estimulación de BM-MSCs con citoquinas proinflamatorias, estos mismos autores, no observaron cambios en la expresión de las moléculas coestimuladoras *CD80*, *CD86* y *CD40L*, lo que sugiere que las BM-MSCs podrían conservar su estatus inmunoevasivo tras la estimulación cuando esto se acompaña de un aumento en la expresión de las moléculas inmunomoduladoras.

8.4 Haplotipos del complejo mayor histocompatibilidad

Recientemente se ha destacado la importancia de la compatibilidad para el MHC equino entre donante y receptor en las terapias celulares alogénicas (Pezzanite et al. 2015, 2022; Rowland et al. 2021).

En el caballo, la región del MHC se localiza en el cromosoma 20 y está codificada por varios genes denominados sistema ELA (equine leukocyte antigen) que dan lugar a 19 serotipos diferentes. La determinación serológica requiere antisueros específicos para cada tipo de ELA muy costosos de obtener y cuya especificidad no siempre es óptima (Miller et al. 2017). Debido a estas limitaciones, comenzó a emplearse el análisis directo

de los genes del ELA para detectar las variaciones que daban lugar a los distintos serotipos (Tseng et al. 2010).

Para llevar a cabo estos análisis se utilizaron como marcadores ciertas regiones del DNA (loci) de alto polimorfismo que presentaban secuencias cortas de nucleótidos (microsatélites) que se repetían un número variable de veces, originando fragmentos de DNA de distinta longitud, representando cada uno un alelo diferente. Estos loci intra-MHC están sometidos a desequilibrio de ligamiento al situarse muy cerca en el genoma, por lo que los alelos de cada marcador tienden a heredarse juntos constituyendo esta combinación de alelos un haplotipo. Por tanto, cada animal presenta dos haplotipos al heredar juntos los alelos de su padre y de su madre. En 2010, se desarrolló un panel de 5 marcadores microsatélites intra-MHC para determinar haplotipos ELA y su correlación con varios serotipos (Tseng et al. 2010), que posteriormente se amplió a 10 marcadores (Sadeghi et al. 2018) (**Figura 8**). En los últimos años se han descrito nuevos marcadores en la especie equina (Holmes et al. 2019).

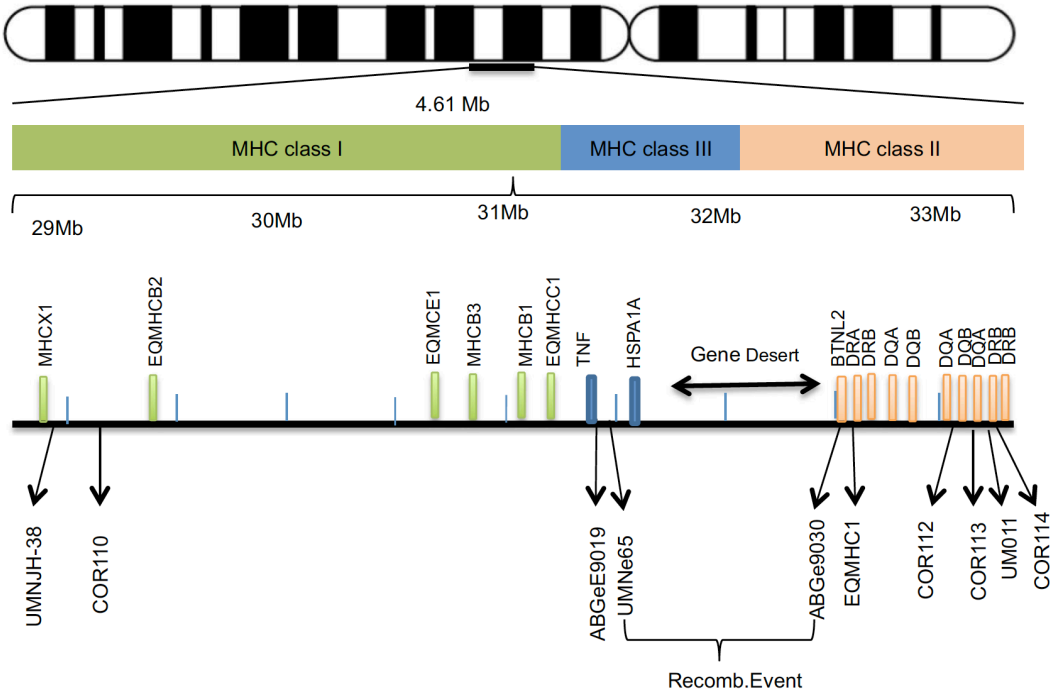


Figura 8. Mapa de los marcadores microsatélites del MHC equino y genes seleccionados del cromosoma 20. Los genes MHC se dividen en tres subgrupos: clase I, clase II y clase III (Sadeghi et al. 2018).

El análisis del tipo de ELA y las variaciones en estos loci del MHC equino pueden proporcionar información sobre la diversidad genética y la capacidad inmunológica de una población. Estudios realizados en caballos árabes (Sadeghi et al. 2018) e islandeses (Holmes et al. 2019) han mostrado una alta variabilidad en estas regiones intra-MHC, estando descritos aproximadamente 500 haplotipos. Otra fuente de variabilidad es la posible recombinación de los dos haplotipos de un progenitor en la descendencia, dando lugar un nuevo haplotipo (**Figura 8**). Esta recombinación se ha observado entre la región que codifica para MHC-I y para MHC-II, proponiéndose un sistema de

hemihaplotipos al observarse también que varios haplotipos diferentes compartían los mismos alelos para marcadores de MHC-I o MHC-II (Sadeghi et al. 2018; Holmes et al. 2019).

Conocer el haplotipo del MHC tanto de los donantes como de los receptores está adquiriendo una creciente importancia en el estudio de la terapia celular alogénica equina. Aunque la gran variabilidad de haplotipos del MHC puede dificultar encontrar dos animales con haplotipos idénticos, esta probabilidad puede aumentar si los animales pertenecen a la misma raza y/o si hay parentesco entre ellos, ya que existen ciertos haplotipos predominantes.

Además, una estrategia muy interesante que están abordando diferentes grupos de investigación en la especie equina es la de utilizar donantes homocigotos para un haplotipo (dos copias iguales, ej. A/A) y receptores heterocigotos compartiendo una copia con el donante (ej. A/B, A/C, A/D) (Rowland et al. 2021; Pezzanite et al. 2022). De este modo, caballos homocigóticos podrían actuar como donantes de MSCs, ya que serían compatibles con el MHC de los animales heterocigóticos. La premisa de utilizar individuos homocigóticos para emparejarlos con diferentes individuos heterocigóticos es la misma que se ha propuesto para crear haplobancos de iPSCs humanas (Taylor et al. 2012; Sullivan et al. 2020). Por ahora, la utilización de caballos compatibles e incompatibles para el MHC es relevante principalmente para fines de investigación, pero es necesario que se profundice el conocimiento en esta área. En la **Tabla 1** se incluyen los principales estudios llevados a cabo en la especie equina en los que se han determinado los haplotipos para el MHC.

Tabla 1. Diferentes estudios realizados en la especie equina en los que se ha determinado el haplotipo para el MHC. Se señalan los marcadores microsatélites analizados en cada estudio.

				MHC clase I				MHC clase III		MHC clase II							
Autor	**Objetivo**	***n***	**Razas**	UMNJH-38	COR110	TAMU305-93	CZM002	ABGE9019	UMNe65	ABGE9030	TKY3324	EQMHC1	COR112	COR113	UM011	COR114	**Total**
Tseng et al. 2010	Biodiversidad	353	PSI y ST		x								x	x	x	x	5
Pezzanite et al. 2015	Terapia MSCs	11	PSI		x	x		x		x	x		x	x	x	x	9
Sadeghi et al. 2018	Biodiversidad	124	PRá	x	x			x	x	x		x	x	x	x	x	10
Rowland et al. 2018	Terapia MSCs	12	Quarter	x	x			x	x	x		x	x	x	x	x	10
Holmes et al. 2019	Biodiversidad	156	Islandeses	x	x	x	x	x	x	x		x	x	x	x	x	12
Jaworska et al. 2020	Biodiversidad	170	Konik Polski	x	x	x		x	x	x	x		x	x	x	x	11
Rowland et al. 2021	Terapia MSCs	35	Quarter	x	x	x	x	x	x	x		x	x	x	x	x	12
Kamm et al. 2021	Terapia MSCs	10	PSI y ST	x	x	x	x	x	x	x		x	x	x	x	x	12
Berglund et al. 2021	Terapia MSCs	8	PSI y ST	x	x					x		x	x	x	x	x	8
Pezzanite et al. 2022	Terapia MSCs	11	Cruzados	x	x	x	x	x	x	x		x	x	x	x	x	12

PSI: Pura Sangre Inglés, PRá: Pura Raza árabe, ST: Standard bred

Actualmente existen pocos estudios que controlen la compatibilidad para el MHC entre donante y receptor y que tengan en cuenta el nivel de expresión de las moléculas inmunogénicas, lo que podría explicar los resultados discrepantes que se observan en los diferentes estudios (Berglund et al. 2017b). Pero los estudios que sí han tenido en cuenta el haplotipo y la expresión del MHC han demostrado que puede producirse respuesta inmunitaria tanto celular como humoral cuando donante y receptor son incompatibles (Pezzanite et al. 2015, 2022; Berglund et al. 2021; Kamm et al. 2021b; Rowland et al. 2021), lo que podría obstaculizar el uso de dosis repetidas de MSCs alogénicas, estrategia estudiada para aumentar la efectividad de la terapia celular (Schnabel et al. 2014).

Estudios recientes también han descrito el desarrollo de memoria inmunológica frente a las moléculas del MHC en MSCs de diferentes especies (Huang et al. 2016; Berglund et al. 2017b). En la especie murina, se ha demostrado que tras la administración de MSCs en receptores MHC-incompatibles, las MSCs sobrevivían un periodo de tiempo inferior en comparación con los receptores MHC-compatibles y además, el rechazo fue más rápido en el caso de los ratones ya sensibilizados (Zangi et al. 2009). En ensayos realizados en macacos se observó un aumento de células T circulantes y de NK, así como de aloanticuerpos reactivos frente a las MSCs tras la administración de MSCs incompatibles; no observado cuando se administraron MSCs autólogas (Isakova et al. 2014).

Por ello, la incompatibilidad para el MHC entre donante y receptor podría repercutir en el efecto terapéutico deseado tal y como se ha observado tras la administración de MSCs alogénicas frente a autólogas en la especie equina (Berglund et al. 2017b; Rowland et al. 2021). Además, el aloreconocimiento por el receptor incompatible de las moléculas de MHC presentes en la superficie de las MSCs del donante implicaría el aumento del riesgo de reacciones adversas, a través de la activación de sistema inmunitario del receptor (Berglund y Schnabel 2017; Voga et al. 2020).

Los diferentes autores indican que todavía se desconocen las implicaciones clínicas de las respuestas inmunitarias a las MSCs alogénicas con MHC-incompatible y que, para responder a esta pregunta fundamental, son necesarios estudios preclínicos y clínicos que documenten el haplotipo del MHC de los donantes y los receptores y que midan las respuestas inmunitarias tras el tratamiento con MSCs (Pezzanite et al. 2015, 2022; Berglund et al. 2021; Kamm et al. 2021b; Rowland et al. 2021).

9. EQUILIBRIO INMUNOMODULACIÓN-INMUNOGENICIDAD

Tras la revisión de las propiedades inmunomoduladoras e inmunogénicas de las MSCs, resulta fundamental definir qué factores influyen sobre dichas propiedades para poder aumentar el potencial terapéutico y reducir el riesgo de aparición de efectos adversos o rechazo de las propias células, lo que repercutiría en su efectividad. La severidad del rechazo de las MSCs depende del equilibrio entre su capacidad inmunomoduladora y su inmunogenicidad, pero este equilibrio no es dependiente de una sola condición, sino que es el conjunto de muchas lo que hace que las MSCs se comporten de una forma más inmunomoduladora o más inmunogénica (Ankrum et al. 2014) (**Figura 9**). Además, debe tenerse en cuenta que, debido a todas las interacciones que existen entre las diferentes células y moléculas del sistema inmunitario, el comportamiento de las células puede ser muy diferente entre especies e incluso entre diferentes individuos de una misma especie.

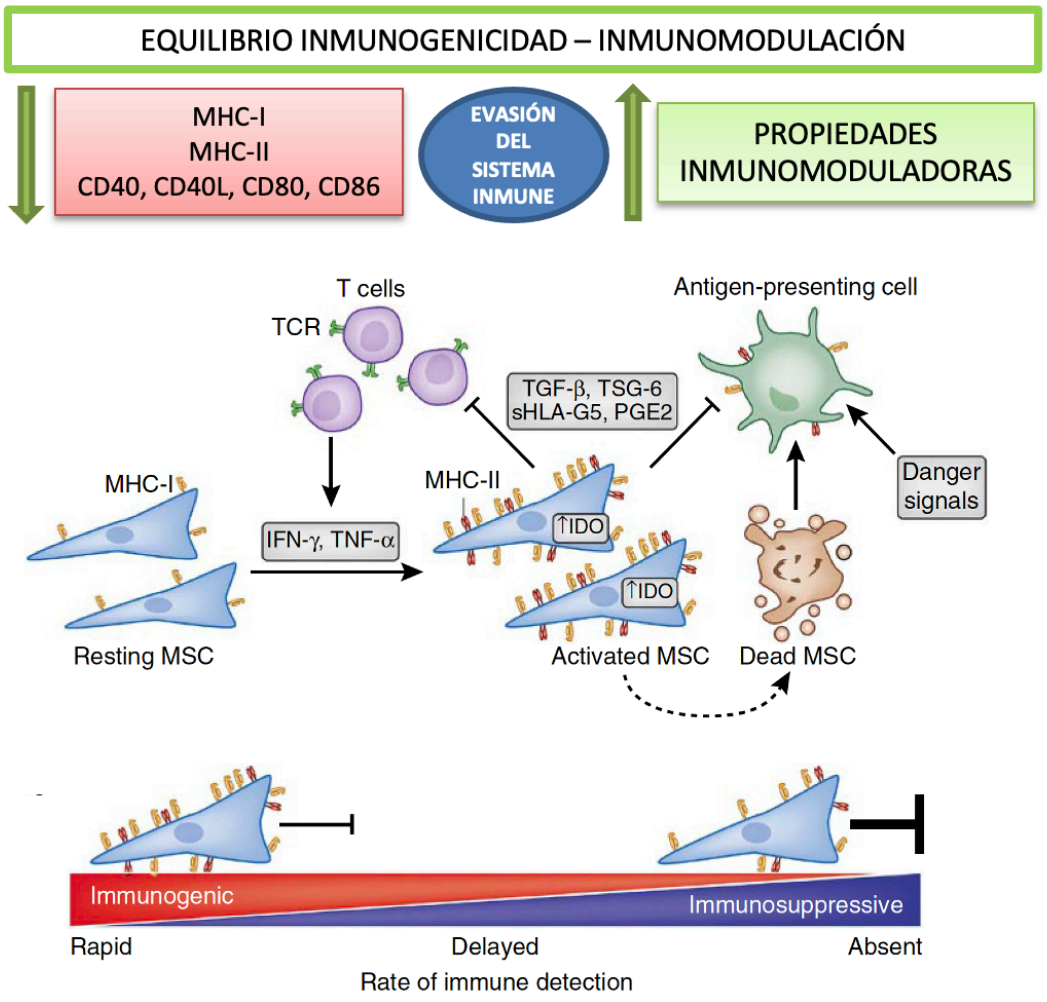


Figura 9. Equilibrio inmunomodulación-inmunogenicidad (Ankrum et al. 2014).

Como se ha desarrollado en la sección anterior, en algunos estudios en la especie equina se ha observado que la administración repetida de MSCs alogénicas puede dar lugar a una respuesta inmunitaria adaptativa, inflamación local y a una menor acción terapéutica de las MSCs (Pezzanite et al. 2015; Rowland et al. 2021). Otros estudios observaron que tras la administración intraarticular repetida de MSCs autólogas y/o alogénicas, se generaba una leve reacción inflamatoria con ambos tipos celulares (Ardanaz et al. 2016; Joswig et al. 2017; Magri et al. 2019; Colbath et al. 2020a). No obstante, esta respuesta local fue leve y transitoria, sin generar problemas graves ni afectar el efecto terapéutico de las MSCs. Estos resultados contradictorios sugieren que existen diferentes factores que podrían influir en la capacidad de las MSCs para evadir el sistema inmunitario y desempeñar sus funciones terapéuticas, así como facilitar el reconocimiento de las MSCs alogénicas.

Entre los diversos factores que influyen en la interacción entre las MSCs y el sistema inmunitario del receptor, se destacan aquellos de mayor relevancia, tales como la compatibilidad del MHC entre donante y receptor, el origen tisular de las MSCs, el estado inflamatorio y la capacidad de diferenciación. Estos dos últimos factores tienen especial importancia por constituir tanto situaciones que pueden darse *in vivo* tras la administración de las MSCs, como por haberse estudiado como estrategias terapéuticas para mejorar la efectividad de las mismas. Asimismo, la exposición de las MSCs a citoquinas proinflamatorias y la administración de MSCs diferenciadas y pre-diferenciadas se han propuesto como estrategias terapéuticas para mejorar su potencial. Sin embargo, estos mismos factores pueden influir sobre el delicado equilibrio inmunomodulación-inmunogenicidad de las MSCs equinas (Barrachina et al. 2018b; Broeckx et al. 2019a; Caffi et al. 2020).

9.1 Estimulación de las células madre mesenquimales

Como se ha desarrollado en las secciones anteriores, en los ambientes inflamatorios que se producen cuando ocurre una lesión, las MSCs son capaces de secretar factores antiinflamatorios y potenciar su efecto inmunomodulador (Ankrum et al. 2014; Foo et al. 2021). Sin embargo, este ambiente inflamatorio puede no ser suficiente para el desarrollo completo de su potencial. Para que ciertos mediadores inmunomoduladores sean secretados y su producción aumente, las MSCs requieren ser activadas, dado que algunos de estos mediadores no se secretan o se liberan en cantidades bajas en condiciones basales (Barrachina et al. 2016).

Para aumentar la secreción de estos mediadores, las MSCs pueden ser estimuladas *in vitro* exponiéndolas a factores proinflamatorios, como TNFα, IFNɣ, IL1 o LPS liberados por bacterias, que promueven tanto la migración de estas células al sitio donde se localiza el daño tisular como la secreción por parte de las mismas de moléculas inmunomoduladoras (MacDonald y Barrett 2020; Foo et al. 2021). Se han investigado estas estrategias con el fin de mejorar la capacidad de las MSCs para inhibir la

proliferación de células T y B, a la vez que se potencian sus propiedades inmunomoduladoras y se reducen sus propiedades inmunogénicas, tanto *in vitro* como *in vivo* en la especie humana (Alvites et al. 2022), murina (Mittal et al. 2022), canina (Wi et al. 2021) y también en la especie equina (Cassano et al. 2018b; Colbath et al. 2020b; Kamm et al. 2021a).

En la especie equina se han utilizado diferentes moléculas como son las citoquinas proinflamatorias TNFα, IFNɣ, TGFβ2 (Berglund et al. 2021), ácido retinoico (Abdelhamid et al. 2017) y la exposición de las MSCs a ácido polinosínico:policidílico (poli I:C) o LPS (Cassano et al. 2018b) con el objetivo de aumentar la capacidad inmunomoduladora de las células (Barrachina et al. 2017; Caffi et al. 2020; Berglund et al. 2021).

La estrategia de utilizar las citoquinas proinflamatorias TNFα e IFNɣ para estimular las MSCs equinas ha sido muy estudiada tanto de forma individual como combinada a diferentes dosis y tiempos de exposición (Cassano et al. 2018c; Caffi et al. 2020).

Tras la exposición de BM-MSCs equinas a concentraciones crecientes de estas citoquinas (5 ng/mL, 20 ng/mL y 50 ng/mL) y tiempos (12 horas, 24 horas y 72 horas) se observó que las BM-MSCs expuestas a 5 ng/mL de TNFα e IFNɣ durante 12 horas aumentaban *in vitro* su capacidad inmunorreguladora y a la vez conservaban su viabilidad y capacidad de diferenciación (Barrachina et al. 2017). Por otro lado, en el estudio realizado por Caffi y colaboradores (2020) se evaluó el efecto de estas citoquinas proinflamatorias en las MSCs equinas, y su impacto en la capacidad de inhibir la proliferación de linfocitos. Para ello, testaron TNFα e IFNɣ individualmente y combinadas a diferentes dosis: 0,1 ng/mL, 1 ng/mL y 10 ng/mL durante 24 horas. En consonancia, también concluyeron que la mejor estrategia para la estimulación de las MSCs era la combinación de ambas citoquinas, pero a dosis de 10 ng/mL.

Así mismo, estos estudios concluyeron que la exposición de las MSCs equinas a un ambiente inflamatorio creado mediante estas citoquinas proinflamatorias aumentaría sus propiedades inmunomoduladoras al aumentar la expresión y/o secreción de las moléculas inmunomoduladoras *in vitro* (Barrachina et al. 2017; Caffi et al. 2020; Berglund et al. 2021). Por ello, la estimulación de las propiedades inmunomoduladoras de las MSCs equinas mediante el uso de citoquinas proinflamatorias antes de su administración se ha propuesto como una estrategia para mejorar su potencial terapéutico (Cuerquis et al. 2014; Barrachina et al. 2018a).

9.1.1 Efecto de las células madre mesenquimales estimuladas sobre la respuesta inmunitaria

El efecto de la estimulación de las MSCs en las moléculas implicadas en el equilibrio entre la inmunomodulación y la inmunogenicidad está siendo objeto de creciente investigación en la especie equina. Estos estudios se han centrado tanto en la expresión génica de estas moléculas en las MSCs como en la secreción de factores por parte de estas células *in vitro* (Barrachina et al. 2017; Caffi et al. 2020; Berglund et al. 2021). No

obstante, la valoración de estas moléculas después de la administración de las MSCs se ve obstaculizada por la dificultad de recuperar las células una vez que han sido administradas en el animal.

De manera similar a lo observado en la especie humana (Ma et al. 2014; Montesinos et al. 2020; Alvites et al. 2022), se ha demostrado que la expresión de *IL6*, *COX2* y *VCAM1* aumenta en las MSCs equinas tras la exposición a la combinación de IFNɣ y TNFα, lo que mejora su capacidad inhibidora de la proliferación de linfocitos *in vitro* (Barrachina et al. 2017; Caffi et al. 2020). En el caso de las moléculas inducibles como iNOS2, la estimulación mediante citoquinas proinflamatorias resulta también muy interesante con el objetivo de potenciar el efecto inmunomodulador de las MSCs tanto en caballos como en otras especies de mamíferos (Su et al. 2014; Noronha et al. 2019; Han et al. 2022).

Respecto a la enzima IDO1, se ha señalado por parte de algunos autores que aparentemente no está involucrada en el proceso de inmunomodulación ni en la capacidad de las MSCs equinas para inhibir la proliferación de linfocitos (Colbath et al. 2017a). Sin embargo, tras estimular las MSCs equinas con citoquinas proinflamatorias, la actividad de IDO1 parece ser también esencial para mantener el efecto inhibidor de la proliferación de linfocitos (Caffi et al. 2020).

Asimismo, en el contexto de una lesión, el ambiente inflamatorio naturalmente aumentaría las propiedades inmunomoduladoras de las MSCs y estas propiedades podrían potenciarse mediante el uso de citoquinas proinflamatorias (Sivanathan et al. 2017; Voga et al. 2020). Sin embargo, dicha estimulación puede generar también un aumento de su inmunogenicidad y una respuesta inmunitaria por parte del receptor alogénico. Se ha observado que la estimulación proinflamatoria de las MSCs puede inducir un aumento de la expresión de las moléculas del MHC-I y MHC-II (Schnabel et al. 2014; Barrachina et al. 2017) lo que limitaría potencialmente su uso alogénico, especialmente en administraciones repetidas por el posible desarrollo de mecanismos de memoria inmunológica.

En la especie equina, la estimulación con IFNɣ en MSCs negativas para el MHC-II resultó en un notable aumento de su expresión (Schnabel et al. 2014). Además, en este mismo estudio, cuando se enfrentaron *in vitro* MSCs positivas para el MHC-II (sin estimulación previa) con células T alogénicas, se observó que la incompatibilidad entre el donante y el receptor inducía un aumento en la proliferación de células T, en comparación con las MSCs negativas para MHC-II o las positivas pero compatibles. Los autores señalaron que la expresión de MHC-II debería ser comprobada antes del uso alogénico de MSCs e, idealmente, también debería serlo la compatibilidad para el MHC entre donante y receptor. Además, es importante considerar que en un entorno inflamatorio donde el IFNɣ esté presente de forma natural, podría producirse un cambio en el perfil inmunogénico de las MSCs equinas (Schnabel et al. 2014). Sin embargo, otros estudios señalan que las MSCs equinas podrían seguir conservando su estatus inmunoevasivo

debido a la ausencia de cambios en la expresión de las moléculas coestimuladoras *CD80*, *CD86* y *CD40L*, junto con el aumento de las moléculas inmunorreguladoras (Barrachina et al. 2017).

En la especie equina solo hay un estudio *in vivo* en el que se hayan administrado MSCs estimuladas con citoquinas proinflamatorias de forma repetida en un modelo de OA (Barrachina et al. 2018a). En este estudio se observó que el uso de administraciones repetidas de MSCs alogénicas, tanto estimuladas como sin estimular, producía efectos beneficiosos en la evolución de la OA equina, principalmente a corto plazo, sugiriendo un mayor potencial regulador y antiinflamatorio con las MSCs estimuladas. Sin embargo, aunque la administración repetida de MSCs alogénicas no produjo reacciones adversas, en el grupo de animales que recibió MSCs estimuladas se apreció una inflamación local, ligera y transitoria tras la segunda administración. Este hallazgo podría estar relacionado con un aumento en la inmunogenicidad de las MSCs derivado de su activación, pudiendo estar implicados mecanismos de memoria inmunológica (Barrachina et al. 2018a).

Tras los hallazgos encontrados, en un estudio posterior concluyeron que la memoria inmunológica podía limitar la duración de los efectos terapéuticos y que las MSCs estimuladas podrían ser más atacadas por el sistema inmunitario del receptor por la mayor expresión del MHC en ellas. Sin embargo, cabe destacar que tras realizar la segunda administración de MSCs estimuladas también se observó un aumento de sus propiedades inmunomoduladoras, hecho que podría contribuir a regular más eficazmente la inflamación y a provocar una menor respuesta humoral cuando se administran por segunda vez (Barrachina et al. 2020).

En el ámbito clínico, es deseable que las propiedades inmunomoduladoras de las MSCs superen su potencial inmunogénico. No obstante, los mecanismos precisos para lograr este equilibrio aún no están claramente definidos, y se requiere de investigaciones adicionales en este campo para comprender cómo ciertos factores pueden modificar este equilibrio de manera óptima.

9.2 Diferenciación de las células madre mesenquimales

Inicialmente se demostró que las MSCs humanas eran capaces de diferenciarse, como mínimo en osteoblastos, condroblastos y adipocitos aunque se ha observado que tienen capacidad de diferenciarse a muchos más tipos celulares como hepatocitos, cardiomiocitos y miocitos, entre otros (Ullah et al. 2015). Asimismo, también se han realizado numerosos estudios en las MSCs de diferentes especies domésticas que han demostrado su capacidad de diferenciarse a múltiples tipos celulares cuando se cultivan en las condiciones adecuadas *in vitro* (Prządka et al. 2021). Como se ha mencionado anteriormente, esta capacidad llevó a pensar que las MSCs podían desarrollar su potencial terapéutico a través de la diferenciación a distintos tipos celulares. Sin embargo, numerosos estudios posteriores tanto en la especie humana como en la equina y demás especies domésticas han demostrado que el principal mecanismo de

acción de las MSCs se debe a sus propiedades inmunomoduladoras y de reparación tisular (Gugjoo et al. 2019; Mishra et al. 2020).

No obstante, varios autores coinciden en que las MSCs obtenidas del mismo tejido que se quiere tratar serían la fuente más adecuada para la terapia, aunque el aislamiento de MSCs de ciertos tejidos resulta complicado y poco factible (Voga et al. 2020). En este sentido, como se ha comentado anteriormente, la capacidad de diferenciación de las MSCs *in vitro* está estrechamente relacionada con el tipo de tejido del cual provienen. Por tanto, es posible que ciertos tipos de MSCs sean más adecuados que otros para tratar determinadas patologías (Iacono y Merlo 2022).

En este sentido, se ha observado que las BM-MSCs tienen mayor potencial condrogénico y osteogénico que las AT-MSCs en la especie equina (Vidal et al. 2008; Branly et al. 2017), por lo que las BM-MSCs podrían ser más eficaces en la reparación de tejidos cartilaginosos u óseos (Gugjoo et al. 2019). Sin embargo, se ha demostrado que las CB-MSCs equinas tienen mayor potencial para diferenciarse en células del cartílago en comparación con las AT-MSCs y BM-MSCs (Burk et al. 2013), aunque éstas últimas se diferencian más rápidamente que las CB-MSCs (Barberini et al. 2014).

Recientemente, se ha planteado el uso de MSCs diferenciadas o prediferenciadas en diversas especies, como la humana y la equina, como una estrategia terapéutica. Esta aproximación no solo busca facilitar la integración de las MSCs en el lugar de la lesión, sino también estimular a las MSCs para que secreten las moléculas paracrinas específicas necesarias para el tratamiento de dicha lesión (Ham et al. 2015; Broeckx et al. 2019b).

En un estudio realizado en un modelo de conejo evaluaron el efecto de la reparación de defectos osteocondrales al prediferenciar las BM-MSCs en condrocitos u osteocitos, encapsularlas en hidrogeles y posteriormente implantarlas en los defectos del cóndilo femoral (Lam et al. 2014). Los resultados de este estudio mostraron que la reparación del cartílago en los defectos osteocondrales fue significativamente mejor cuando se utilizaron MSCs prediferenciadas en condrocitos u osteocitos en comparación con las MSCs no prediferenciadas. Actualmente en la especie humana no existen en el mercado productos que contengan MSCs prediferenciadas *in vitro* (Childs et al. 2020), aunque ALLOB, producto basado en MSCs estimuladas osteogénicamente, se encuentra en ensayo clínico de fase IIB (Jayankura et al. 2021).

En la especie equina, existen en el mercado dos productos con MSCs derivadas de sangre periférica prediferenciadas a condrocitos (ci-MSCs, Arti-Cell Forte) y a tenocitos (tp-MSCs, RenuTend) para el tratamiento de patologías articulares y tendinopatías, respectivamente. El grupo de investigación detrás del desarrollo de estos dos productos ha publicado y divulgado los resultados preclínicos de sus experimentos durante los últimos años (Broeckx et al. 2019a; Depuydt et al. 2021; Van Hecke et al. 2021).

En uno de los primeros estudios llevados a cabo por el grupo de investigación creador de Arti-Cell Forte, observaron que un mayor porcentaje de pacientes volvían al trabajo tras el tratamiento con una combinación de ci-MSCs y plasma (86%) en comparación con los caballos tratados con una combinación de MSCs sin diferenciar y plasma (78%), aunque la diferencia no fue estadísticamente significativa (Broeckx et al. 2014). Posteriormente, el mismo grupo observó en un estudio *in vitro* que la inducción condrogénica de las MSCs mejoraba de manera significativa su capacidad de adherirse a la superficie del cartílago, así como su capacidad de penetración en las lesiones de cartílago en cultivos de explantes de cartílago (Spaas et al. 2015).

Aunque estos primeros estudios se centraron principalmente en valorar la eficacia de las ci-MSCs para el tratamiento de la OA en pacientes equinos, en estudios posteriores han valorado también la seguridad de estas terapias en profundidad (Broeckx et al. 2018). Sin embargo, aún no se conoce de forma certera cómo puede variar el efecto terapéutico de las MSCs prediferenciadas dependiendo del tejido de origen en la especie equina.

9.2.1 Efecto de las células madre mesenquimales diferenciadas sobre la respuesta inmunitaria

El efecto de la diferenciación o prediferenciación de las MSCs sobre el equilibrio inmunomodulación-inmunogenicidad y las interacciones con las células del sistema inmunitario *in vitro* e *in vivo* ha sido muy poco estudiado en las diferentes especies. Además, la falta de estandarización y las diferencias en el tipo de fuentes de tejido celular, métodos de obtención y protocolos de diferenciación podría explicar los diferentes resultados observados (Gugjoo et al. 2019; Voga et al. 2020).

Estudios realizados en la especie humana han demostrado que la diferenciación de las MSCs a osteoblasto aumenta la expresión de las moléculas coestimuladoras *CD80* y *CD86* e induce la proliferación de células T cuando son administradas de forma alogénica, sugiriendo que el proceso de diferenciación de las MSCs las hace más inmunogénicas (Shi et al. 2010). Sin embargo, otros estudios han observado que las MSCs humanas son capaces de mantener el mismo perfil regulador tanto si están diferenciadas a condrocito como si no (Technau et al. 2011). Así mismo, estudios más recientes han observado que no se producen cambios en las proteínas de superficie celular tras la diferenciación condrogénica (Lee et al. 2016) y miogénica (Joo et al. 2014) de las MSCs. Sin embargo, otros estudios en la especie murina y humana han señalado que tras la diferenciación, las BM-MSCs podrían perder sus propiedades inmunomoduladoras y ya no serían capaces de evadir al sistema inmunitario ni *in vitro* ni *in vivo* (Lohan et al. 2014; Ryan et al. 2014).

En la especie murina se ha observado que la diferenciación condrogénica de las MSCs altera su inmunogenicidad al aumentar la expresión del *MHC* y la proliferación de los linfocitos B alogénicos, aumentando su susceptibilidad a ser lisadas por linfocitos T

citotóxicos aloespecíficos (Ryan et al. 2014). Además, la implantación *in vivo* de condrocitos alogénicos derivados de MSCs murinas encapsulados en hidrogeles de alginato también indujo una respuesta local y sistémica de memoria de las células T, con lo que sus capacidades inmunomoduladoras se redujeron respecto a las MSCs indiferenciadas (Ryan et al. 2014). En este estudio también observaron que tras la diferenciación condrogénica, las MSCs perdían su capacidad de inhibir la proliferación de las células T *in vitro*, lo que se vio asociado a la disminución de la secreción de NO y PGE2.

En las investigaciones que se están llevando a cabo actualmente con MSCs pre-diferenciadas en la especie equina, los autores coinciden en que es importante preservar una baja inmunogenicidad para poder realizar terapias alogénicas y evitar el reconocimiento inmunitario por parte del receptor (MacDonald y Barrett 2020; Depuydt et al. 2022).

Broeckx y colaboradores (2019) realizaron un estudio con 12 caballos a los que indujeron OA en la articulación metacarpofalángica. En este estudio se administraron de forma intraarticular ci-MSCs con baja expresión de MHC-I y negativas para MHC-II y la respuesta inflamatoria local fue muy ligera, similar a la producida por la administración de solución salina. Aunque en este estudio no se tuvieron en cuenta los haplotipos de los donantes y receptores, lo que podría haber aportado información sobre la compatibilidad /incompatibilidad genética, los autores señalan que la baja expresión del MHC observada en las ci-MSCs podría haber permitido a estas células evadir el sistema inmunitario.

En cambio, en otro estudio en la especie equina se observó que tanto la osteogénesis, la adipogénesis como la condrogénesis aumentaban la expresión del *MHC-I* y *MHC-II* (Barrachina et al. 2018b). Estos resultados indican que la diferenciación de las MSCs equinas podría tener un impacto en su inmunogenicidad y desempeñar un papel en la respuesta inmunitaria del receptor tras su administración (MacDonald y Barrett 2020).

En la especie equina, solo existe un estudio en el que se haya analizado la interacción de las ci-MSC alogénicas con los linfocitos de los animales receptores de estas células (Van Hecke et al. 2021). En este estudio retrospectivo se realizaron tres administraciones de ci-MSCs alogénicas a 10 caballos con diferentes patologías naturales y no se observó aumento de la inmunogenicidad tras la diferenciación condrogénica de las MSCs al cocultivarlas con linfocitos alogénicos. Cabe destacar que no observaron ningún tipo respuesta inmunitaria celular, ni efecto inmunomodulador ni inmunogénico de las ci-MSCs. Asimismo, en el análisis de los sueros llevado a cabo desde 5 días hasta un año y medio después de tratar a 10 caballos, no se observó presencia de anticuerpos alo-ci-MSC mediante citometría de flujo, indicando que tampoco se desarrollaba respuesta inmunitaria humoral (Van Hecke et al. 2021).

En un estudio reciente en la especie equina se evaluó si tras administraciones únicas y/o repetidas de tp-MSCs se inducía una respuesta inmunitaria celular y humoral en caballos que sufrían lesiones tendinosas (Depuydt et al. 2022). Los resultados mostraron que tras la administración de tp-MSCs, los animales no desarrollaban ni respuesta inmunitaria celular ni humoral tras el tratamiento intralesional único o repetido (dos consecutivos) con tp-MSCs alogénicas.

Cabe destacar que los cambios que se producen en el perfil secretor de las MSCs tras la diferenciación aún no se han estudiado en la especie equina. Aunque la bibliografía actual sobre la diferenciación de las MSCs presenta resultados contradictorios entre diferentes especies y estudios, se ha observado que la modificación del perfil secretor de las MSCs alogénicas una vez diferenciadas puede tener consecuencias negativas en su supervivencia *in vivo*, lo que potencialmente puede afectar a su efecto terapéutico (Barrachina 2017). Además, la respuesta de rechazo inmune podría tener efectos adversos en el individuo, y diversos estudios indican que un aumento en la expresión de moléculas inmunogénicas podría desencadenar esta reacción. Sin embargo, otros estudios han demostrado que no hay un incremento en la inmunogenicidad ni en la expresión de moléculas *MHC* y moléculas coestimuladoras (Barrachina 2017).

Es necesario realizar más investigaciones para comprender mejor las implicaciones de la diferenciación en las propiedades inmunológicas de las MSCs. Es fundamental evaluar si la respuesta inmunitaria hacia las MSCs alogénicas afecta su potencial terapéutico, así como comprender cómo la diferenciación influye en las propiedades paracrinas de las MSCs *in vivo* (Lohan et al. 2017).

10. INTERACCIÓN ENTRE EL SISTEMA INMUNITARIO DEL RECEPTOR Y LAS CÉLULAS MADRE MESENQUIMALES EQUINAS

Como se ha desarrollado anteriormente, puede generarse respuesta inmunitaria celular y humoral contra las MSCs alogénicas y en consecuencia desarrollarse mecanismos de memoria inmunológica, lo que podría limitar la posibilidad de administraciones repetidas de MSCs alogénicas en la especie equina (Schnabel et al. 2014; Kamm et al. 2021a). Se han llevado a cabo diferentes ensayos que permiten valorar cómo interaccionan las MSCs con el sistema inmunitario del receptor (Berglund et al. 2017b). Estos ensayos permiten conocer cómo las MSCs son capaces de regular el sistema inmunitario y qué estrategias se pueden utilizar para potenciar su capacidad inmunomoduladora o reducir su poder inmunogénico.

Sin embargo, es importante tener en cuenta que se trata de ensayos *in vitro* y por tanto, pueden no reflejar fielmente los procesos biológicos que ocurren *in vivo* (Colbath et al. 2020b). A pesar de ello y aunque los modelos *in vitro* no sean capaces de modelizar la complejidad del sistema inmunitario y la interacción célula-célula, pueden contribuir a

conocer de manera preliminar los complejos mecanismos de reconocimiento y/o evasión del sistema inmune.

10.1 Diálogos inmunológicos: la respuesta del receptor ante las células madre mesenquimales

Existen diferentes tipos de ensayos que permiten valorar la respuesta inmunitaria celular y humoral que se puede producir contra las MSCs. A continuación, se presentan los conocimientos actuales sobre las respuestas inmunitarias que se generan frente a las MSCs alogénicas, así como los principales ensayos que podrían emplearse en futuros estudios clínicos y preclínicos para identificar y evaluar de manera precisa las respuestas inmunitarias. Comprender cómo y en qué situaciones el sistema inmunológico del receptor responde a las MSCs alogénicas permitirá desarrollar estrategias que mejoren la eficacia y seguridad de estas células.

10.1.1 Explorando la respuesta inmune celular del receptor a las células madre mesenquimales

Los ensayos *in vitro* utilizados para evaluar la capacidad inmunomoduladora e inmunogénica de las MSCs consisten en sistemas de cocultivo de MSCs con células inmunitarias. Estos ensayos pueden realizarse con células mononucleares de sangre periférica (PBMCs, de sus siglas en ingles *peripheral blood mononuclear cells*) o con linfocitos de sangre periférica (PBLs, de sus siglas en inglés *peripheral blood lymphocytes*) (Carrade Holt et al. 2014; Paterson et al. 2014; Remacha et al. 2015; Ranera et al. 2016; Colbath et al. 2017a), pero también pueden llevarse a cabo utilizando otras células inmunitarias como los monocitos/macrófagos (Scharf et al. 2016; Berglund et al. 2017b; Cassano et al. 2018c, b). Estos enfoques experimentales tienen como objetivo común determinar los cambios producidos en la proliferación de células T y en los subconjuntos de linfocitos, así como determinar cuáles son las moléculas secretadas por las MSCs y cuáles por las células inmunitarias. Para llevar a cabo estas determinaciones se requieren técnicas como los ELISAs y la citometría de flujo.

10.1.1.1 Valoración de la capacidad inmunomoduladora de las células madre mesenquimales

Como se ha mencionado, la capacidad de las MSCs de inhibir *in vitro* la proliferación de células T activadas se considera una característica clave de sus propiedades inmunomoduladoras. Este mecanismo se debe principalmente a la secreción paracrina, pero el contacto directo célula-célula desempeña también un papel muy importante (Paterson et al. 2014; Kamm et al. 2021a).

Los ensayos inmunomoduladores *in vitro* pueden realizarse en sistemas de cocultivo directo, en los que las células inmunitarias activadas se añaden directamente a los pocillos que contienen MSCs, lo que permite tanto el contacto directo como las acciones paracrinas (Kamm et al. 2021a). Por otro lado, también pueden utilizarse sistemas

"*transwell*" cuando el objetivo es estudiar las acciones secretoras (Paterson et al. 2014), aunque algunos autores consideran que estos sistemas pueden no reflejar correctamente todas las interacciones celulares existentes (Gao et al. 2017; Luk et al. 2017). Algunos autores incluso han señalado que para modular la respuesta inmunitaria se podría prescindir de la actividad paracrina de las MSCs, e indican que el contacto de las células inmunes con las MSCs podría ser suficiente para que ejerzan algunos de sus efectos inmunomoduladores (Luk et al. 2016).

En estos sistemas de cocultivo, se permite que las MSCs y las células inmunitarias interactúen y se respondan mutuamente (enfoque bidireccional). En cambio, para dilucidar cómo las moléculas bioactivas secretadas por las MSCs pueden modificar las células inmunitarias activadas, éstas pueden ser expuestas al medio de las MSCs (Paterson et al. 2014) (enfoque unidireccional).

En los diferentes estudios en los que se ha valorado la capacidad inmunomoduladora de las MSCs equinas, se ha demostrado que tanto las MSCs alogénicas como las autólogas son capaces de inhibir la proliferación de células T en mayor o menor medida. Sin embargo, las pequeñas variaciones que existen entre las metodologías llevadas a cabo por los diferentes estudios podrían explicar los diferentes resultados observados. Estas diferencias en las metodologías se deben al tipo de mitógeno utilizado para estimular a los PBLs, el ratio MSCs:PBLs, los controles empleados y los días de cocultivo, entre otros. En la **Tabla 2** se presentan las principales diferencias en las metodologías empleadas para valorar la capacidad inmunomoduladora de las MSCs en la especie equina.

Tabla 2. Estudios en los que se ha valorado la capacidad inmunosupresora de las MSCs equinas (Valoración de la inhibición de la proliferación)

Autores	Fuente celular	Mitógeno	Estado MSCs	Ratios MSCs : Tcells	Días	Controles positivos	Controles negativos
Carrade et al. 2012	BM-MSCs, AT-MSCs, CB-MSCs y CT-MSCs	5 µg/mL PHA	Irradiadas 10 Gy	1:1 1:5 1:10	4	PBMCs + PHA	PBMCs solos
Aarya Hari et al. 2013	AT-MSCs	10 µg/mL PHA	5 µg/mL actinomycin D	1:100 1:500	3	PBMCs + PHA	PBMCs solos
Carrade et al. 2014	BM-MSCs, AT-MSCs, CB-MSCs y CT-MSCs	5 µg/mL PHA	Irradiadas 10 Gy	1:5	4	PBMCs + PHA	PBMCs solos
Paterson et al. 2014	BM-MSCs	5 µg/mL PHA	125 µg/mL Mitomicina C	1:10 1:66 1:200 1:400	3	PBMC + PHA	PBMCs solos
Williams et al. 2014	CB-MSCs	PBLs alogénicos irradiados	Irradiadas 20 Gy	1:10	6	PBLs + PBLs alogénicos irradiados	PBLs + PBLs autólogos irradiados
Remacha et al. 2015	AT-MSCs y BM-MSCs	PHA 20 µg/mL	-	1:1 1:10 1:20 1:50	3	PBMCs + PHA	PBMCs solos
Tessier et al. 2015	CB-MSCs	PBLs alogénicos irradiados	Irradiadas 20 Gy	1:10	6	PBLs + PBLs alogénicos irradiados	PBLs + PBLs autólogos irradiados
Ranera et al. 2016	BM-MSCs	PHA 2,5 µg/mL	-	1:10 1:50 1:100 1:250 1:500	4	PBMCs + PHA	PBMCs solos
Clark et al. 2016	BM-MSCs	5 µg/mL PHA	Irradiadas 10 Gy	1:5	4	PBMCs + PHA	PBMCs solos
Maumus et al. 2016	AT-MSCs	5 µg/mL PHA	-	1:1 1:10 1:100 1:1000	5	PBMCs + PHA	PBMC solos
Colbath et al. 2017	BM-MSCs	2,5 µg/mL ConA	-	1:10 1:100 1:1000	4	PBMCs + ConA	PBMC inactivados 1 µM hidroxurea
Cassano et al. 2018	BM-MSCs	5 µg/ml ConA	-	1:15	3	PBLs + ConA	PBLs solos
Longhini et al. 2019	PB-MSCs	2 µg/mL PHA	-	1:10	3	PBMCs + PHA	PBMCs solos
Lepage et al. 2019	CT-MSCs y CB-MSCs	ConA	Irradiadas 20 Gy	1:10	5	PBMCs + ConA	PBMC solos
Caffi et al. 2020	BM-MSCs	5 µg/mL ConA	-	1:1 1:4 1:16 1:32 1:64	4	PBMCs + ConA	PBMCs solos
Espinosa et al. 2020	BM-MSCs	5 µg/mL ConA	-	1:16	4	PBMCs + ConA	PBMCs solos
Van Hecke et al. 2021	ciMSCs	5 µg/mL ConA	-	1:10	4	PBMCs + ConA	PBMCs solos
Connard et al. 2021	BM-MSCs	5 µg/mL ConA	-	1:50	5	PBMCs + ConA	PBMCs solos
Even et al. 2022	BM-MSCs	5 µg/mL ConA	-	1:50	5	PBMCs + ConA	PBMCs solos

10.1.1.2 Valoración del potencial inmunogénico de las células madre mesenquimales

El cocultivo de MSCs con linfocitos inactivados, lo que se conoce como reacción mixta de linfocitos (MLRs, de sus siglas en inglés *mixed lymphocytes reactions*), y los ensayos citotóxicos, proporcionan una interesante aproximación para conocer la inmunogenicidad de las MSCs (Berglund et al. 2017b). En los MLRs unidireccionales, los linfocitos no se activan previamente y las MSCs actúan como estimuladoras para evaluar su potencial para inducir una respuesta proliferativa en los linfocitos (Schnabel et al. 2014). En los ensayos de citotoxicidad, los linfocitos T citotóxicos se generan *in vitro* en un MLR clásico (exposición a otra población de linfocitos con MHC incompatible) y luego se utilizan como células efectoras para probar su capacidad de lisar las MSCs que presentan el mismo MHC al que han sido expuestas (Berglund et al. 2017b).

A pesar de las limitaciones de estos enfoques para predecir la inmunogenicidad *in vivo*, se ha demostrado su utilidad en varios estudios con MSCs equinas, aunque los resultados observados tras los MLRs unidireccionales son contradictorios (Carrade Holt et al. 2014; Paterson et al. 2014; Schnabel et al. 2014; Colbath et al. 2017a). Algunos autores han señalado que tras el cocultivo de MSCs con PBLs no se observaba respuesta inmunitaria celular por parte de los PBLs alogénicos (Pigott et al. 2013; Paterson et al. 2014). En cambio, otros estudios han observado que tanto las MSCs autólogas como las alogénicas inducen niveles leves pero similares de activación linfocitaria *in vitro* (Colbath et al. 2017a; Depuydt et al. 2022). Asimismo, se ha observado que cuando aumenta la expresión de MHC-II en las MSCs equinas, se produce un aumento significativo en la proliferación de linfocitos respondedores (Schnabel et al. 2014).

La valoración de la capacidad inmunogénica también puede realizarse *ex vivo*, obteniendo PBLs de animales que han recibido MSCs alogénicas y utilizándolos como respondedores en un MLR unidireccional (Carrade et al. 2011) o como efectores en un ensayo de citotoxicidad (Isakova et al. 2014). El aumento de la respuesta proliferativa o citotóxica reflejaría la generación de memoria inmunológica celular y la capacidad del sistema inmunitario del receptor para rechazar las MSCs del donante, respectivamente. Además, la secreción de diferentes mediadores al reestimular los PBLs *ex vivo* puede reflejar la expansión de las células T efectoras o de memoria y podría ayudar a predecir la inmunogenicidad *in vivo* (Berglund et al. 2017b).

En la especie equina hay pocos estudios que hayan analizado la capacidad de las MSCs para inducir una respuesta inmunitaria sin la presencia de activadores externos (Schnabel et al. 2014; Colbath et al. 2017a; Kamm et al. 2021b). En la **Tabla 3** se presentan los estudios que han analizado la capacidad inmunogénica de las MSCs equinas y las principales diferencias entre las metodologías utilizadas.

Tabla 3. Estudios en los que se ha valorado el potencial inmunogénico de las MSCs equinas (Valoración de la proliferación).

Bibliografía	Fuente celular	Ratios MSCs : T cells	Días	Controles positivos	Controles negativos
Pigott et al. 2013	BM-MSCs	1:20	1, 3 y 6	-	PBMCs solos
Paterson et al. 2014	BM-MSCs y ESCs	1:2	5	PBMCs + PBMCs alogénicos inactivados con mitomicina-C (1:2)	PBMCs + PBMCs autólogos inactivados con mitomicina-C (1:2)
Schnabel et al. 2014	BM-MSCs	1:30 1:50	5	PBLs + PBLs MHC-incompatibles (irradiados)	PBLs + PBLs MHC-compatibles (irradiados)
Colbath et al. 2017	BM-MSCs	1:10 1:50 1:100 1:500 1:1000 1:2000	4	PBMCs + 2,5 µg/mL ConA	PBMC inactivados con 1 µM hidroxurea
Berglund et al. 2021	BM-MSCs	1:50	5	PBLs + PBLs MHC-incompatibles (irradiados)	PBLs + PBLs MHC-compatibles (irradiados)
Kamm et al. 2021	BM-MSCs	1:1 1:10 1:100	3 y 5	PBMCs + 2,5 µg/mL PWM	PBMC solos
Van Hecke et al. 2021	ciMSCs	1:10	4	PBMCs + 5 µg/mL ConA	PBMC solos
Depuydt et al. 2022	tpMSCs	1:10	4	PBMCs + 5 µg/mL ConA	PBMC solos

10.1.1.3 Herramientas para valorar la capacidad inmunomoduladora e inmunogénica de las células madre mesenquimales

Dada la complejidad del sistema inmunitario, junto con los ensayos *in vitro* y *ex vivo*, son necesarios más experimentos *in vivo* para dilucidar cómo las MSCs modulan las células inmunitarias en una lesión o en qué medida provocan una respuesta inmunitaria en un escenario alogénico (Consentius et al. 2015).

La evaluación de las respuestas celulares sistémicas (Kol et al. 2015) y locales (Barrachina et al. 2018a; Colbath et al. 2020b), incluido el estudio de los cambios en las poblaciones de células inmunitarias y las subpoblaciones linfocitarias (Van Hecke et al. 2021; Depuydt et al. 2022), también proporcionaría información sobre la capacidad de las MSCs para regularlas o activarlas. Estas determinaciones requieren técnicas como los ELISAs y la citometría de flujo, pero el análisis de las propiedades inmunológicas en las MSCs equinas presenta una dificultad añadida, que es la falta de reactivos especie-específicos (Guest et al. 2022). Este es un problema común a muchas especies veterinarias como el perro, el cerdo, la cabra o la oveja, a pesar de su creciente valor como pacientes y modelos de enfermedades humanas (Hunka et al. 2020).

Cuando se valora la interacción de las MSCs y las células inmunitarias, los subconjuntos linfocitarios son los que presentan mayor interés (Hillmann et al. 2019). Como se ha desarrollado anteriormente, los linfocitos pueden dividirse en dos tipos principales: T y B. Además, pueden distinguirse dos tipos principales de linfocitos T: T citotóxicos y T colaboradores. Dentro de los linfocitos T colaboradores, los linfocitos Treg son una subpoblación de especial interés en lo que respecta a la regulación ejercida por las MSCs (Kamm et al. 2021b). Se presentan en la **Tabla 4** los anticuerpos de citometría de flujo disponibles actualmente para estudiar las poblaciones linfocitarias equinas.

Tabla 4. Resumen de los anticuerpos disponibles para citometría de flujo de linfocitos equinos en estudios de MSCs.

Tipo celular	Marcador	Clon	Tipo e Isotipo	Especie hospedadora	Reactividad	Referencia y proveedor	Bibliografía
Linfocitos T	CD3	CD3-12	Monoclonal IgG1	Rata	Anti-humano	MCA1477 - Bio-Rad	Colbath et al. 2017 Hillmann et al. 2019 Pfeiffenberger et al. 2019
	CD3	UC F6G-3.3	Monoclonal IgG1	Ratón	Anti-caballo	University of California Davis, (USA)	Berglund et al. 2021 Cassano et al. 2018 Schnabel et al. 2014 Van Hecke et al. 2021
	CD5	CVS5	Monoclonal IgG1	Ratón	Anti-caballo	MCA1079 - Bio-Rad	Colbath et al. 2017
Células T citotóxicas	CD8	CSV21	Monoclonal IgG2a	Ratón	Anti-caballo	MCA1080 - Bio-Rad	Hillmann et al. 2019 Pfeiffenberger et al. 2019 Ranera et al. 2016
		CVS8	Monoclonal IgG1	Ratón	Anti-caballo	MCA2385 - Bio-Rad	Kamm et al. 2021 Tomlinson et al. 2018
Células T colaboradoras	CD4	CVS4	Monoclonal IgG1	Ratón	Anti-caballo	MCA1078 - Bio-Rad	Hillmann et al. 2019 Kamm et al. 2021 Pfeiffenberger et al. 2019 Pigott et al. 2013 Ranera et al. 2016 Tessier et al. 2015
		HB61A		Ratón	Anti-caballo	VMRD	Kol et al. 2015
Células T reguladoras	CD25	-	Policlonal	Cabra	Anti-humano	AF-223 - R&D Systems	Kamm et al. 2021 Kol et al. 2015
	Foxp3	PCH101	Monoclonal IgG2a	Rata	Anti-humano	14-4776 - eBioscience, Thermofisher	Kol et al. 2015
		FJK-16s	Monoclonal IgG2a	Rata	Anti-ratón	14-5773-80 - eBioscience, Thermofisher	Hillmann et al. 2019
Linfocitos B	Pan-Ig	CVS36	Monoclonal IgG1	Ratón	Anti-caballo	MCA1899 - Bio-Rad	Kol et al. 2015
	CD21	CA2.1D6	Monoclonal IgG1	Ratón	Anti-perro	MA5-16610 - Thermofisher	Kamm et al. 2021
	CD21	B-ly4	Monoclonal IgG1	Ratón	Anti-humano	561357 - BD Pharmigen	Kol et al. 2015

Tras la realización de ensayos basados en los cocultivos de MSCs y linfocitos, resulta de gran interés estudiar los cambios en las moléculas producidas por los linfocitos en respuesta a las MSCs. La dos moléculas más estudiadas tras el cocultivos de MSCs y linfocitos en la especie equina son IFNɣ (Carrade et al. 2012; Pigott et al. 2013; Carrade Holt et al. 2014; Paterson et al. 2014; MacDonald 2015; Clark et al. 2016; Colbath et al. 2017a) y TNFα (Carrade et al. 2012; Carrade Holt et al. 2014; Paterson et al. 2014; MacDonald 2015; Clark et al. 2016; Scharf et al. 2016; Kornicka et al. 2018).

10.1.2 Explorando la respuesta inmune humoral del receptor ante las células madre mesenquimales

Los avances en el conocimiento de las propiedades inmunes de las MSCs hacen necesario un estudio más detallado de sus implicaciones en la terapia celular alogénica, ya que el reconocimiento de las MSCs por el sistema inmune del receptor podría conllevar una pérdida de su eficacia y posibles efectos adversos para el receptor (Berglund et al. 2017b).

En la especie humana, mientras que algunos estudios han evidenciado la inducción de aloinmunidad (Ankrum et al. 2014; Lohan et al. 2017), otros han mostrado una aplicación segura y efectiva de las MSCs alogénicas (Hatsushika et al. 2014; Vega et al. 2015), lo que sugiere que bajo ciertas condiciones éstas son capaces de evadir al sistema inmunitario y desarrollar sus funciones. Una cuestión estrechamente relacionada con el

reconocimiento alogénico de las MSCs es la compatibilidad para el MHC entre donante y receptor. El aloreconocimiento de las moléculas de MHC incompatibles presentes en la superficie de las MSCs del donante y la consiguiente respuesta de rechazo frente a estas células conllevaría la disminución de su efecto terapéutico y aumentaría el riesgo de reacciones adversas (Huang et al. 2016). Así mismo, además de poder inducir una respuesta inmunitaria de tipo celular, las MSCs alogénicas también pueden inducir la producción de anticuerpos aloespecíficos (Lohan et al. 2017), lo que podría obstaculizar el uso de dosis repetidas de MSCs alogénicas (Hatsushika et al. 2014).

El desarrollo de respuesta inmunitaria humoral y la producción de aloanticuerpos tras la administración de MSCs alogénicas ha sido descrita en humanos, macacos (Isakova et al. 2014; García-Sancho et al. 2017; Lohan et al. 2017) y también en el caballo (Pezzanite et al. 2015; Berglund y Schnabel 2017; Rowland et al. 2021).

En el primer estudio que analizó la respuesta humoral en la especie equina, se observó que tras la administración intradérmica de MSCs MHC-incompatibles se producía una reacción inflamatoria local y anticuerpos con capacidad citotóxica frente a los leucocitos del mismo MHC que las MSCs que habían sido administradas, aunque la respuesta humoral fue variable entre individuos (Pezzanite et al. 2015). En una continuación de este estudio, se observó que los anticuerpos anti-MHC generados después de la exposición a MSCs con MHC-incompatible eran citotóxicos contra las MSCs del mismo tipo de MHC que el del donante, pero no tenían ese efecto cuando se administraban MSCs alogénicas MHC-compatibles. La ausencia de desarrollo de anticuerpos citotóxicos contra las MSCs del mismo tipo de MHC indicaría que tanto la compatibilidad para el MHC como la respuesta inmunitaria humoral frente a las MSCs deberían considerarse en la terapia alogénica (Berglund y Schnabel 2017).

En otro estudio en el que se observó que las MSCs podían generar respuesta humoral aloespecífica en la especie equina (Owens et al. 2016), se detectó producción de anticuerpos anti-MSCs en el 40% de los caballos que recibieron MSCs (BM-MSCs o AT-MSCs) alogénicas por diferentes vías (intravenosa, intraarterial, intratendinosa e intraocular).

Cabe destacar que el significado clínico de estos hallazgos y de su efecto sobre la supervivencia de las MSCs *in vivo* sigue sin estar claro. Además, debe añadirse a esta consideración que el ambiente inflamatorio que encuentren las MSCs al ser administradas puede aumentar o inducir la expresión de las moléculas *MHC-I* y *II*, así como también puede suceder por efecto de la diferenciación de las MSCs (Remacha et al. 2015).

Como se ha mencionado anteriormente, la producción de anticuerpos tras la administración de MSCs diferenciadas a diferentes tipos celulares ha sido investigada recientemente en la especie equina (Van Hecke et al. 2021; Depuydt et al. 2022). Los resultados de ambos estudios indicaron que ni las ci-MSCs ni las tp-MSCs inducían la

producción de anticuerpos anti-MSCs tras administraciones repetidas. Sin embargo, Depuydt y colaboradores (2022) sí detectaron altos niveles anticuerpos anti-albúmina de suero bovino (BSA, de sus siglas en inglés *bovine serum albumin*) en varios de los animales.

El desarrollo de anticuerpos anti-BSA podría ser debido a que la mayoría de las vacunas contienen BSA, por lo que la revacunación equina contra la gripe, rinoneumonitis y tétanos, puede conducir a la inducción de una respuesta inmunitaria a proteínas xenogénicas presentes en estas vacunas (Depuydt et al. 2022). El principal problema de la BSA es que es el componente principal del FBS (aproximadamente 2,5 mg/mL en FBS) (Francis 2010). Durante el cultivo, las MSCs pueden internalizar proteínas xenogénicas presentes en el FBS y en consecuencia, cuando las MSCs son administradas, pueden inducir una respuesta inmunitaria y generarse anticuerpos contra estos compuestos del FBS (Joswig et al. 2017).

En el estudio de Joswig y colaboradores (2017) se demostró que tanto las inyecciones intraarticulares repetidas de MSCs autólogas con FBS como las de MSCs alogénicas sin FBS provocaban una respuesta clínica adversa tras la segunda inyección. Además, en un estudio posterior del mismo grupo se observó que cuando la inflamación había sido inducida por el FBS, había una fuerte correlación entre la activación de linfocitos *in vitro* con la respuesta adversa *in vivo*, lo que confirmaba que los MLR *in vitro* reflejan la actividad inmunomoduladora de las MSC *in vivo* (Rowland et al. 2018) .

Es importante destacar la importancia de agentes xenogénicos como el FBS en los ensayos *in vitro* cuando se valora la respuesta inmunitaria humoral, ya que, si las células tienen antígenos del FBS y en el suero de los receptores hay anticuerpos frente a esos antígenos, puede haber citotoxicidad contra las células sin que sea problema de la compatibilidad para el MHC (Pezzanite et al. 2015). Para conseguir que las MSCs estén libres de antígenos xenogénicos se han propuesto protocolos de depleción progresiva del FBS y su sustitución por suero equino los días anteriores al experimento o administración (Berglund y Schnabel 2017).

La respuesta inmunitaria desarrollada contra las MSC alogénicas equinas, tanto compatibles como incompatibles para el MHC y los factores que influyen en ella, deben estudiarse en profundidad para desarrollar terapias celulares más eficaces y seguras (Pezzanite et al. 2015; Berglund y Schnabel 2017; Rowland et al. 2021).

10.2 Diálogos inmunológicos: la respuesta de las células madre mesenquimales ante el sistema inmunitario del receptor

Como se ha explicado en apartados anteriores, en la especie equina, existen varios trabajos que han analizado cómo cambia el perfil inmune y la secreción de las moléculas inmunomoduladoras e inmunogénicas de las MSCs *in vitro* tras ser expuestas a distintos estímulos y tras ser cocultivadas con PBLs. Sin embargo, hay muy pocos trabajos acerca

de cómo cambian estas propiedades tras la administración *in vivo* de MSCs por la dificultad que supone recuperarlas una vez administradas al animal.

Diferentes autores han valorado a dónde se dirigen las MSCs equinas tras ser administradas y los estudios realizados señalan que su ubicación final depende en gran medida de la vía de administración, pero también del tipo de lesiones a tratar (Burk et al. 2016; Geburek et al. 2016). Sin embargo, qué hacen o qué les ocurre a las MSCs equinas una vez administradas, no ha sido estudiado en profundidad.

Estudios realizados en otras especies han abordado este tema valorando la viabilidad y la proliferación de las MSCs tras su administración. Por ejemplo, en un modelo de OA ovina en el que se marcaron las MSCs para valorar su distribución durante 12 semanas, la evaluación inmunohistológica mostró que las MSCs administradas desarrollaban un fenotipo condrogénico (Delling et al. 2015). En un modelo de OA en rata tratada con MSCs humanas se demostró que las MSCs humanas eran capaces de integrarse y proliferar en el menisco y el cartílago de la articulación de las ratas (Li et al. 2016).

Sin embargo, como se ha mencionado anteriormente, el mecanismo inmunomodulador ejercido por las MSCs puede no requerir la supervivencia a largo plazo de las MSCs (Mund et al. 2020; Kamm et al. 2021a). De esta manera, los efectos inmunomoduladores de las MSCs se pueden seguir viendo a largo plazo a través de la apoptosis y la fagocitosis por parte de las células inmunitarias, aunque las MSCs mueran (Voga et al. 2020).

10.2.1 Valoración de las moléculas inmunomoduladoras e inmunogénicas secretadas por las células madre mesenquimales

En la evaluación *in vivo* de las propiedades inmunitarias de las MSCs, diferentes autores coinciden en que se debería incluir la medición de los mediadores secretados por las MSCs o biomarcadores específicos a nivel sistémico (Kol et al. 2015) o en fluidos biológicos (Colbath et al. 2020a) que reflejen la regulación del entorno de la lesión. Por ello, conocer qué moléculas han secretado las MSCs en el lugar de la lesión resulta de gran interés para potenciar sus efectos terapéuticos.

En los estudios en los que se ha valorado la capacidad de las MSCs de inhibir la proliferación linfocitaria, las técnicas ELISAs han permitido conocer cuáles son las implicaciones de las diferentes moléculas paracrinas secretadas por las MSCs. Por otro lado, en los ensayos de inmunogenicidad cuyo objetivo es estudiar si las MSCs pueden inducir una respuesta inmunitaria y, por tanto, estimular la proliferación de los linfocitos, el estudio de las moléculas secretadas por las MSCs para evitar esta proliferación puede aportar mucha información. En la **Tabla 5** se presentan las principales moléculas inmunomoduladoras presentes en el sobrenadante de los cocultivos y en el líquido sinovial.

Tabla 5. Principales moléculas analizadas mediante ELISAs en la especie equina.

Moléculas	Bibliografía cocultivos	Bibliografía líquido sinovial
PGE2	Carrade et al. 2012, 2014, Clark et al. 2016, Scharf et al. 2016 Colbath et al. 2017, Berglund et al. 2021	Maninchedda et al. 2015, Bertoni et al. 2019 Broeckx et al. 2019, Colbath et al. 2020
IL6	Carrade et al. 2012, 2014, Pigott et al. 2013, Paterson et al. 2014 MacDonald et al. 2015, Clark et al. 2016, Kornicka et al. 2018	Broeckx et al. 2019, Rowland et al. 2021
IL10	Pigott et al. 2013, Carrade et al. 2014, Paterson et al. 2014 MacDonald et al. 2015, Clark et al. 2016, Scharf et al. 2016 Kornicka et al. 2018, Longhini et al. 2019	Broeckx et al. 2019, Rowland et al. 2021
TGFβ1	Carrade et al. 2012, Colbath et al. 2017, Berglund et al. 2021	
IFNγ	Carrade et al. 2012, 2014, Pigott et al. 2013, Paterson et al. 2014 MacDonald, 2015, Clark et al. 2016, Colbath et al. 2017	Broeckx et al. 2019, Rowland et al. 2021
TNFα	Carrade et al. 2012, 2014, Paterson et al. 2014, MacDonald et al. 2015 Clark et al. 2016, Scharf et al. 2016, Kornicka et al. 2018	Broeckx et al. 2019, Rowland et al. 2021

Cabe destacar que para poder demostrar que son las MSCs, y no los PBLs, la principal fuente de estas moléculas es necesario que se utilicen inhibidores de estas moléculas para valorar su influencia en los cambios linfocitarios. Mientras que hay un gran consenso en relación a que las MSCs son la principal fuente de PGE2, existe cierta controversia sobre TGFβ1 (Berglund et al. 2021). Se ha sugerido la utilización de marcajes intracelulares o análisis de la expresión génica en las MSCs y en los PBLs para dilucidar cuál es la principal fuente.

Como se ha mencionado anteriormente, es muy importante identificar cómo puede cambiar la expresión de las moléculas en la superficie de las MSCs equinas cuando son expuestas a diferentes estímulos *in vitro* e *in vivo*. Schnabel et al. (2014) señalaban que se debe tenerse en cuenta que incluso las MSCs negativas para MHC-II podrían expresarlo si se administraban en una zona de inflamación activa, como demostraron mediante la estimulación de las MSCs *in vitro* con IFNɣ.

En un estudio reciente se valoró como las expresión del MHC-II en las MSCs equinas cambiaba debido a la exposición a un entorno proinflamatorio, que reflejaría la situación de una articulación inflamada (Hill et al. 2017). Se comparó la expresión del MHC-II tanto por citometría de flujo como por expresión génica y en ambos casos no se observó aumento de la expresión de MHC-II. Además, los resultados obtenidos indicaron que los ensayos mediante RT-qPCR eran comparables a los obtenidos por citometría de flujo, y que, por tanto, los resultados de RT-qPCR podían utilizarse como una medida precisa de la expresión de la superficie celular del MHC-II (Hill et al. 2017).

En la especie equina actualmente solo hay un estudio de modelo de OA en el que se haya analizado la expresión génica de las diferentes moléculas inmunomoduladoras e inmunogénicas en el cartílago y membrana sinovial tras la administración repetida de MSCs alogénicas (Barrachina et al. 2018a).

Con el fin de comprender mejor el comportamiento de las MSCs in vivo, una estrategia prometedora consiste en administrar las MSCs utilizando materiales biocompatibles que permitan su colocación en el sitio de la lesión (Santos et al. 2019). Estos materiales no solo protegerán y asegurarán la viabilidad de las MSCs una vez administradas, sino que también les permitirán conservar sus actividades paracrinas (Colbath et al. 2017b).

Además, la utilización de estos materiales ofrece la posibilidad de recuperarlos tras ser administrados para estudiar a diferentes tiempos cómo ha cambio el perfil inmune de las MSCs (Ceccaldi et al. 2012; Ryan et al. 2014). A continuación, se describen materiales específicos utilizados para la encapsulación de las MSCs para su aplicación *in vivo*.

10.2.1.1 Encapsulación de las células madre mesenquimales

El uso de soportes para vehiculizar las MSCs ha sido estudiado como estrategia para el tratamiento de defectos focales articulares tanto en la especie equina como en otras especies (Maihöfer et al. 2021; Ribitsch et al. 2021).

En un modelo de defecto osteocondral en rodillas de conejos se valoró el efecto de la administración de CB-MSCs humanas encapsuladas en implantes de ácido hialurónico (HA). Observaron que la combinación de las CB-MSCs y HA resultó en un tejido de reparación de mejor calidad y cantidad en comparación a solo la administración del implante de HA. También observaron que la arquitectura celular y la disposición del colágeno transcurridas 16 semanas eran similares a las del tejido cartilaginoso articular sano circundante (Park et al. 2017).

Utilizando el modelo equino para valorar la cicatrización focal del cartílago, las MSCs han sido administradas en diferentes soportes con el objetivo de retener las células dentro de los lugares de la lesión (Colbath et al. 2017b).

En un modelo de defecto troclear medial del fémur en la especie equina, se utilizaron células progenitoras del cartílago articular, que son una subpoblación de células multipotentes con potencial condrogénico. Se comparó el efecto de la administración de implantes de fibrina, células condroprogenitoras autólogas encapsuladas en fibrina y células condroprogenitoras alogénicas encapsuladas en fibrina (Frisbie et al. 2015). En las artroscopias y en las histologías realizadas observaron que los defectos de los animales tratados con células autólogas presentaban un tejido de reparación significativamente mejor en comparación con los defectos de los caballos tratados sólo con fibrina. En cambio, la calidad del tejido en el grupo que recibió MSCs alogénicas fue menor que la del grupo de sólo fibrina (Frisbie et al. 2015). Sin embargo, en otro estudio en el que se utilizaron soportes de fibrina generados a partir de plaquetas, no observaron diferencias entre el uso del soporte por sí solo o con MSCs autólogas embebidas para mejorar la reparación del cartílago equino (Goodrich et al. 2016).

En general las MSCs encapsuladas en soportes y/o combinadas con el uso de cirugía han mostrado buenos resultados en lesiones focales del cartílago equino, aunque los resultados observados dependen en gran medida del material utilizado. Se debe seguir investigando con el objetivo de aclarar el efecto de los implantes en la viabilidad, calidad del tejido reparado, así como los soportes pueden alterar la capacidad de migración, las propiedades paracrinas y la interacción con el entorno local de la articulación (Colbath et al. 2017b).

10.2.1.1.1 Tipos de materiales para la encapsulación celular

Actualmente existen diferentes polímeros de origen natural que han sido estudiados por su potencial para la encapsulación de células (**Tabla 6**). Existen diferentes mecanismos para su fabricación ya que pueden gelificarse mediante estímulos externos (temperatura, fuerza iónica) o mediante reticulación permanente. Además, algunos de estos materiales son biodegradables y otros no (Gasperini et al. 2014; Liu et al. 2022).

Tabla 6. Resumen de las principales propiedades de los materiales más utilizados para la encapsulación celular (Gasperini et al. 2014).

Nombre	Fuente	Tipo	Gelificación	Biodegradable
Agarosa	algas marinas	polisacárido	térmica	no
Carragenina	algas marinas	polisacárido	térmica/ionotrópica	no
Alginato	algas marinas	polisacárido	ionotrópica	no
Chitosán	crustáceos	polisacárido	ionotrópica	sí
Goma gellan	algas marinas	polisacárido	térmica/ionotrópica	incierta
Ácido hialurónico	matriz extracelular	polisacárido	térmica	sí
Colágeno	matriz extracelular	proteína	térmica/cambio pH	sí
Gelatina	matriz extracelular/colágeno	proteína	térmica	sí
Elastina	matriz extracelular/sintético	proteína	térmica	sí
Fibrina	sangre	proteína	enzimática	sí
Fibra de seda	seda	proteína	térmica/cambio pH	variable

De entre los diferentes materiales, se ha descrito que los hidrogeles son los materiales cuyas características proporcionan un entorno tridimensional más similar al de las matrices extracelulares naturales de las células (Moshtagh et al. 2018). Además, una de las principales ventajas de los hidrogeles frente a los biomateriales más sólidos es que, por lo general, se les puede dar forma para que se adapten al defecto y, algunos incluso permiten la gelificación in situ, lo que puede dar lugar a un relleno óptimo de un defecto de cartílago (Hernández et al. 2010; Andersen et al. 2015).

La encapsulación de las MSCs en diferentes hidrogeles (naturales o sintéticos) ha sido descrita como una forma de administración que a su vez proporciona la estimulación mecánica y molecular necesarias para la restauración morfofuncional del cartílago articular dañado (Santos et al. 2018). Entre los diferentes hidrogeles se encuentran el alginato de sodio, quitosano, ácido hialurónico, condroitín sulfato, colágeno, fibrina, gelatina, poliácido láctico-co-glicólico (PLGA) y polietilenglicol, entre otros (Liu et al. 2022). Sin embargo, el alginato parece ser el biomaterial más prometedor en los enfoques que pretenden mejorar la reparación del cartílago articular (Maihöfer et al. 2021).

10.2.1.1.1.1 Hidrogeles de alginato

La utilización de hidrogeles de alginato está siendo cada vez más estudiada para la encapsulación de MSCs al ser uno de los polímeros más adecuados para la fabricación de cápsulas debido a su fácil gelificación, su adecuada estabilidad mecánica y su biocompatibilidad (Moshtagh et al. 2018; Liu et al. 2022). En la **Figura 10** se presentan los estudios llevados a cabo con alginato en los últimos años.

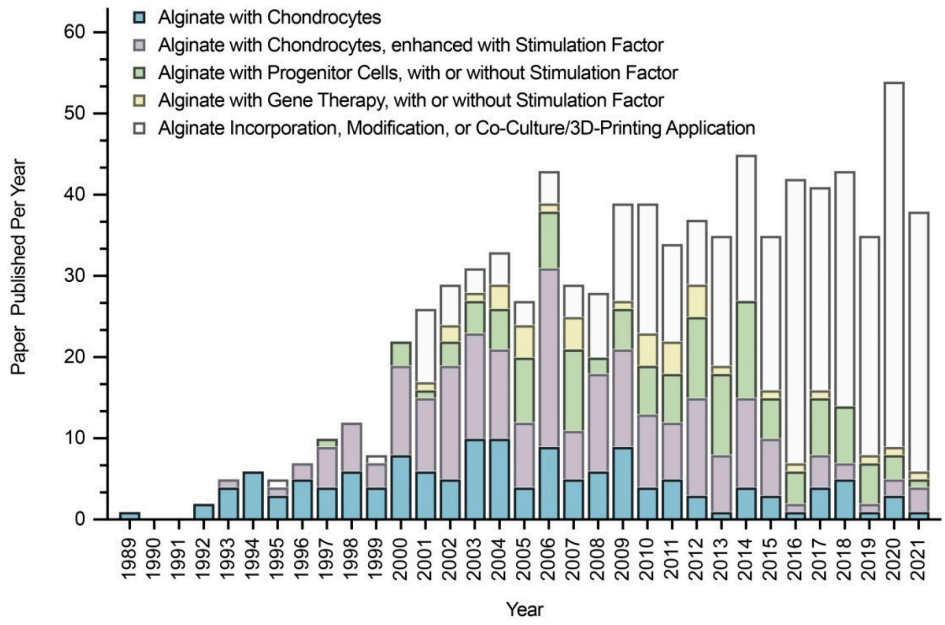


Figura 10. Utilización del alginato para la regeneración del cartílago (Liu et al. 2022).

El alginato es un polisacárido aniónico e hidrófilo de origen natural que proviene de las algas marinas pardas y está compuesto por copolímeros lineales no ramificados de ácido β-D-manurónico (M) y ácido α-L-gulurónico (G) enlazados 1→4 (Liu et al. 2022).

Su estructura molecular viene definida por cómo se encuentren distribuidas estas regiones: regiones compuestas por una sola unidad de monómeros G o M consecutivos (-GGG- o -MMM-) o bien por regiones en las que los monómeros se aproximan a una secuencia alternante (-MGMG-) (**Figura 11**) (Andersen et al. 2015; Liu et al. 2022).

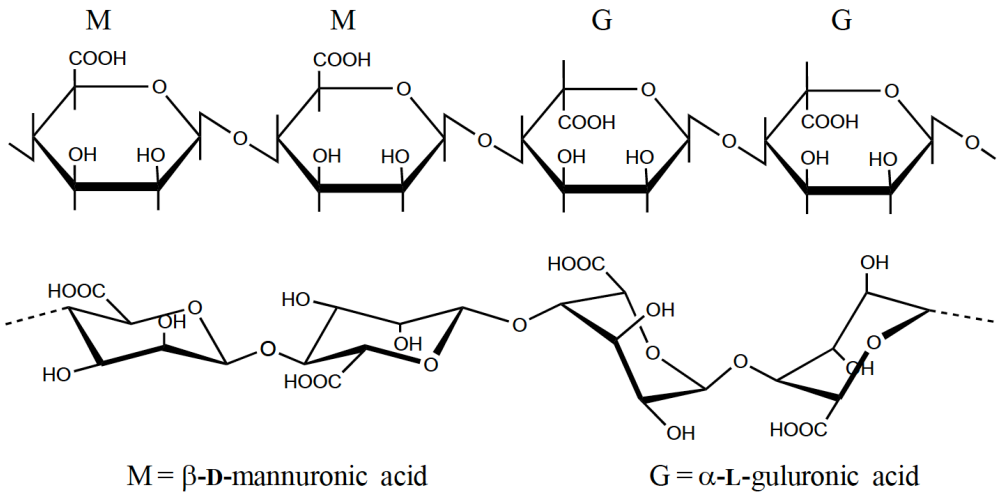


Figura 11. Estructura del hidrogel de alginato (Andersen et al. 2015).

El contenido de G en la mayoría de los alginatos se encuentra entre el 30% y el 70% y las regiones varían considerablemente de longitud y distribución dependiendo de qué especie y parte del alga se extrae el alginato. Además, la composición química y la

distribución de las regiones en la molécula de alginato desempeñan un papel importante en su capacidad para formar geles iónicos (Andersen et al. 2015; Liu et al. 2022).

Los cationes multivalentes, como el calcio, al enlazarse con los grupos de ácido carboxílico de los bloques G de las cadenas de alginato forman una red de gel flexible que puede utilizarse en el proceso de encapsulación celular (Moshtagh et al. 2018).

10.2.1.1.1.1.1 Propiedades de los hidrogeles de alginato

El alginato es un compuesto biocompatible cuya porosidad y viscosidad pueden modificarse. Estas propiedades viscoelásticas pueden ajustarse cambiando el peso molecular del polímero, la composición (relación G/M), y/o la concentración utilizada (Bidarra y Barrias 2019).

El alginato puede ser de baja (comúnmente utilizada), media o alta viscosidad (Heiligenstein et al. 2011), y estudios recientes indican que la viabilidad de las células encapsuladas es mayor al utilizar alginato de baja viscosidad (Liu et al. 2022).

La proporción G/M debe tenerse en cuenta dependiendo de la aplicabilidad que se vaya a dar a las cápsulas, ya que, como se ha mencionado, estas moléculas juegan un papel muy importante en la capacidad de formar geles iónicos (Andersen et al. 2015).

En un estudio en el que compararon alginatos con mayor proporción de guluronato con alginatos con mayor proporción de munuronato concluyeron que los implantes de alginato de tipo M eran más difíciles de manipular debido a su falta de cohesión y menor rigidez en comparación con los implantes de alginato de tipo G (Ceccaldi et al. 2012). Además, también se realizaron diferentes valoraciones *in vivo* colocando estos implantes en la superficie del ventrículo izquierdo en ratones y observaron que los implantes tipo G mantenían su forma y morfología transcurridos 28 días desde su colocación. Asimismo, los alginatos con una relación G/M alta proporcionan una menor resistencia a la tracción (Ceccaldi et al. 2012; Liu et al. 2022), ofreciendo un sustrato contra el que se puede ejercer tracción y, por tanto, que facilita la adhesión celular y permite una óptima proliferación celular (Heiligenstein et al. 2011; Liu et al. 2022).

Cabe destacar que la proporción G/M también puede repercutir en la actividad de los condrocitos tras la encapsulación, influyendo en su proliferación, adhesión celular, colonización, migración, difusión de los nutrientes (Zhang et al. 2015b; Liu et al. 2022).

En relación a la concentración a utilizar para la formación de implantes, se han descrito concentraciones de alginato de 0,5-4% (p/v) y concentraciones de Ca^{2+} de 15-144 mM para la investigación en regeneración del cartílago (Jang et al. 2014; Swioklo et al. 2017). Aunque la mayoría de las investigaciones se desarrollan con cápsulas de alginato al 1-1,5% (p/v) y Ca^{2+} a 102 mM (Samuel et al. 2018; Liu et al. 2022).

10.2.1.1.1.1.2 Interacciones entre hidrogeles de alginato y células madre mesenquimales: un vínculo prometedor en la investigación

Gracias a las características que presenta, el uso de alginato de sodio se ha utilizado en diferentes trabajos para encapsular células, como si se tratara de una matriz extracelular sintética (Ho et al. 2022), y resulta una buena opción para encapsular en ellos las MSCs y así poder recuperarlas para estudiarlas con detalle (Santos et al. 2019).

Cabe destacar que las células de los mamíferos no pueden interactuar con los polisacáridos del alginato, lo cual es crucial para que el hidrogel se mantenga íntegro y las MSCs permanezcan en su interior. Además, permite el paso de nutrientes y metabolitos del organismo a las células del interior del gel y viceversa, lo que es idóneo para estudiar la interacción entre la respuesta inmunitaria del animal y las MSCs (Watts et al. 2013).

Se ha demostrado que las MSCs humanas encapsuladas en geles de alginato, transcurridos 30 días de cultivo *in vitro*, pueden ser estimuladas con citoquinas inflamatorias y ser capaces de secretar factores inmunomoduladores y tróficos así como inhibir la proliferación de células T (Leijs et al. 2017). Además, diferentes autores coinciden en que la encapsulación de las MSCs en geles de alginato ya sean MSCs indiferenciadas o diferenciadas *ex vivo*, puede ser necesaria para retener las células en el lugar de la lesión y para protegerlas de la respuesta inmunitaria alogénica *in vivo* (Ryan et al. 2014).

En otro estudio realizado en MSCs humanas en el que compararon la viabilidad y el potencial condrogénico de las MSCs en tres tipos de hidrogeles (alginato, fibrina y hialuronano-poli(N)-isopropilacrilamida), concluyeron que las MSCs encapsuladas en geles de alginato fueron las que mostraron mayor capacidad condrogénica tanto *in vitro* como *in vivo* tras ser implantados por vía subcutánea en ratones desnudos durante 12 semanas (de Vries-van Melle et al. 2014).

En la especie equina hay pocos trabajos en los que se hayan encapsulado MSCs en hidrogeles de alginato. En un estudio en el que se compararon diferentes sistemas 3D para inducir la condrogénesis de las MSCs (cultivo en pellet clásico, agarosa, alginato y alginato con fibrina), en todos los tipos de soporte se pudo corroborar la diferenciación condrogénica de las MSCs equinas (Watts et al. 2013). Además, observaron que los mejores sistemas de diferenciación condrogénica en esta especie eran los basados en alginato, tanto en términos de morfología como de expresión génica.

En otro estudio cuyo objetivo fue cultivar y evaluar la viabilidad y capacidad de diferenciación de MSCs encapsuladas en soportes tridimensionales de hidrogel de alginato, observaron que a los 21 días la diferenciación condrogénica era evidente y que además las MSCs sobrevivían 28 días dentro de los geles (tiempo final del estudio) (Santos et al. 2018). Concluyeron que los hidrogeles de alginato mantenían las MSCs vivas y además estimulaban la diferenciación de las MSCs a condrocitos cuando se

aportaban los suplementos necesarios. Sin embargo, destacaron que eran necesarios más estudios *in vivo* para validar el potencial terapéutico de la aplicación intraarticular de las MSCs encapsuladas, aunque los resultados obtenidos hasta entonces eran prometedores.

En un estudio reciente llevado a cabo con AT-MSCs de mulas se evaluó el efecto de la encapsulación con alginato a lo largo de 7 días. En este estudio se analizó la morfología de la cápsula y la viabilidad celular, el inmunofenotipo y la liberación tras la encapsulación (de Souza et al. 2021). Mediante microscopia electrónica observaron que todas las cápsulas presentaban poros circulares e irregulares en la superficie del hidrogel en los que las MSCs seguían presentes y vivas. Los autores señalaban que la alta viabilidad de las MSCs observada durante los siguientes 7 días se debía a la gran capacidad de absorción de líquidos y difusión de nutrientes que mostraron las cápsulas. Además, a las 24 horas y 7 días se comparó el inmunofenotipo de MSCs sin encapsular y encapsuladas tras disolver los alginatos. La citometría de flujo mostró que la encapsulación no había alterado su inmunofenotipo ya que las MSCs que habían sido encapsuladas seguían siendo positivas a CD90 y negativas a CD11b, CD45 y MHC-II. En este estudio también observaron que a partir de las 72 horas algunas MSCs migraban desde el alginato a la placa de cultivo volviendo a pegarse y expandirse, siendo más evidente a los 7 días. Los autores concluyeron que la encapsulación de las MSCs en hidrogeles de alginato era una técnica viable que no comprometía la viabilidad de las células y les permitía preservar su estado indiferenciado, convirtiéndose en una opción muy relevante para futuros estudios de sistemas de cultivo tridimensionales y la administración de agentes bioactivos *in vivo* (de Souza et al. 2021).

En relación a la administración *in vivo*, en un caso clínico administraron AT-MSCs tratadas con resveratrol encapsuladas en alginato a una yegua de 5 años que presentaba una cojera de la grupa izquierda provocada por un quiste óseo subcondral (Golonka et al. 2020). La polimerización se llevó a cabo *in situ* administrando primero las MSCs y el alginato líquido y luego administrando $CaCl_2$ en la lesión. El tratamiento se combinó con flunixin meglumine IV durante 3 días, rehabilitación y ejercicio controlado. El análisis radiológico mostró una remodelación ósea completa y la yegua pudo volver a trabajar a los seis meses sin observarse recidivas durante los siguientes once meses. Aunque solo es un caso, los resultados observados reafirman el potencial terapéutico de las MSCs encapsuladas en alginato en el tratamiento de trastornos ortopédicos en caballo (Golonka et al. 2020).

En un estudio reciente en la especie equina se evaluó la viabilidad y capacidad de migración de las MSCs encapsuladas en alginato y sin encapsular en un modelo de defecto condral en el carpo durante 21 días (Santos et al. 2019). En el análisis histológico a los 7 días se observó que el uso de MSCs encapsuladas e implantadas intraarticularmente permitía que las células permanecieran durante un período de tiempo más largo dentro de la articulación. También evaluaron si las MSCs habían sido

capaces de migrar del implante a la articulación mediante el análisis del líquido sinovial por citometría de flujo y en todos los tiempos encontraron MSCs que habían migrado y sobrevivido. Este hallazgo podría ser debido a que las MSCs habían sido capaces de salir por los poros de las cápsulas. De este modo, el efecto que ejercerían las MSCs encapsuladas podría ser doble, por contacto directo y por secreción de moléculas inmunomoduladoras.

Como se ha desarrollado, la encapsulación de las MSCs en implantes de alginato es un sistema muy interesante para su aplicación en diversas patologías musculoesqueléticas con un componente inflamatorio, como son la artrosis o las tendinopatías (Leijs et al. 2017). Sin embargo, se necesitan más estudios que valoren la interacción de las MSCs con el sistema inmunitario del receptor y es necesario optimizar los implantes para que puedan ser administrados en localizaciones específicas.

11. EXPLORANDO LOS HORIZONTES DE LA INVESTIGACIÓN CON LAS CÉLULAS MADRE MESENQUIMALES EQUINAS: PRESENTE Y PERSPECTIVAS FUTURAS

En los últimos años se han logrado muchos avances en el desarrollo de terapias con MSCs equinas, especialmente en enfermedades musculoesqueléticas, pero también en otro tipo de enfermedades. Los resultados observados en muchos de estos estudios son muy prometedores para el futuro de las terapias con MSCs en diversas enfermedades, pero aún quedan muchas cuestiones por resolver.

Actualmente, la heterogeneidad existente entre los diseños experimentales y protocolos terapéuticos empleados en los distintos estudios llevados a cabo en las MSCs equinas para el tratamiento de patologías del aparato locomotor hacen que resulte complicado realizar revisiones sistemáticas de las mismas. Además, la ausencia de publicaciones con seguimiento a largo plazo de los animales tratados dificulta la valoración de su efectividad en el ámbito clínico (Durgam y Stewart 2017; Pratley 2020).

Existen además otras cuestiones clínicas que deben abordarse en cuanto al lugar, momento de administración de las MSCs, dosis terapéutica, además del uso alogénico y actualmente no existe un consenso para todas estas cuestiones.

El lugar de aplicación de las MSCs varía en función de la lesión de tratar. Se recomienda inyectarlas directamente en la zona de la lesión, de forma ecoguiada, en las lesiones del centro del tendón o ligamento afectado, o bien mediante inyección intrasinovial en el caso de articulaciones (Knott et al. 2022). En cuanto al número de células necesarias para su uso terapéutico, este ha sido y sigue siendo un punto de discusión importante y la recomendación actual es la aplicación de 10×10^6 MSCs por lesión en tejidos blandos y de 20×10^6 MSCs suspendidas en HA en articulaciones (Schnabel et al. 2013; Ferris et al. 2014; Gugjoo et al. 2019; Colbath et al. 2020b).

Otro punto importante en el protocolo terapéutico es el número de dosis. Se ha sugerido la aplicación de múltiples inyecciones de MSCs en función de la evolución de la lesión tendinosa o ligamentosa, generalmente evaluando previamente el caso transcurridos 30 días desde la primera administración (Schnabel et al. 2013; Colbath et al. 2020b), pero todavía no hay pautas estandarizadas al respecto.

Respecto al momento del tratamiento, la administración temprana de MSCs podría resultar más útil que su aplicación cuando ya se ha formado el tejido fibroso cicatricial (Schnabel et al. 2013; Voga et al. 2020), por lo que en muchas ocasiones no sería posible utilizar terapia autóloga dado el tiempo que requiere la obtención de estas células. Como se ha desarrollado anteriormente, esta limitación, podría verse resuelta mediante la utilización de MSCs alogénicas, que evitarían el retraso temporal del tratamiento asociado a la expansión de MSCs autólogas.

Asimismo, el empleo de la terapia celular alogénica supone una gran ventaja para la aplicación de las MSCs en la clínica equina debido a la posibilidad de creación de bancos celulares que permitan disponer de MSCs para la administración temprana del tratamiento (Colbath et al. 2020b). Sin embargo, las MSCs alogénicas pueden ser reconocidas por la respuesta innata y adaptativa del sistema inmunitario y el desarrollo de anticuerpos probablemente comprometería su eficacia terapéutica al repetirse el tratamiento (Berglund et al. 2017b; Rowland et al. 2021).

Aunque los resultados de varios estudios han demostrado que la terapia con MSCs alogénicas es segura para el paciente y que no conlleva complicaciones a corto plazo, la respuesta inmunitaria generada tras la administración repetida de MSCs no debe menospreciarse (Voga et al. 2020; Kamm et al. 2021b).

En la actualidad siguen sin estar claros todos los mecanismos de acción de las MSCs y existen diversos factores que pueden influir sobre sus propiedades inmunes, algunos aumentando su capacidad inmunomoduladora y otros, por el contrario, su capacidad inmunogénica (Ankrum et al. 2014; Berglund et al. 2017b).

Entre los factores más relevantes que pueden influir en el proceso de reconocimiento de las MSCs alogénicas se encuentran la compatibilidad para el MHC entre donante y receptor, la inflamación y la diferenciación. Estos dos últimos tienen una especial importancia por constituir tanto situaciones que pueden darse *in vivo* tras la administración de las células, como por haberse estudiado como estrategias terapéuticas para mejorar la efectividad de las mismas (Barrachina et al. 2018a; Van Hecke et al. 2021).

De esta forma, nos encontramos ante diversas situaciones que pueden influir sobre el delicado equilibrio inmunomodulación-inmunogenicidad de las MSCs, aspecto clave para la administración segura y efectiva de MSCs alogénicas. Las condiciones terapéuticas para lograr que primen las propiedades inmunomoduladoras sobre las inmunogénicas no se conocen con exactitud, lo que en buena parte se debe a la

dificultad de realizar estudios que esclarezcan qué sucede con las MSCs *in vivo*, principalmente por la dificultad que supone recuperarlas una vez aplicadas. Dicha limitación podría superarse encapsulando las MSCs equinas en un soporte que permita su recuperación y posterior estudio (Ryan et al. 2014; Santos et al. 2019).

Sólo conociendo las interacciones de las MSCs con la respuesta inmunitaria del receptor se podrán diseñar estrategias terapéuticas y optimizar los tratamientos alogénicos, siendo el caballo un modelo animal de gran valor, tanto en el aspecto locomotor como en el inmunológico. A pesar de los considerables avances en medicina regenerativa equina en los últimos años, se necesita mucho más trabajo para resolver todas estas cuestiones. La investigación en este campo permitirá encontrar tratamientos efectivos y seguros con MSCs para los pacientes, tanto en medicina veterinaria como humana.

II. HIPÓTESIS DE PARTIDA Y OBJETIVOS

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Durante los últimos años, ha habido importantes progresos en el campo de la terapia celular en la especie equina, fundamentalmente en la utilización de MSCs en el tratamiento de enfermedades relacionadas con el aparato locomotor. Aunque las patologías relacionadas con el sistema musculoesquelético poseen gran relevancia en el ámbito clínico equino, existen otras patologías sin un tratamiento definitivo establecido. Este escenario, sumado al valor del caballo como modelo animal de enfermedades humanas, ha impulsado la búsqueda de nuevas opciones terapéuticas y las MSCs podrían desempeñar un papel fundamental en este contexto. Los resultados obtenidos en diferentes estudios son muy prometedores para el avance de las terapias con MSCs equinas en diversas enfermedades, aunque todavía existen numerosos aspectos por resolver, siendo de gran relevancia el estudio de las interacciones de las MSCs alogénicas con el sistema inmune del receptor.

La compatibilidad para el MHC entre donante y receptor, la inflamación y la diferenciación pueden influir sobre el equilibrio inmunomodulación-inmunogenicidad de las MSCs equinas, constituyendo el conocimiento y manejo de estos factores potenciales herramientas para optimizar las terapias celulares alogénicas.

La inducción de la capacidad inmunomoduladora de las MSCs mediante la estimulación con citoquinas proinflamatorias (IFNɣ y TNFα), permitiría a las mismas evadir al sistema inmunitario del receptor de forma que serían capaces de ejercer sus funciones reguladoras al ser administradas de forma alogénica, independientemente de la compatibilidad para el MHC.

En cambio, el efecto de la diferenciación de las MSCs a condrocito influiría sobre las propiedades inmunomoduladoras de las MSCs equinas, pudiendo generarse una respuesta inmunitaria frente a las células diferenciadas cuya severidad podría además verse influenciada por la incompatibilidad para el MHC.

En definitiva, nuestra hipótesis de partida es que distintas situaciones que se pueden utilizar como estrategia terapéutica podrían influir sobre las interacciones de las MSCs alogénicas con el sistema inmunitario del receptor, siendo el conocimiento y manejo de dichos factores fundamentales para optimizar la terapia celular alogénica.

El objetivo general planteado en la presente memoria de Tesis Doctoral es profundizar en el estudio del efecto de la inflamación y la diferenciación condrogénica sobre el equilibrio inmunomodulación-inmunogenicidad de las MSCs, teniendo en cuenta además la influencia de la compatibilidad para el MHC y escogiendo la especie equina por su importancia como paciente y como modelo animal para diferentes enfermedades.

Para ello, se estudiará la respuesta inmunitaria frente a las MSCs alogénicas, compatibles o incompatibles para el MHC, tanto a nivel celular como humoral y utilizando ensayos tanto *in vitro* como *in vivo* para caracterizar de forma completa dicha respuesta frente a las MSCs alogénicas estimuladas o diferenciadas, ya que la relación de las MSCs con el sistema inmunitario y la desviación del equilibrio inmunomodulación-inmunogenicidad resultan claves para lograr tratamientos alogénicos efectivos y seguros.

Para alcanzar dicho objetivo general se plantearon los siguientes objetivos específicos:

OBJETIVOS ESPECÍFICOS

1. Revisar el estado actual, las aplicaciones y las limitaciones de la utilización de las MSCs equinas en diferentes patologías, junto a su potencial traslación como modelo animal de estas patologías en el ámbito de la medicina humana.

2. Determinar *in vitro* el perfil inmunomodulador e inmunogénico de las MSCs equinas sin estimular (MSC-SE), estimuladas con citoquinas proinflamatorias (MSC-E) y diferenciadas a condrocito (MSC-C); así como la respuesta frente a ellas de linfocitos de sangre periférica (PBLs) alogénicos MHC-compatibles y MHC-incompatibles.

3. Valorar *in vivo* la respuesta inmunitaria celular a nivel local frente a las diferentes condiciones de las MSCs (MSC-SE, MSC-E y MSC-C) tras su implantación subcutánea en receptores alogénicos tanto MHC-compatibles como MHC-incompatibles.

4. Estudiar *in vivo* la respuesta inmunitaria sistémica, tanto a nivel celular como humoral, frente a las diferentes condiciones de las MSCs (MSC-SE, MSC-E y MSC-C) tras su implantación subcutánea en receptores alogénicos tanto MHC-compatibles como MHC-incompatibles.

5. Analizar la generación de mecanismos de memoria inmunológica *in vivo*, tanto a nivel celular como humoral, estudiando la respuesta inmunitaria completa descrita en los objetivos anteriores tras la reexposición de los animales receptores MHC-compatibles y MHC-incompatibles a las mismas MSCs (MSC-SE, MSC-E o MSC-C).

III. PRESENTACIÓN DE LAS INVESTIGACIONES REALIZADAS

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Fruto de la investigación llevada a cabo en el marco de esta Tesis Doctoral, se han publicado 3 artículos en revistas indexadas en el Journal Citation Reports (JCR). Además, de dichos artículos, en esta Memoria se han incluido 3 manuscritos más, los cuales se encuentran en etapas avanzadas de preparación. Asimismo, en la presente Tesis Doctoral se incluyen tanto una breve sección de Material y Métodos como una sección de Discusión que engloban de forma general todos los manuscritos. A continuación, se indican las referencias completas de las publicaciones que forman parte de esta Tesis Doctoral.

MANUSCRITO I

Cequier, A.; Sanz, C.; Rodellar, C.; Barrachina, L. The usefulness of mesenchymal stem cells beyond the musculoskeletal system in horses. Animals. 11 - 4, pp. 931 [28 pp]. 2021. ISSN 2076-2615. DOI: 10.3390/ani11040931
Factor de impacto JCR**: 3 (Q1)**

MANUSCRITO II

Cequier, A.; Romero, A.; Vázquez, F.J; Vitoria, A.; Bernad, E.; Fuente, S; Zaragoza, P; Rodellar, C.; Barrachina, L. Equine mesenchymal stem cells influence the proliferative response of lymphocytes: effect of inflammation, differentiation and MHC-compatibility. Animals. 12 - 8, pp. 984 [25 pp]. 2022. ISSN 2076-2615. DOI: 10.3390/ani12080984
Factor de impacto JCR**: 3 (Q1)**

MANUSCRITO III

Cequier, A.; Vázquez, F.J; Romero, A.; Vitoria, A.; Bernad, E.; García-Martínez, M.; Gascón, I.; Barrachina, L.; Rodellar, C. The immunomodulation-immunogenicity balance of equine mesenchymal stem cells (MSCs) is differentially affected by the immune cell response depending on inflammatory licensing and major histocompatibility complex (MHC) compatibility. Frontiers in Veterinary Science. 9, 2022. ISSN 2297-1769. DOI: 10.3389/fvets.2022.957153
Factor de impacto JCR**: 3.2 (Q1)**

MANUSCRITO IV

Cequier, A.; Serrano M.B.; Soler-Monsó M.T.; Romero, A.; Bernad, E.; Vázquez, F.J; Vitoria, A.; Fuente, S; García-Martínez, M.; Albareda, J.; Barrachina, L.; Rodellar, C. Local immune response induced by allogenic equine mesenchymal stem cells (MSCs) and their gene expression changes after repeated administration to MHC-matched and MHC-mismatched recipients.

MANUSCRITO V

Cequier, A.; Barrachina, L.; Vázquez, FJ.; Vitoria, A.; Bernad, E.; Fuente, S.; Serrano, M.B.; Zaragoza, P.; Romero, A.; Rodellar, C. Effect of inflammation, differentiation, and MHC compatibility on the immunogenicity of allogeneic equine mesenchymal stem cells (MSCs)

MANUSCRITO VI

Cequier, A.; Bernad, E.; Romero, A.; Vitoria, A.; Fuente, S.; Fernández, I.; Zaragoza, P.; Serrano, M.B.; Rodellar, C.; Vázquez, FJ.; Barrachina, L. Allo-antibody production in response to repeated administration of equine mesenchymal stem cells (MSCs) in MHC-matched and MHC-mismatched recipients

Es importante resaltar que, como resultado de su participación en los procesos de desarrollo y publicación, la doctoranda ha sido coautora de dos estudios previos que, si bien no están directamente vinculados con los objetivos específicos de esta Tesis Doctoral, guardan una estrecha relación con su tema central. La colaboración en estos estudios, además de contribuir a la formación de la doctoranda en el ámbito de investigación abordado en su Tesis, ha permitido obtener resultados que han sido de gran relevancia para el posterior desarrollo de la investigación presentada en esta Tesis Doctoral. Estos estudios también han sido publicados y se encuentran indexados en revistas incluidas en el JCR. Las referencias completas de los mismos se detallan a continuación:

Barrachina, L.; **Cequier, A.**; Romero, A.; Vitoria, A.; Zaragoza, P.; Vázquez, F.J.; Rodellar, C. Allo-antibody production after intraarticular administration of mesenchymal stem cells (MSCs) in an equine osteoarthritis model: effect of repeated administration, MSC inflammatory stimulation, and equine leukocyte antigen (ELA) compatibility. Stem Cell Research & Therapy. 11, pp. 52 [12 pp.]. 2020. ISSN 1757-6512. DOI: 10.1186/s13287-020-1571-8
Factor de impacto JCR**: 7.5 (Q1)**

Yaneselli, K.; Barrachina, L.; Remacha, A.R.; Algorta, A.; Vitoria, A.; **Cequier, A.**; Romero, A; Vázquez, F.J; Maisonnave, J.; Rodellar C. Effect of allogeneic platelet lysate on equine bone marrow derived mesenchymal stem cell characteristics, including immunogenic and immunomodulatory gene expression profile. Veterinary Immunology and Immunopathology. 217, pp. 109944 [8 pp.]. 2019. ISSN 0165-2427. DOI: 10.1016/j.vetimm.2019.109944
Factor de impacto JCR**: 1.8 (Q2)**

IV. MATERIAL Y MÉTODOS

IV. MATERIAL Y MÉTODOS

A continuación, se procederá a describir brevemente y de forma general los diseños experimentales, técnicas y materiales utilizados durante el desarrollo de la presente Tesis Doctoral, indicando para la consecución de qué objetivos se han empleado y en qué Manuscritos han sido incluidos. En cada manuscrito se proporciona la información detallada sobre el material y la metodología utilizados, incluyendo el diseño experimental, el análisis estadístico y otros aspectos específicos de cada estudio.

Para la consecución del **Objetivo 1**, se elaboró el **Manuscrito I**, que consiste en una revisión bibliográfica acerca de la utilización de MSCs en patologías equinas no relacionadas con el aparato locomotor.

En la presente sección de Material y Métodos se proporcionará una breve descripción de los tipos de ensayos que se llevaron a cabo en esta Tesis Doctoral para la consecución de los **Objetivos 2**, **3**, **4** y **5**: ensayos *in vitro* y ensayos *in vivo*. Cabe destacar que la mayoría de las técnicas moleculares y celulares utilizadas fueron las mismas en ambos ensayos, aunque se emplearon con propósitos distintos. Por tanto, en las secciones correspondientes se describirán las características principales de cada una de estas técnicas y se indicará en qué ensayos específicos se utilizaron.

Todas las técnicas moleculares y celulares fueron previamente optimizadas y validadas antes de llevar a cabo los ensayos *in vitro*. Así mismo, antes de iniciar los ensayos *in vivo,* también se llevaron a cabo diferentes ensayos para poner a punto y optimizar diferentes técnicas. Estas consideraciones se encuentran recogidas en el **Anexo VI**.

1. DISEÑOS EXPERIMENTALES

A continuación, se detallan los diseños experimentales llevados a cabo en los ensayos *in vitro* e *in vivo*, incluyendo información sobre los enfoques metodológicos utilizados, las variables evaluadas y los procedimientos específicos utilizados en cada etapa del estudio.

1.1 Diseño experimental de los ensayos *in vitro*

Para la consecución del **Objetivo 2** y la elaboración de los **Manuscritos II** y **III** de la presente Tesis Doctoral se estudió la capacidad inmunomoduladora e inmunogénica de las MSCs en tres condiciones: sin estimular (MSC-SE), estimuladas con citoquinas proinflamatorias (MSC-E) y diferenciadas a condrocito (MSC-C). Las MSCs de tres caballos homocigotos para determinados haplotipos del MHC en las distintas condiciones se cocultivaron con linfocitos de sangre periférica (PBL) autólogos (*n* = 3) o alogénicos de animales MHC-compatibles (*n* = 8) y MHC-incompatibles (*n* = 7) utilizando las combinaciones mostradas en la **Figura 12**.

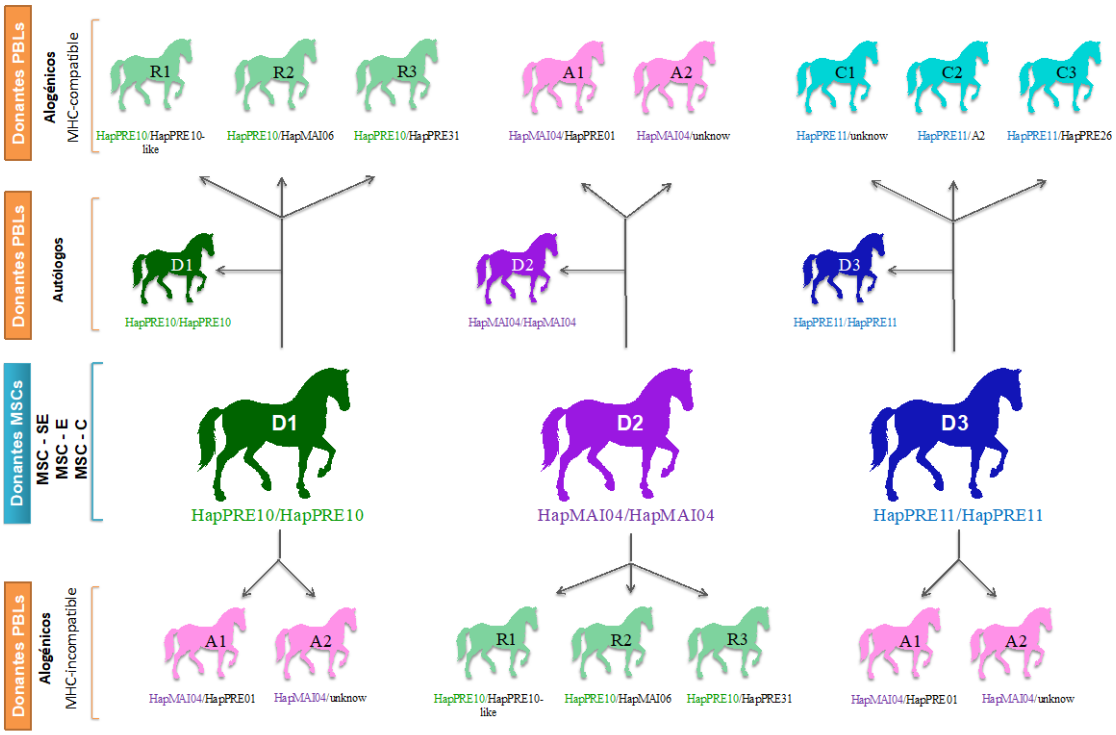


Figura 12. Diseño experimental ensayos *in vitro*: se utilizaron células madre mesenquimales (MSCs) y donantes de linfocitos de sangre periférica (PBL) para establecer cocultivos autólogos y alogénicos del complejo mayor de histocompatibilidad (MHC)-compatible y MHC-incompatible para estudiar la capacidad inmunomoduladora y el potencial inmunogénico de las MSCs. Se utilizaron MSCs sin estimular (MSC-SE), estimuladas con citoquinas proinflamatorias (MSC-E) o diferenciadas a condrocito (MSC-C).

Se llevaron a cabo dos tipos de ensayos: ensayos inmunosupresores y ensayos de reacciones linfocitarias mixtas unidireccionales modificadas (Modified one-way MLR, de sus siglas en inglés *mixed lymphocytes reactions*) (**Figura 13A**). Mediante estos ensayos se valoró la capacidad de supresión de la proliferación de los PBLs por parte de los distintos tipos de MSCs y en las distintas combinaciones (capacidad inmunomoduladora) y se analizó la posible respuesta proliferativa por parte de los PBLs al enfrentarlos a los distintos tipos de MSCs (capacidad inmunogénica). Al finalizar cada ensayo de cocultivo, los PBLs se marcaron con diferentes anticuerpos para estudiar los cambios en la frecuencia y proliferación de cada subconjunto de linfocitos mediante citometría de flujo (**Figura 13B**). Además del análisis linfocitario, la secreción de IFNɣ en los sobrenadantes de los cocultivos se evaluó mediante ELISA como reflejo de la activación de las células T (**Manuscrito II**).

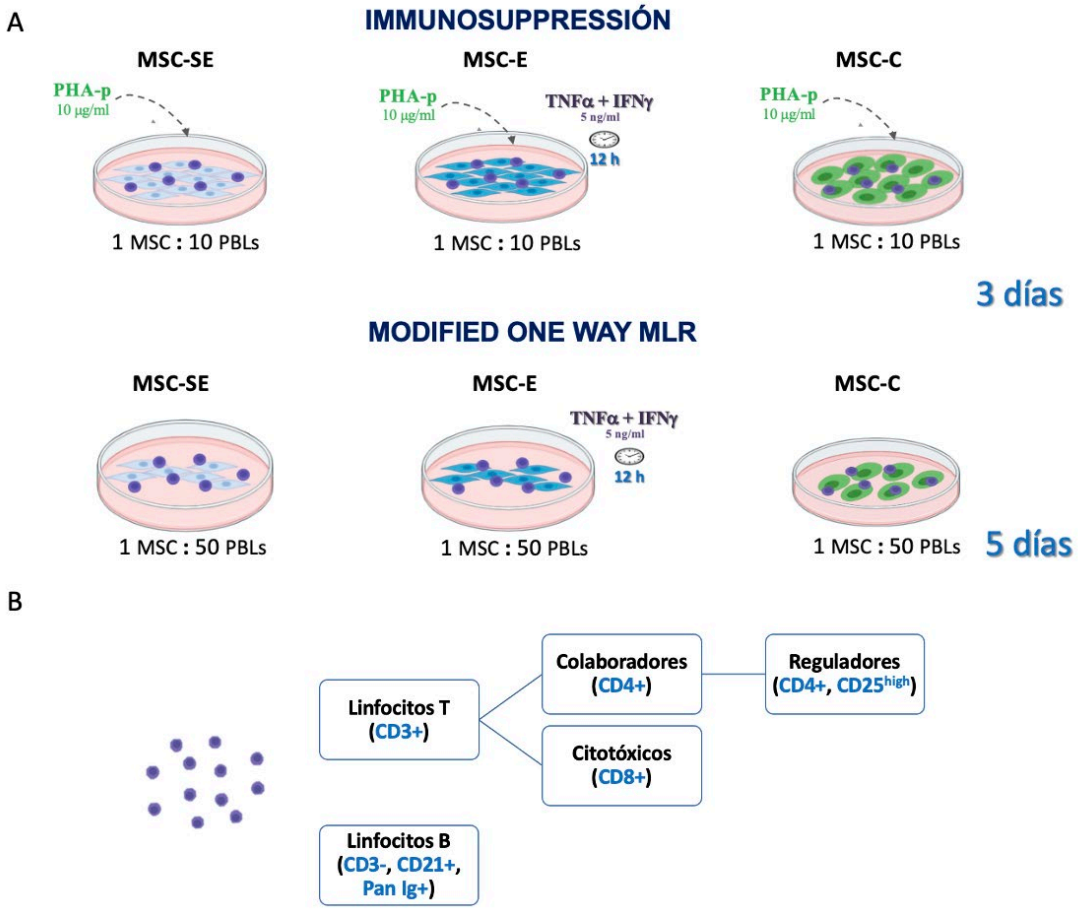


Figura 13. Representación esquemática (**A**) de los ensayos llevados a cabo en el **Manuscrito I** para valorar la capacidad inmunomoduladora e inmunogénica de las MSCs en distintas condiciones: sin estimular (MSC-SE), estimuladas con citoquinas proinflamatorias (MSC-E) o diferenciadas a condrocito (MSC-C) al enfrentarlas a PBLs autólogos, MHC-compatibles y MHC-incompatibles. (**B**) Subpoblaciones linfocitarias analizadas por citometría de flujo tras los cocultivos.

Tras ambos ensayos, para la elaboración del **Manuscrito III**, se analizó el perfil inmune de las MSC-SE y MSC-E tras la exposición a los PBLs, incluyendo la expresión génica (RT-qPCR) de las diferentes moléculas inmunomoduladoras e inmunogénicas y la secreción de distintos mediadores inmunomoduladores al medio de cultivo (ELISA) (**Figura 14**).

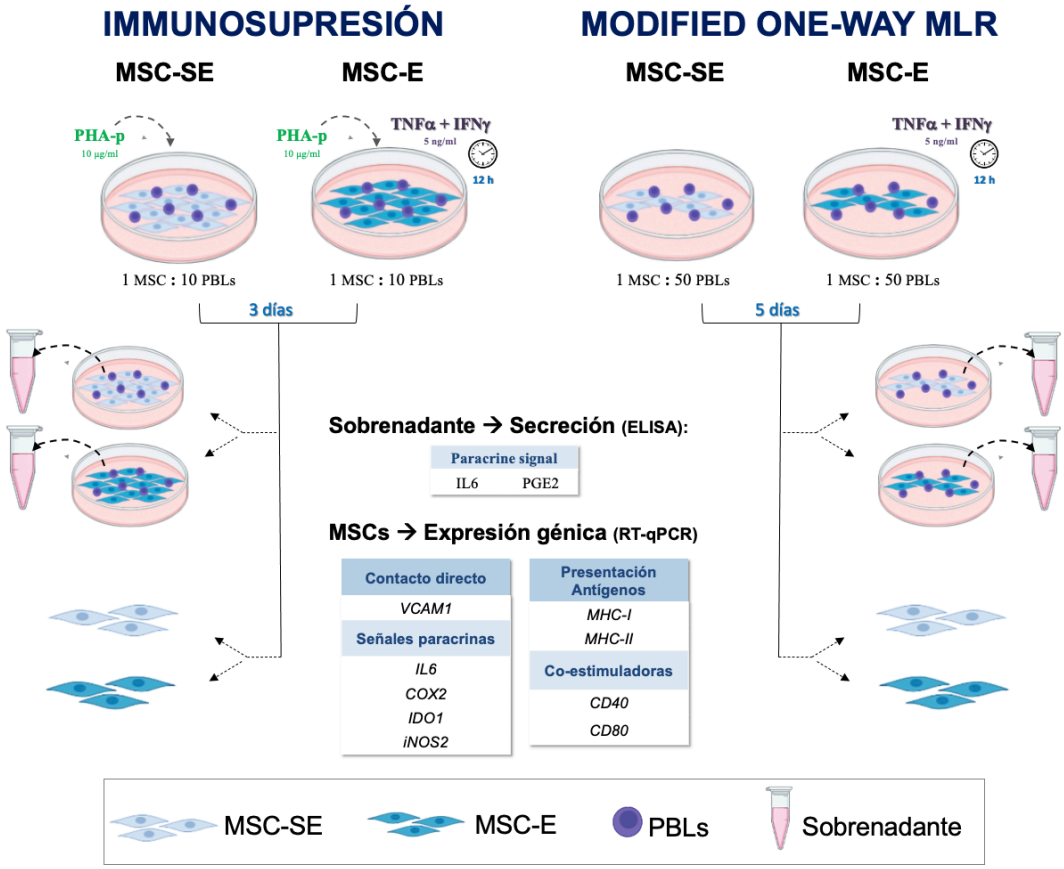


Figura 14. Representación esquemática de los cocultivos llevados a cabo en el **Manuscrito III.** Se cocultivaron MSCs equinas no estimuladas (MSC-SE) y estimuladas con citoquinas proinflamatorias (MSC-E) tanto en los ensayos de inmunosupresión como en los de reacciones linfocitarias mixtas unidireccionales modificadas (Modified one-way MLR), utilizando PBLs activados con fitohemaglutinina isoforma P (PHA) o linfocitos en reposo, respectivamente. Tras los cocultivos, se recogieron los sobrenadantes y las MSCs para evaluar, respectivamente, la secreción y expresión génica de diferentes mediadores implicados en la capacidad inmunomoduladora y el potencial inmunogénico de estas células.

1.2 Diseño experimental de los ensayos *in vivo*

Para la consecución de los **Objetivos 3**, **4** y **5** y la elaboración de los **Manuscritos IV**, **V** y **VI** se utilizaron un total de 20 caballos (12 machos castrados, 7 hembras y 1 semental). Para llevar a cabo los ensayos *in vivo*, se utilizó el grupo de 11 caballos que previamente había participado en los ensayos *in vitro* (autólogos y MHC-compatibles), y se seleccionaron 10 nuevos animales para el estudio *in vivo* (9 MHC-incompatibles y 1 animal control). Los grupos de caballos MHC-compatibles y MHC-incompatibles se dividieron a su vez en tres subgrupos de 2-3 caballos cada uno y cada subgrupo recibió un tipo de MSCs: MSC-SE, MSC-E y MSC-C (**Figura 15**).

Autólogos (Donantes MSCs)	Receptores alogénicos MHC-compatibles			Receptores alogénicos MHC-incompatibles		
D1 MSC-C	R1	R2	R3	X1	X2	X3
HapPRE10/HapPRE10	HapPRE10/HapPRE10-1	HapPRE10/HapMAI06	HapPRE10/HapPRE31	HapMai50/desconocido	A3b/A3e	Desconocido/desconocido
D2 MSC-E	A1	A2		Y1	Y2	Y3
HapMAI04/HapMAI04	HapMAI04/HapPRE01	HapMAI04/desconocido		COR71/desconocido	HapPRE09/COR182	HapPSL01/HapPSL01
D3 MSC-SE	C1	C2	C3	Z1	Z2	Z3
HapPRE11/HapPRE11	HapPRE11/desconocido	HapPRE11/A2	HapPRE11/HapPRE26	HapPRE10/ZAR14	A9a/desconocido	A8/A10b

Figura 15. Distribución de los grupos de caballos (autólogos, receptores alogénicos MHC-compatibles y receptores alogénicos MHC-incompatibles) y tipos de MSCs recibidas en los ensayos *in vivo*: sin estimular (MSC-SE), estimuladas con citoquinas proinflamatorias (MSC-E) o diferenciadas a condrocito (MSC-C).

A cada animal se le colocaron tres implantes subcutáneos de alginato en las tablas del cuello (T0), conteniendo cada uno 5×10^6 MSCs del mismo tipo (MSC-SE, MSC-E o MSC-C). A la semana (T1), tres semanas (T2) y seis semanas (T3) se recuperaron los implantes y, transcurrido un mes tras retirar el último, se volvió a exponer a cada animal al mismo tipo de MSCs (T4) en las tablas contralaterales del cuello. Los implantes fueron retirados a los mismos tiempos (T5, T6, T7; respectivamente) para estudiar el efecto de una segunda administración y el posible desarrollo de mecanismos de memoria inmunológica (**Figura 16**).

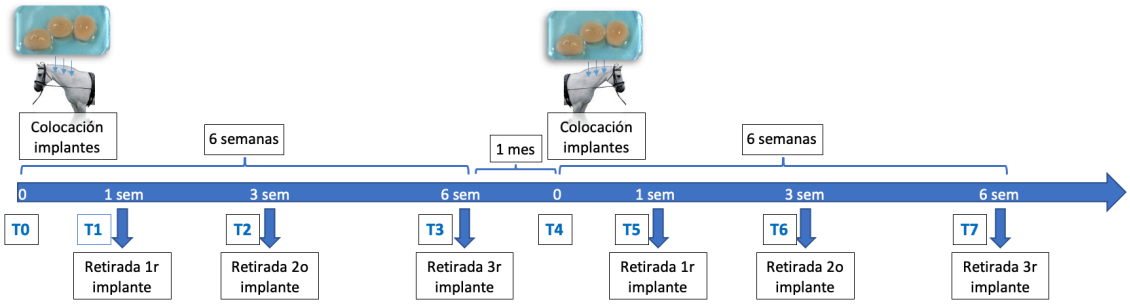


Figura 16. Representación esquemática del diseño experimental *in vivo*.

A los tiempos mencionados se valoró la respuesta inmune tanto celular como humoral y tanto a nivel local como sistémico, mediante la obtención seriada de sangre de la vena yugular y el análisis de los implantes retirados.

Con el fin de evaluar la respuesta inmunitaria celular local, se llevaron a cabo análisis histológicos de los implantes extraídos, así como análisis de expresión génica de moléculas relacionadas con la capacidad inmunomoduladora e inmunogénica de las MSCs equinas (**Manuscrito IV**). La respuesta inmune celular sistémica y la generación de respuesta celular de memoria se evaluó reexponiendo *in vitro* PBLs de los distintos receptores al tipo de MSCs que recibió de su donante (MSC-SE, MSC-E o MSC-C) (**Manuscrito V**). Con el objetivo de valorar la respuesta inmune humoral, se realizaron ensayos de microcitotoxicidad para detectar la presencia de aloanticuerpos (**Manuscrito VI**). En la **Figura 17** se representan de forma esquemática las diferentes valoraciones llevadas a cabo en los ensayos *in vivo* de la presente Tesis Doctoral.

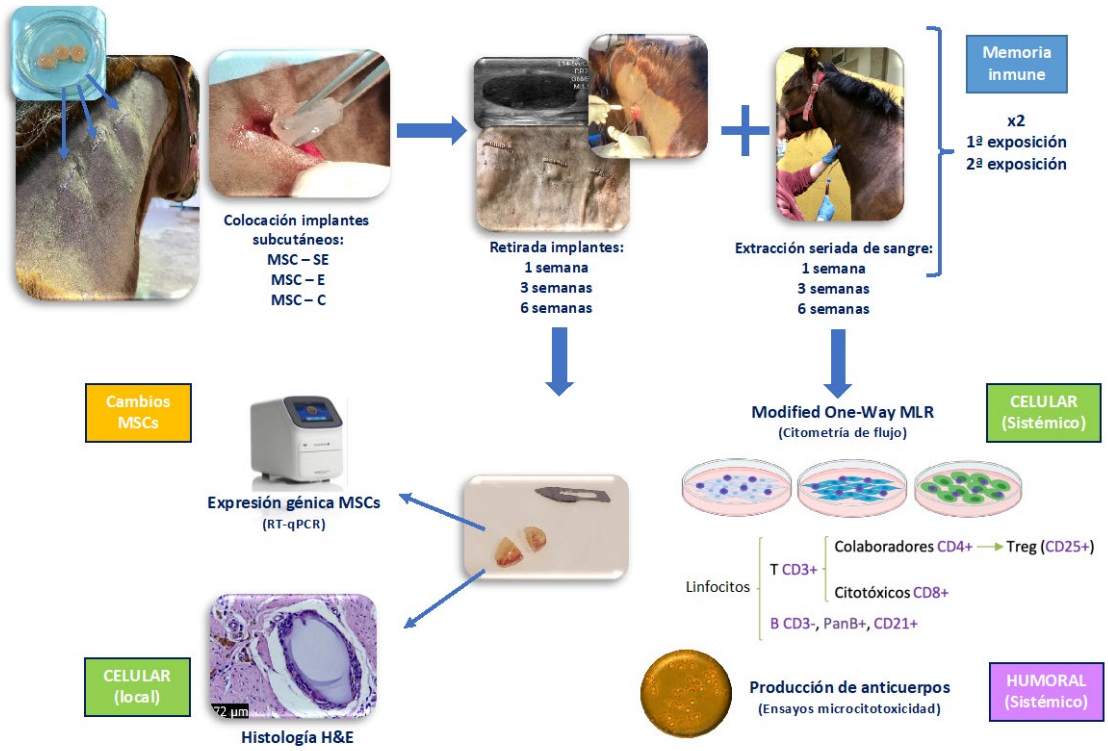


Figura 17. Representación esquemática de los ensayos *in vivo*.

2. SELECCIÓN DE CABALLOS CON HAPLOTIPOS MHC-COMPATIBLES Y MHC-INCOMPATIBLES

En primer lugar, se buscaron 3 caballos homocigotos para determinados haplotipos del MHC equino que fueron los donantes de MSCs de todos los ensayos *in vitro* e *in vivo*.

Para llevar a cabo los ensayos *in vitro* y para la consecución del **Objetivo 2** se seleccionaron un total de 11 caballos heterocigotos de entre 2 y 12 años para el estudio: 7 machos castrados, 3 hembras y 1 semental. Se conformaron tres grupos de animales que incluían de dos a tres caballos heterocigóticos que compartían un haplotipo con uno de los 3 donantes de MSCs. En cada grupo, el caballo homocigótico se utilizó como donante de MSCs, ya que era compatible con el MHC de los animales heterocigóticos del grupo, pero MHC-incompatible con los animales de los otros grupos, lo que permitió conformar combinaciones autólogas, MHC-compatibles y MHC-incompatibles.

Para llevar a cabo los ensayos *in vivo* y la consecución de los **Objetivos 3**, **4** y **5**, se utilizaron los mismos 11 caballos que en los ensayos *in vitro* (autólogos y MHC-compatibles), y se seleccionaron 10 animales nuevos MHC-incompatibles (10 machos castrados de 7-12 años).

Todos los experimentos con animales se llevaron a cabo cumpliendo con la Licencia de Proyecto PI 15/16, aprobada por el Comité Ético de Investigación Animal de la Universidad de Zaragoza. El cuidado y uso de los animales se realizó siguiendo estrictamente la Política Española de Protección Animal RD53/2013, la cual está en total conformidad con la Directiva 2010/63 de la Unión Europea sobre la protección de los animales utilizados para fines científicos.

2.1 Reacción en cadena de la polimerasa multiplex (PCR multiplex): determinación de los haplotipos del MHC equino

Se analizaron 10 marcadores microsatélites intra-MHC mediante PCR multiplex para establecer los haplotipos asociados a determinados antígenos de ELA. Estos marcadores habían sido previamente utilizados tanto en estudios que evaluaban la variabilidad de haplotipos en diferentes razas equinas (Tseng et al. 2010; Sadeghi et al. 2018) como en investigaciones que evaluaban la relevancia de la compatibilidad donante-receptor en la terapia celular en la especie equina (Pezzanite et al. 2015; Berglund y Schnabel 2017).

El DNA genómico se obtuvo a partir de sangre de la vena yugular utilizando el kit Quick-gDNA™ Miniprep Kit (Zymo Research), siguiendo las instrucciones del fabricante. Se utilizaron cebadores marcados con fluorescencia y se realizaron electroforesis capilares en el secuenciador automático ABI Prism 3130 para la identificación de los genotipos. Las secuencias de oligonucleótidos (**Tabla 7**) utilizadas para amplificar los microsatélites asociados con serotipos de ELA equino habían sido previamente descritas (Tseng et al. 2010; Sadeghi et al. 2018).

Tabla 7. Secuencias de oligonucleótidos para amplificar microsatélites intra-MHC. Las etiquetas FAM, VIC, NED y PET hacen referencia a los fluorocromos producidos por Applied Biosystems.

Región MHC	Microsatélite	Secuencia de oligonucleótidos
MHC - I	UMNJH-38	F: **FAM**-TGTGTGTGCACCTGTCCTTT R: GATGGGAGGGAATGAGGAAT
	COR110	F: TTTGGTCTTTGCAGGTATGG R: **VIC**-TCTCCCTTCCTCTTTGTTCC
MHC - III	ABGe9019	F: **FAM**-CTGAGAGAGACAGCATTTGTGG R: GAAAGGTGTCTCCATTCTTGCT
	UMNe65	F: NED-TCGCAAAACCCACAGACTAC R: TTCTCCTTTCCTTCCACTCC
MHC - II	ABGe9030	F: **PET**-CCAGCAGACCTGCAAGAGTA R: AGCATGAGAGCCATGAAGGT
	EQMHC1	F: **VIC**-ATGCATACCGGGAAAGACAG R: AGAGACTTCAGTCTCTGTGGTG
	COR112	F: TTACCTGGTTATTGGTTATTTGG R: **NED**-TCACCCACTAAATCTCAAATCC
	COR113	F: TGTTTAGAACTCGCCAGGAG R: **FAM**-TCATCAGTTCCTTGCCTAGC
	UM011	F: TGAAAGTAGAAAGGGATGTGG R: **FAM**-TCTCAGAGCAGAAGTCCCTG
	COR114	F: TCAAAATCCACACTCCCTTC R: **PET**-TCCATAAAGAGTGGGACACTG

3. OBTENCIÓN DE CÉLULAS MADRE MESENQUIMALES DE MÉDULA ÓSEA

Estos procedimientos se han utilizado en los **Manuscritos II**, **III**, **IV**, **V** y **VI** con el objetivo de disponer de MSCs equinas caracterizadas tanto para los ensayos *in vitro* como *in vivo*. Toda la metodología referente al cultivo celular de MSCs equinas había sido puesta a punto por el equipo de investigación LAGENBIO (Ranera et al. 2011), así como utilizada en los sucesivos estudios llevados a cabo (Remacha et al. 2015; Barrachina et al. 2017, 2018a).

La metodología que se describe a continuación fue la misma en todos los estudios y se encuentra detallada en el **Manuscrito II**. De cada uno de los 3 caballos homocigotos se obtuvieron 40 mL de médula ósea del esternón en jeringas heparinizadas utilizando una aguja Jamshidi de 11G con los caballos bajo sedación (romifidina 0,04 mg/kg y butorfanol 0,02 mg/kg). Las células mononucleares se aislaron por gradiente de centrifugación con Lymphoprep (Atom) y se sembraron a 37 °C y 5% de CO_2 en medio de cultivo para MSCs compuesto por *Dulbecos´s modified Eagle´s médium* (DMEM) bajo en glucosa suplementado con 1% de L-glutamina, 1% de penicilina/estreptomicina y 10% de suero fetal bovino (FBS) (todo de Sigma-Aldrich).

A las 24, 48 y 72 horas tras la extracción, las células se lavaron con tampón fosfato salino (PBS, de sus siglas en inglés *phosfate buffered saline*) (Gibco) y se reemplazó el medio de cultivo para eliminar las células no adherentes al plástico.

Las células se expandieron cambiando el medio de cultivo cada 3 días hasta alcanzar aproximadamente el 80% de confluencia. Al llegar a esta confluencia, las células se separaron de la superficie de la placa de cultivo utilizando 0,25% tripsina/EDTA (Sigma-Aldrich) y se contaron con un hemocitómetro (cámara de Neubauer) utilizando azul de tripano para excluir las células muertas. Las células volvieron a sembrarse y se expandieron en las mismas condiciones hasta el pase 3, momento en el que se caracterizaron como MSCs equinas por su inmunofenotipo y su capacidad de diferenciación trilinaje.

El inmunofenotipo se estudió mediante el análisis de la expresión génica de los marcadores *CD90*, *CD105*, *CD44*, *CD73*, *CD34*, *CD45*, *MHC-I* y *MHC-II* utilizando la técnica de reacción en cadena de la polimerasa en tiempo real (RT-qPCR) y por la expresión en la superficie celular de las moléculas CD90, CD105, CD44, MHC-I y MHC-II mediante citometría de flujo. Las técnicas de RT-qPCR y citometría de flujo se verán con mayor detalle en los apartados correspondientes.

La diferenciación a los linajes osteogénico, adipogénico y condrogénico se indujo utilizando medios de cultivo suplementados con las moléculas apropiadas y se comprobó mediante tinciones específicas y expresión génica de marcadores propios de cada linaje. Las MSCs caracterizadas en pase 3 se criopreservaron a -80 °C en un medio consistente en 10% de dimetilsulfóxido (DMSO) (Sigma-Aldrich) y 90% de FBS hasta el momento de su utilización.

3.1 Preparación de las células madre mesenquimales para los ensayos *in vitro*

La metodología que se detalla a continuación se empleó en el desarrollo de los experimentos *in vitro* incluidos en los **Manuscritos II** y **III** y en los experimentos *in vivo* incluidos en el **Manuscritos V**.

Antes de los cocultivos, las MSCs de cada donante se descongelaron y se sembraron a 5.000 células/cm^2 en medio convencional de MSCs, a 37 °C y 5% de CO_2 durante 72 horas para recuperarse de la congelación. Posteriormente, 24 horas antes de los cocultivos, las MSCs se sembraron en placas de 24 pocillos a 100.000 MSCs por pocillo para los ensayos de inmunosupresión (**Manuscritos II** y **III**), y a 20.000 MSCs por pocillo para los ensayos modified one-way MLR (**Manuscritos II**, **III** y **V**).

Para realizar la estimulación inflamatoria de la condición de MSC-E (**Manuscritos II**, **III** y **V**), la concentración de citoquinas proinflamatorias que se añadió al medio basal de las MSCs, antes de añadir los PBLs, fue de 5 ng/mL IFNɣ y 5 ng/mL TNFα (R&D Systems) durante 12 horas. Está concentración fue elegida en base a los resultados previos del grupo LAGENBIO (Barrachina et al. 2017).

La diferenciación condrogénica para la condición MSC-C se indujo con el medio comercial StemPro™ Chondrogenesis Differentiation Kit (Thermofisher) utilizando el sistema micromass durante 14 días antes de añadir los PBLs. El protocolo de siembra se

adaptó para adecuarse a la concentración celular requerida en cada diseño experimental (**Manuscritos II** y **V**).

4. COCULTIVO DE CÉLULAS MADRE MESENQUIMALES Y LINFOCITOS

Los ensayos para valorar la capacidad inmunomoduladora e inmunogénica de las MSCs equinas *in vitro* se realizaron en la investigación desarrollada en los **Manuscritos II** y **III** para la consecución del **Objetivo 2** de las Tesis Doctoral. En estos experimentos *in vitro* se aislaron PBLs de 11 caballos (3 caballos homocigotos y 8 caballos heterocigotos) para establecer combinaciones autólogas, alogénicas MHC-compatibles y MHC-incompatibles, cocultivando los PBLs con las MSCs en las tres condiciones (MSC-SE, MSC-E y MSC-C). Además de valorar la capacidad inmunogénica de las MSCs en términos de inducir una respuesta proliferativa por parte de los PBLs, también se valoró la producción de IFNɣ por parte de las células T.

En los ensayos *in vivo*, tras la administración de las MSCs encapsuladas a los distintos receptores, se realizaron cocultivos a diferentes tiempos para valorar la capacidad inmunogénica de las MSCs al reexponer *in vitro* los PBLs de los distintos receptores con las MSCs del donante en las condiciones que las recibió cada receptor (MSC-SE, MSC-E y MSC-C). Estos ensayos se encuentran desarrollados en el **Manuscrito V** y fueron diseñados para valorar la respuesta inmune celular sistémica tras la exposición *in vivo* a MSCs de distintos tipos (**Objetivo 4**) y el efecto de la reexposición de los animales receptores a estas células (**Objetivo 5**).

A continuación, se describe brevemente la metodología llevada a cabo en los ensayos de inmunosupresión e inmunogenicidad. En ambos ensayos los linfocitos de todos los caballos se marcaron con 2,5 µM 5(6)-carboxifluoresceína diacetato N-succinimidil éster (CFSE, Sigma-Aldrich) para posteriormente medir su proliferación y número de generaciones (picos de intensidad de fluorescencia decreciente) mediante citometría de flujo.

4.1 Ensayos de inmunosupresión

La función inmunomoduladora de las MSCs se determinó por su capacidad para modular la proliferación de linfocitos estimulados por mitógenos. Las MSCs se sembraron previamente en placas de 24 pocillos, con 100.000 células por pocillo por duplicado y se prepararon para cada condición (MSC-SE, MSC-E, MSC-C). Los linfocitos marcados con CFSE de caballos autólogos, MHC compatibles e incompatibles se sembraron a 1×10^6 PBL por pocillo (proporción 1:10 MSC:PBL), con medio de PBLs (RPMI1640 suplementado con 10% de FBS, 0,1 mM de 2-mercaptoetanol (Sigma-Aldrich), 100 U/mL de penicilina y 0,1 mg/mL de estreptomicina) y 10 µg/mL del mitógeno fitohemaglutinina isoforma P (PHA). Además, como controles se sembraron 500.000 células no marcadas (control de ruido de fondo) y marcadas con CFSE (con y sin PHA, controles proliferantes y no proliferantes, respectivamente). Tras 3 días de cultivo se llevaron a cabo los correspondientes análisis linfocitarios (linfocitos T, T colaboradores,

T citotóxicos, T reguladores y B) mediante citometría de flujo y de secreción de IFNɣ mediante ELISAs.

4.2 Ensayos de inmunogenicidad

Para evaluar la capacidad de las MSCs de estimular una respuesta proliferativa en los linfocitos, se realizaron ensayos modified one-way MLR. Las MSCs se sembraron previamente a 20.000 células por pocillo en placas de 24 pocillos por duplicado para cada condición (MSC-SE, MSC-E, MSC-C). Los PBLs teñidos con CFSE de animales autólogos, MHC-compatibles y MHC-incompatibles se sembraron a 1×10^6 PBL por pocillo (proporción MSC: PBL de 1:50).

Asimismo, se establecieron los correspondientes controles como los descritos para los ensayos de inmunosupresión (PBLs sin CFSE para extraer la señal de fondo y PBLs marcados con CFSE, con y sin PHA, como controles internos de proliferación y no proliferación). También se establecieron controles MLRs clásicos para todos los PBLs respondedores. Para ello, se utilizaron PBLs MHC-compatibles y MHC-incompatibles como estimuladores tratándolos con 50 µg/mL de mitomicina C (Sigma-Aldrich) (incubación a 37 °C durante 30 minutos seguido de dos lavados con PBS) para inhibir su proliferación. Los PBLs estimuladores se sembraron a 500.000 PBL/pocillo en placas de 96 pocillos inmediatamente antes de la adición de 500.000 PBL respondedores teñidos con CFSE (proporción 1:1) para crear MLRs MHC-compatibles e incompatibles para todos los PBLs analizados. Todos los cultivos se mantuvieron durante 5 días sin cambiarles el medio, y luego se llevaron a cabo los correspondientes análisis linfocitarios (linfocitos T, T colaboradores, T citotóxicos, T reguladores y B) mediante de citometría de flujo y de secreción de IFNɣ mediante ELISAs.

4.3 Citometría de flujo

La citometría de flujo se utilizó en la investigación desarrollada en el **Manuscrito II** como parte de la caracterización del inmunofenotipo de las MSCs. Además, como se ha desarrollado en la sección anterior, esta técnica se utilizó tanto para determinar el perfil inmunomodulador e inmunogénico de las MSCs en distintas situaciones *in vitro* (**Manuscrito II**) como para el análisis de los cambios en las poblaciones linfocitarias de los PBLs reexpuestos a los distintos tipos de MSCs que habían recibido *in vivo* (**Manuscrito V**). Los análisis se realizaron en el Servicio Científico Técnico – Separación celular y Citometría del Centro de investigación biomédica de Aragón (CIBA), utilizando el citómetro de flujo FACSAria (BD Biosciences).

La citometría de flujo es una técnica de análisis celular que se basa en el uso de anticuerpos dirigidos específicamente a moléculas extracelulares situadas en la superficie de las células o a moléculas intracelulares. Para ello se utilizan anticuerpos primarios que pueden estar directamente conjugados con un fluorocromo (tinción directa) o no conjugados, lo que requiere un anticuerpo secundario unido a un

fluorocromo con reactividad contra la especie huésped del anticuerpo primario (tinción indirecta) (Adan et al. 2017).

Los citómetros de flujo utilizan una variedad de láseres con diferentes longitudes de onda para excitar fluorocromos específicos que se encuentran unidos a las células mediante anticuerpos. La señal fluorescente generada por la excitación de los fluorocromos es captada por un detector y se convierte en una señal eléctrica. Esta señal eléctrica se traduce digitalmente en porcentajes de células positivas para marcadores específicos y en intensidad de fluorescencia, proporcionando así información sobre las características de las células analizadas (Adan et al. 2017).

Para la caracterización del inmunofenotipo de las MSCs equinas se utilizaron los anticuerpos monoclonales de ratón anti-humanos CD90-PE (clon 5E10, BD Pharmingen), CD105-FITC (clon SN6, R&D Systems), HLA-ABC-FITC (Beckman Coulter) y HLA-DR-APC (Immunostep). La reactividad anti-caballo de los anticuerpos había sido previamente validada (Barrachina et al. 2016, 2018a).

Para la elaboración de los **Manuscritos II** y **V**, en la presente Tesis Doctoral se diseñaron dos paneles multicolor de marcadores que permitieron evaluar las diferentes subpoblaciones linfocitarias junto a la proliferación de estas subpoblaciones tras los cocultivos con MSCs. En el primer panel se utilizaron anticuerpos anti-CD3 para evaluar los linfocitos T y se utilizaron anticuerpos anti-CD21 y anti-Pan B-cells para evaluar las células B. En el segundo panel, se utilizaron anticuerpos anti-CD8, anti-CD4 y anti-CD25 para evaluar las células T citotóxicas, colaboradoras y reguladoras, respectivamente. Todos los anticuerpos primarios se utilizaron directamente conjugados o en combinación con anticuerpos secundarios. La información relativa a las características, la dilución y los fluorocromos conjugados para la valoración de las poblaciones linfocitarias se presenta en la **Tabla 8**.

Tabla 8. Anticuerpos utilizados en citometría de flujo para la valoración de las poblaciones linfocitarias.

Panel	Células marcadas	Anticuerpo primario	Anticuerpo secundario	Canal y fluorocromo	Dilución	Características
Panel 1	**Linfocitos T**	**CD3** Rata anti-humano; clon CD3-12, monoclonal isotipo IgG1	Ratón anti-rata; clon R1-12D10, monoclonal isotipo IgG1	FL-2 (PE)	1º: 1:100 2º: 1:100	Intracelular
	Linfocitos B	**CD21** Ratón anti-humano; clon B-ly4, monoclonal isotipo IgG1	-	FL-6 (APC)	1:40	Superficie Conjugado directo
	Linfocitos B	**Pan B-cells** Ratón anti-caballo; clon CVS36, monoclonal isotipo IgG1	Cabra anti-ratón; policlonal isotipo IgG H+L	FL-7 (Alexafluor 700)	1º: 1:100 2º: 1:200	Superficie
Panel 2	**Linfocitos T citotóxicos**	**CD8** Ratón anti-caballo; clon CVS8, monoclonal isotipo IgG1	-	FL-2 (RPE)	1:5	Superficie Conjugado directo
	Linfocitos T colaboradores	**CD4** Ratón anti-caballo; clon CVS4, monoclonal isotipo IgG1	Rata anti-ratón; clon RMG1-1, monoclonal isotipo IgG1	FL-8 (APC - Cy7)	1º: 1:200 2º: 1:200	Superficie
	Linfocitos T reguladores	**CD25**/IL-2R Cabra anti-humano; clon AF-223, policlonal isotipo IgG	Burro anti-cabra; policlonal isotipo IgG H+L	FL-6 (AlexaFluor 647)	1º: 1:50 2º: 1:400	Superficie

5. ANÁLISIS DE MOLÉCULAS RELACIONADAS CON LA INMUNOMODULACIÓN E INMUNOGENICIDAD DE LAS CÉLULAS MADRE MESENQUIMALES EQUINAS

El análisis de las principales moléculas relacionadas con la capacidad inmunomoduladora y potencial inmunogénico de las MSCs equinas se estudió con diferentes perspectivas tanto en los ensayos *in vitro* (**Objetivo 2**, **Manuscrito III**) como *in vivo* (**Objetivo 3** y **5**, **Manuscrito IV**).

Las moléculas analizadas en ambos estudios fueron las siguientes:

- Moléculas inmunomoduladoras:
  - Implicadas en los mecanismos paracrinos: *COX2* (PGE2), *IDO1*, *iNOS2*, IL6
  - Implicadas en los mecanismos de contacto directo: *VCAM1/CD106*
- Moléculas inmunogénicas:
  - MHC: MHC-I y MHC-II
  - Coestimuladoras: *CD40* y *CD80*

En los ensayos *in vitro*, la expresión de los genes que codifican las moléculas relacionadas con la inmunomodulación y la inmunogenicidad de las MSCs se evaluó en situación basal para las MSC-SE y MSC-E y después de ser cocultivadas con PBLs autólogos, MHC-compatibles o MHC-incompatibles, tanto en los ensayos de inmunosupresión como en los ensayos modified one-way MLR mediante RT-qPCR. Asimismo, también se analizó la secreción de IL6 y PGE2 mediante ELISAs en estos mismos ensayos (**Manuscrito III**).

Tras la administración de las MSCs *in vivo* a los distintos receptores se valoró de forma seriada la respuesta inmunitaria celular local postimplantación. Los implantes de cada uno de los caballos se retiraron a diferentes tiempos para estudiar, mediante RT-qPCR, la expresión génica de moléculas relacionadas con la inmunomodulación y la inmunogenicidad de los distintos tipos de MSCs (MSC-SE, MSC-E y MSC-C) tras su exposición al sistema inmune *in vivo* (**Manuscrito IV**).

5.1 Reacción en cadena de la polimerasa cuantitativa en tiempo real (RT-qPCR)

Esta metodología se ha empleado en el desarrollo de los experimentos incluidos en los **Manuscritos II**, **III** y **IV** con distintas finalidades. En el **Manuscrito II** se utilizó en el proceso de caracterización de las MSCs para estudiar su fenotipo y su capacidad de diferenciación a los distintos linajes. En el **Manuscrito III** esta técnica se empleó para valorar cambios en el perfil de expresión génica de las moléculas inmunomoduladoras e inmunogénicas de las MSCs tras enfrentarse a PBLs autólogos, MHC-compatibles y MHC-incompatibles.

Asimismo, esta técnica se utilizó en el **Manuscrito IV** para valorar cambios en la expresión de las moléculas inmunomoduladoras e inmunogénicas en las MSCs encapsuladas en los implantes de alginato tras ser administradas en los caballos.

La técnica de la RT-qPCR se basa en el uso de fluorocromos que se intercalan en la molécula de ADN, de forma que, a mayor número de copias de un fragmento durante la amplificación, mayor fluorescencia se registrará. La metodología utilizada para la extracción del ARN mensajero (ARNm) y su posterior retrotranscripción a ADN complementario (ADNc) dependieron del tipo de muestra.

Para la extracción de ARNm a partir de cultivos celulares (**Manuscrito II** y **III**), se utilizó el kit Cells-to-cDNA II (Ambion). En el caso de los tejidos sólidos como pellets de condrocitos (**Manuscrito II**) e implantes de alginato (**Manuscrito IV**), se empleó la misma metodología en ambos casos. Cada muestra se colocó en un tubo eppendorf de 2 mL y se añadieron 500 µL de TRI Reagent® (R2050, Zymo Research) y una perla de acero inoxidable (Qiagen). Las muestras se disgregaron y homogenizaron con el instrumento TissueLyser LT (Qiagen) a 50 Hz durante 6 minutos. Para la extracción de ARN se utilizó el kit comercial Direct-zol RNA Miniprep Kit (R2052, Zymo Research) y posteriormente se midió la concentración de ARN de cada muestra con el espectrofotómetro NanoDrop™ ND-1000 (Thermo Fisher). Además, se evaluó la calidad mediante los ratios 260/280 y 260/230 de absorbancia. El primero de ellos indica el grado de contaminación de la muestra con proteínas (absorbancia a 280 nm) mientras que el segundo indica el grado de presencia de sales y otros contaminantes que tienen una absorbancia a 230 nm. El kit empleado para la retrotranscripción a ADNc fue qScript cDNA Supermix (Quanta Biosciences).

El análisis de expresión génica se llevó a cabo por triplicado en el equipo QuantStudio3 Real Time PCR System (Applied Biosystems) utilizando Fast SYBR Green Master Mix (Applied Biosystem) y los cebadores específicos que se encuentran detallados en el **Manuscrito III**. El programa de amplificación empleado consistió en una activación inicial a 95 °C durante 20 segundos seguida de 40 ciclos de 3 segundos a 95 °C y 30 segundos a 60 °C y finalmente una curva de disociación para comprobar la especificidad de la amplificación.

Los niveles de expresión génica se obtuvieron mediante cuantificación relativa empleando el método $2^{-\Delta\Delta Ct}$. El factor de normalización para cada muestra se calculó como la media geométrica de la expresión de los valores Ct de los genes normalizadores Gliceraldehído-3-fosfato deshidrogenasa (*GAPDH*) y beta-2 microglobulina (B_2M).

5.2 Ensayo de inmunoadsorción ligado a enzima (ELISA)

La técnica ELISA se utilizó en la investigación *in vitro* desarrollada en el **Manuscrito III** para valorar el perfil de secreción de IL6 y PGE2 de las MSCs en distintas situaciones, así como en el **Manuscrito II** para valorar la producción de IFNɣ por parte de las células T al ser enfrentadas a MSCs en distintas condiciones. La secreción de estas moléculas de interés se analizó en el sobrenadante de cultivo de MSCs (MSC-SE, MSC-E y MSC-C), antes (basal) y después de enfrentarlas a linfocitos autólogos o alogénicos MHC-compatibles e incompatibles. Asimismo, también se analizó la secreción de IFNɣ en los

cocultivos realizados tras la administración de las MSCs *in vivo* a los distintos receptores (**Manuscrito V**).

La técnica ELISA permite detectar la presencia de antígenos en muestras biológicas a través de un anticuerpo enlazado a una enzima. De la misma manera que otros tipos de inmunoensayos, esta técnica se basa en la utilización de interacciones anticuerpo-antígeno altamente específicas. En un ensayo ELISA, el antígeno se inmoviliza directamente o mediante el uso de un anticuerpo de captura inmovilizado en la superficie. A continuación, el antígeno debe formar un complejo con un anticuerpo de detección conjugado con una molécula susceptible de detección, como una enzima o un fluoróforo. Existen cuatro tipos principales de ELISA: ELISA directo, ELISA indirecto, ELISA sándwich y ELISA competitivo y cada uno de ellos presenta ventajas, desventajas y características únicas. Sin embargo, en la especie equina existe una limitada disponibilidad de kits ELISAs especie-específicos, por lo que en la presente Tesis Doctoral se utilizaron kits ELISAs tipo sándwich para estudiar IFNɣ (Equine IFN-gamma DuoSet ELISA, R&D Systems, REF: DY1586) e IL6 (Equine IL6 DuoSet ELISA, R&D Systems, REF: DY1886) y tipo competitivo para estudiar PGE2 (Prostaglandin E2 Parameter Assay Kit, R&D Systems, Ref: KGE004B).

6. IMPLANTACIÓN DE CÉLULAS MADRE MESENQUIMALES EQUINAS *IN VIVO*

Todos los procedimientos relacionados con la fabricación, colocación y extracción de los implantes junto con la preparación de las MSCs para los ensayos *in vivo* se encuentran detallados en el **Manuscrito IV**.

6.1 Fabricación de los implantes de alginato

Para la construcción de cada implante, se mezcló 1 mL de una suspensión celular con 5 × 10^6 MSCs con 1 mL de una solución de alginato (NovaMatrix) al 3%. El alginato se polimerizó por inmersión en una solución estéril de $CaCl_2$ 102 mM durante 5 minutos. A continuación, los implantes se incubaron durante 30 minutos a 37 °C y 5% CO_2. Tras la incubación, se retiró la solución de $CaCl_2$ y los implantes se lavaron 3 veces con PBS.

6.2 Preparación de las células madre mesenquimales para los ensayos *in vivo*

Los implantes de MSC-SE y MSC-E se mantuvieron en el medio de cultivo de MSCs descrito previamente durante 48 horas. En los implantes de MSC-E, 12 horas antes de la colocación, se añadieron 5 ng/mL de cada una de las citoquinas proinflamatorias: TNFα e IFNɣ (R&D Systems).

En el caso de las MSC-C, la diferenciación condrogénica se realizó en los mismos implantes con medio de inducción condrogénico durante 14 días a 37 °C y 5% de CO_2. Tras la polimerización, los implantes se lavaron tres veces con PBS y una vez con medio de diferenciación condrogénica incompleta (ICM) que consistía en DMEM con alto contenido en glucosa (Sigma-Aldrich) suplementado con 10% de FBS, 100 U/mL de penicilina y 0,1 mg/mL de estreptomicina, 2 mM de L-glutamina, 1% de insulina, solución

de suplementos de transferrina y selenio (ITS+ premix, Beckton Dickinson), 1 mM de piruvato sódico (Gibco), 100 nM de dexametasona, 50 µg/mL de ácido ascórbico-2-fosfato y 40 µg/mL de prolina (Sigma-Aldrich). Los implantes se cultivaron a 37 °C y 5% CO_2 durante 14 días en 2 mL de medio condrogénico completo (CCM) suplementado con 10 ng/mL de TGFβ3 (Peprotech) por implante, cambiando el medio cada dos días.

Todos los implantes (MSC-SE, MSC-E y MSC-C), 24 horas antes de su colocación, se lavaron 2 veces con PBS y se sustituyó el FBS del medio de cultivo por suero autólogo de cada receptor para que las MSCs estuvieran libres de antígenos xenogénicos que pudieran interferir con la respuesta inmunitaria. Posteriormente, justo antes de la colocación de los implantes, se lavaron de nuevo tres veces con PBS.

6.3 Colocación y retirada de los implantes

En cada animal se colocaron de forma subcutánea tres implantes de alginato con 5×10^6 MSCs por implante. Se colocaron a lo largo de las tablas del cuello, de craneal a caudal, dejando aproximadamente 10 cm entre ellos utilizando la técnica del bolsillo subcutáneo (Bellas et al. 2015) (**Figura 18**). Cada implante se retiró a un tiempo distinto: 1, 3 y 6 semanas. El mismo procedimiento se repitió en las tablas del cuello contralaterales un mes después de la retirada del último implante retirándolos a los mismos tiempos. Las MSCs de estos implantes fueron del mismo donante y tipo que las colocadas en la primera intervención con la finalidad de valorar la capacidad de memoria del organismo frente a las MSCs.

Para llevar a cabo la intervención quirúrgica, que se realizó en estación, los caballos se sedaron con detomidina (0,01 mg/kg, Sedaquick, Fatro) y butorfanol (0,02 mg/kg, Torbugesic, Pfizer). Además, se les administró una única dosis de flunixin meglumine IV (1,1 mg/Kg, Niglumine, Calier). Tras la preparación aséptica del campo quirúrgico, se inyectó lidocaína al 5%, (Anesvet, Laboratorios Ovejero) en las zonas de incisión.

A cada caballo se le hicieron tres incisiones horizontales de aproximadamente 3 cm de longitud y se diseccionó el tejido subcutáneo de toda la zona ventral a la incisión creando un bolsillo subcutáneo de 1,5x1,5 cm. A continuación, se colocaron los implantes dentro de los bolsillos y se suturó cada incisión en dos planos. Además, en los bordes ventral, caudal y rostral de cada bolsillo también se colocaron grapas para delimitar la zona de implantación y evitar la migración de los implantes. Durante los siguientes 3 días, los caballos se mantuvieron con cobertura antibiótica (penicilina procaína IM a 15 mg/kg una vez al día, Procapen, Livisto).

Para la extracción de los implantes, que se llevó a cabo a los tiempos mencionados, se siguió el mismo procedimiento de preparación del campo quirúrgico que en la colocación de los implantes. Para extraer cada implante se hizo una incisión en el borde rostral de cada bolsillo de unos 3 cm de longitud y se diseccionó el tejido subcutáneo (**Figura 18**). La incisión se cerró como se ha mencionado anteriormente y tras la intervención, los caballos fueron monitorizados durante las siguientes 72 horas.

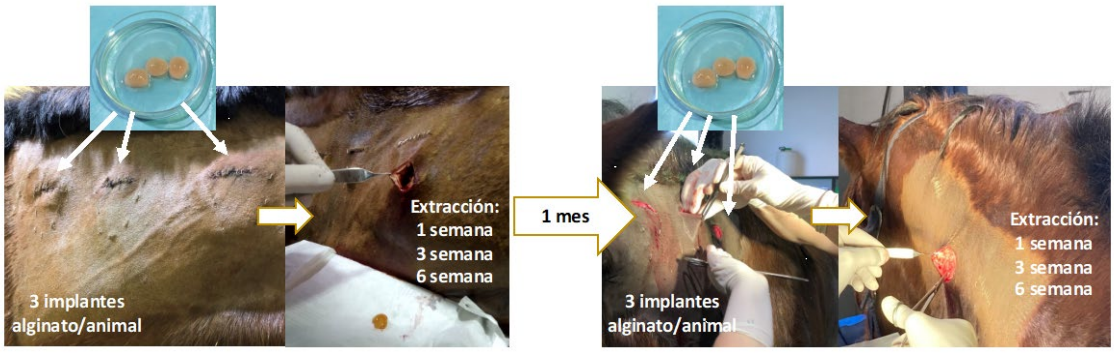


Figura 18. Representación esquemática de la colocación y retirada de los implantes

7. VALORACIÓN CLÍNICA DE LOS ANIMALES EN LOS ENSAYOS *IN VIVO*

El seguimiento clínico local y sistémico se realizó como parte de los ensayos *in vivo* incluidos en el **Manuscrito IV**. La valoración clínica de los animales se realizó antes de la intervención y dos veces al día durante los primeros 3 días postoperatorios y luego una vez al día para las primeras 2 semanas post-implantación. Se llevaron a cabo los siguientes controles clínicos: frecuencia cardiaca, frecuencia respiratoria, temperatura rectal, color de mucosas, tiempo de relleno capilar, sonidos intestinales, apetito, sed y producción de heces.

En estos mismos tiempos también se exploraron y evaluaron los sitios de implantación, incluyendo temperatura de superficie de estos y sensibilidad a la palpación. También se realizaron controles ecográficos de los lugares de implantación utilizando una sonda ecográfica lineal de baja frecuencia inmediatamente antes y después de la colocación de los implantes, y previamente a la extracción.

8. VALORACIÓN HISTOLÓGICA DE LOS IMPLANTES

Para llevar a cabo la valoración seriada de la respuesta inmunitaria celular a nivel local post-implantación (**Objetivo 3**), cada implante se dividió en dos piezas tras su retirada para realizar tanto el estudio de expresión génica como análisis histopatológico, estando ambos recogidos en el **Manuscrito IV**.

En análisis histológico consistió en el estudio de los implantes, y en los caballos que presentaron un aumento del tejido subcutáneo, se llevó a cabo una disección de las cápsulas fibrosas que rodeaban los implantes con el fin de analizarlas de manera simultánea. Un evaluador ciego valoró todos los parámetros recogidos en la **Tabla 9** asignó y a cada muestra se le asignó una puntuación utilizando un sistema de puntuación de 0 a 3. La puntuación que obtuvo cada caballo para cada parámetro se normalizó respecto a la puntuación obtenida en los implantes acelulares utilizados en el estudio.

Tabla 9. Escala utilizada para la valoración de las preparaciones histológicas. Se incluyen 4 grados denominados de 0 a 3 para cada uno de los parámetros estudiados y la descripción de cada categoría.

Puntuación histológica		
Linfocitos	0	Ausencia de linfocitos en la sección
	1	Linfocitos ocasionales a lo largo de la sección
	2	Leve presencia de linfocitos con pequeños focos
	3	Presencia difusa de linfocitos en toda la sección
Células polimorfonucleadas (PMN)	0	Ausencia de PMN en la sección
	1	PMN ocasionales a lo largo de la sección
	2	Leve presencia de PMN con pequeños focos
	3	Presencia difusa de PMN en toda la sección
Eosinófilos	0	Ausencia de eosinófilos en la sección
	1	Eosinófilos ocasionales a lo largo de la sección
	2	Leve presencia de eosinófilos con pequeños focos
	3	Presencia difusa de eosinófilos en toda la sección
Histiocitos	0	Ausencia de histiocitos en la sección
	1	Histiocitos ocasionales a lo largo de la sección
	2	Leve presencia de histiocitos con pequeños focos
	3	Presencia difusa de histiocitos en toda la sección
Proliferación vascular	0	Ausencia de proliferación vascular
	1	Proliferación vascular leve y focal
	2	Proliferación vascular moderada
	3	Proliferación vascular destacada y difusa en toda la sección
Proliferación fibroblástica	0	Ausencia de proliferación fibroblástica
	1	Proliferación fibroblástica leve y focal
	2	Proliferación fibroblástica moderada
	3	Proliferación fibroblástica destacada y difusa en toda la sección
Fibrosis	0	Ausencia de fibrosis
	1	Fibrosis leve y focal
	2	Fibrosis moderada
	3	Fibrosis destacada y difusa en toda la sección
Hemorragia	0	Ausencia de hemorragia
	1	Hemorragia leve y focal
	2	Hemorragia moderada
	3	Hemorragia destacada y difusa en toda la sección
Cantidad de implante en la cápsula	0	Ausencia de implante
	1	Depósito superficial por debajo de la cápsula interna
	2	Implante que afecta a menos de la mitad de la pared
	3	Implante que afecta a más de la mitad de la pared
Células multinucleadas	0	Ausencia de células multinucleadas alrededor del implante
	1	Células multinucleadas ocasionales alrededor del implante
	2	Varias células multinucleadas alrededor del implante
	3	Múltiples células multinucleadas alrededor del implante
Metaplasia sinovial	0	Ausencia de metaplasia sinovial
	1	Metaplasia sinovial leve y focal
	2	Metaplasia sinovial moderada
	3	Metaplasia sinovial difusa a lo largo de la cápsula
Necrosis grasa	0	Ausencia de necrosis grasa

	1	Necrosis grasa leve y focal con adipocitos necróticos ocasionales.
	2	Pequeños grupos de adipocitos necróticos
	3	Grandes grupos de adipocitos necróticos
Hemosiderina	0	Ausencia de pigmento
	1	Depósitos focales y aislados de pigmento
	2	Pequeños depósitos de pigmento
	3	Grandes depósitos de pigmento
Grasa	0	Ausencia de grasa en la sección histológica
	1	Presencia leve y focal de grasa
	2	Presencia moderada de grasa, menos del 50% de la sección
	3	Presencia de grasa, más del 50% de la sección
Fascia muscular	0	Ausencia
	1	Presencia

9. ENSAYOS DE MICROCITOTOXICIDAD

Estos ensayos se realizaron de forma seriada a lo largo del estudio recogido en el **Manuscrito VI** con el objetivo de valorar la respuesta inmunitaria humoral a lo largo del tiempo tras la colocación de los implantes (**Objetivos 4** y **5**). Se realizaron ensayos de microcitotoxicidad enfrentando el suero de cada receptor a las MSCs de cada donante (MHC-compatibles o incompatibles) para detectar la presencia de anticuerpos citotóxicos en el suero de los receptores siguiendo la metodología previamente descrita (Pezzanite et al. 2015; Berglund y Schnabel 2017). Las MSCs de cada donante se diluyeron en PBS a concentración de 1×10^6 células/mL y el suero correspondiente de cada receptor se utilizó sin diluir y diluido en PBS a 1:2 y 1:16. En placas Terasaki (One Lambda) se enfrentó 1 µL de cada dilución de suero con 1 µL de la suspensión de MSCs correspondiente y se incubaron durante 30 minutos a temperatura ambiente. A continuación, se añadieron 5 µL de complemento de conejo y las placas Terasaki se incubaron una hora más a temperatura ambiente. Los pocillos se tiñeron con 2 µL de eosina al 5% y se fijaron con 5 µL de formalina al 10%. La citotoxicidad en cada pocillo se valoró observando al microscopio el porcentaje de células muertas y utilizando el siguiente sistema de puntuaciones: grado 1 (<10% células muertas), grado 2 (10-19% células muertas), grado 4 (20-49% células muertas), grado 6 (50-80% células muertas), grado 8 (100% células muertas).

V. RESULTADOS Y DISCUSIÓN

MANUSCRITO I

animals

MDPI

Review

The Usefulness of Mesenchymal Stem Cells beyond the Musculoskeletal System in Horses

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Simple Summary: The main target of mesenchymal stem cell therapy in horses has long been the locomotor system, because these athletic animals commonly suffer from tendon and joint lesions. Originally, mesenchymal stem cells were thought to act by just differentiating into the cells of the injured tissue. However, these cells are also able to regulate and stimulate the body's own repair mechanisms, opening the door to many applications in inflammatory and immune-mediated disorders in both animals and humans. In horses, beyond their traditional application in the musculoskeletal system, these cells have been studied for ophthalmologic pathologies such as corneal ulcers or immune-mediated processes, and for reproductive disorders such as endometritis/endometrosis. Their potential has been explored for equine pathologies very similar to those affecting people, such as asthma, metabolic syndrome, aberrant wound healing, or endotoxemia, as well as for equine-specific pathologies such as laminitis. Current evidence is still preliminary, and further research is needed to clarify different aspects, although research performed so far shows the promising potential of mesenchymal stem cells to treat a wide variety of equine pathologies, some of which are analogous to human disorders. Therefore, advancements in this path will be beneficial for both animals and people.

Abstract: The differentiation ability of mesenchymal stem cells (MSCs) initially raised interest for treating musculoskeletal injuries in horses, but MSC paracrine activity has widened their scope for inflammatory and immune-mediated pathologies in both equine and human medicine. Furthermore, the similar etiopathogenesis of some diseases in both species has advanced the concept of "One Medicine, One Health". This article reviews the current knowledge on the use of MSCs for equine pathologies beyond the locomotor system, highlighting the value of the horse as translational model. Ophthalmologic and reproductive disorders are among the most studied for MSC application. Equine asthma, equine metabolic syndrome, and endotoxemia have been less explored but offer an interesting scenario for human translation. The use of MSCs in wounds also provides a potential model for humans because of the healing particularities in both species. High-burden equine-specific pathologies such as laminitis have been suggested to benefit from MSC-therapy, and MSC application in challenging disorders such as neurologic conditions has been proposed. The available data are preliminary, however, and require further development to translate results into the clinic. Nevertheless, current evidence indicates a significant potential of equine MSCs to enlarge their range of application, with particular interest in pathologies analogous to human conditions.

Keywords: One Medicine; mesenchymal stem cells; immune-mediated disorders; ophthalmology; reproduction; equine asthma; equine metabolic syndrome; wounds; laminitis; neurologic disorders

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1. Introduction

The equine industry is a sector of great economic importance, mainly due to the sporting dedication of the horse. Thus, musculoskeletal conditions have a great relevance in these animals. The limited healing capacity of ligaments, tendons, and cartilage have led to the interest in advanced biological therapies [1] aimed at restoring the structure and functions of tissues or organs using the own mechanisms of the body [2], of which mesenchymal stem cells (MSCs) are of particular relevance.

Initially, MSC therapeutics were predicated to be based on their ability to differentiate into cells of the appropriate tissue type, and thus to directly stimulate regeneration of the damaged structures. However, it has been shown that MSCs exert their effects mainly through the secretion of a wide range of bioactive molecules [1], which significantly increases the scope for their therapeutic applications. Actually, the main focus on musculoskeletal application in equine practice is in contrast with human medicine, where MSC therapies are primarily focused on immune-mediated, inflammatory, and ischemic diseases [3].

Even though musculoskeletal pathologies are among the most frequent in horses, the alteration of other systems is also of great relevance provided their economic and welfare impact [4]. The treatment of some of these pathologies presents important limitations owing to different factors, such as horse anatomy and healing physiology in the case of wounds [5], or intrinsic characteristics of immune-based pathologies such as asthma, where only palliative treatments are available [6]. Thus, the range of therapeutic mechanisms elicited by equine MSCs could extend their application beyond the locomotor system. Moreover, numerous naturally occurring diseases in both animals and humans (developmental, infectious, autoimmune, or allergic) have similar pathophysiological bases [7].

This review aims at revising the current knowledge on the use of MSCs for equine pathologies beyond the locomotor system, including ophthalmic and reproductive pathologies, equine metabolic syndrome, equine asthma, wounds, laminitis, neurological disorders, and systemic inflammatory response syndrome, highlighting the value of the horse as a translational model for developing novel treatments that could benefit both animals and humans.

1.1. "One Medicine, One Health": The Role of the Horse

In comparison with small animals such as rodents, large species such as horses better resemble the anatomy and physiology of humans, and their greater lifespan allows for longer-term follow-up. Several organizations such as the European Medicines Agency, the U.S. Food and Drug Administration, and the International Society for Stem Cell Research are recommending the use of large animal models to evaluate the efficacy, durability, dose response, and safety of advanced therapeutic medicinal products [8]. Horses are already recognized as models for several human diseases, including metabolic syndrome [9], asthma [10], musculoskeletal diseases [11,12], melanoma [13], or autoimmune uveitis [14]. Moreover, other equine conditions may also serve as models, including infectious diseases [15], fertility disorders [16], and even depression and mental conditions [17,18].

All of this led to the development of the "One Health, One Medicine" approach, which, according to the World Health Organization, promotes a vision of the health of humans, animals and the environment as a coherent system, and presumes that diseases in humans and animals require analogous therapeutic approaches [19]. Hence, progress in animal models is mutually beneficial for animals, researchers, and human and veterinary patients. Importantly, the rapid advancement of regenerative medicine in the horse makes this species particularly relevant for translational research [20].

1.2. Properties of Mesenchymal Stem Cells

Mesenchymal stem cells are adult and multipotent stem cells of mesodermal origin, which have raised interest in the field of regenerative medicine due to their unique biological properties [21]. Equine MSC characterization is based on the recommendations of the

International Society for Cell Therapy (ISCT) set for human MSCs, which include cellular plastic adherence, expression of the surface markers cluster of differentiation (CD)90, CD105 and CD73, and the lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and human leukocyte antigen (HLA) class II. In addition, these cells must be able to differentiate at least into osteoblasts, adipocytes and chondrocytes in vitro [22]. Equine MSCs meet the set criteria of plastic adherence and multipotency, but variably express different surface markers depending on tissue source [23,24].

In equine medicine, MSCs are often the choice for advanced therapies, due to their ease of isolation and culture, their multipotency, and their ability to migrate to damaged tissues [25]. The mechanisms of action through which MSCs exert their effects have not been fully characterized [21]. Initially, it was thought that MSCs differentiated directly into cells of the affected tissue or enhanced the activity of resident cells [3], although it has been reported that MSCs can act indirectly by secreting immunomodulatory and bioactive factors [22–24,26,27].

These paracrine effects can be divided into immunomodulatory, anti-scarring, chemoattractant and trophic effects, which can be further subdivided into anti-apoptotic, supportive (stimulation of mitosis, proliferation and differentiation of precursors) and angiogenic. The number of molecules known to mediate the paracrine action of MSCs increases every day [28]; thus indicating that there is a substantial potential to harness these properties to treat several medical conditions in horses.

For example, MSCs secrete different chemokines that recruit and regulate the function of several cell types, as well as growth factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) or the leukemia inhibitory factor (LIF) that promote survival and stimulate the proliferation of resident cells. Some of these growth factors, such as VEGF, also elicit pro-angiogenic effects of great importance, because the restoration of blood supply is essential for the recovery of damaged tissues [25].

In addition, MSCs secrete different cytokines and mediators such as interleukin (IL)-6 and 10, prostaglandin E-2 (PGE-2), transforming growth factor (TGF-β), or nitric oxide (NO), which elicit immunomodulatory actions such as inhibiting the proliferation of T lymphocytes [29], prevent lysis mediated by cytotoxic T cells [30], suppress the activation of natural killer (NK) cells [31] and macrophages [32], or modulate B cell proliferation [33].

2. Applications of Equine MSCs in Ophthalmology

The corneal epithelium contains limbal stem cells (LSCs), whose deficiency may greatly affect corneal transparency and integrity of the ocular surface [34]. Mesenchymal stem cells can differentiate into corneal epithelial cells both in vitro and in vivo in rabbits [35]. In addition, MSC paracrine activity can reduce oxidative stress and inhibit the release of pro-inflammatory cytokines, thereby reducing corneal inflammation and neovascularization [36,37]. The in vivo ophthalmologic studies presented here are summarized in Table 1.

2.1. Corneal Ulcers and Ulcerative Keratitis

Horses are more prone to corneal damage than other species because of their large-sized eyes placed laterally and prominently [38], and the active movements of their head which favors exposure to bacterial or fungal contamination [39]. Some corneal ulcers can be very severe due to protease activity and can lead to vision loss. The therapeutic strategy should be aimed at eradicating the infection and limiting cornea destruction, in order to control pain and minimize scar formation [40]. Current therapies include topical antibiotics, anti-proteases, and mydriatic or cycloplegic drugs [41], but the typically associated pain makes repeated local administration difficult.

The wound healing capacity of equine autologous bone marrow-derived MSCs (BM-MSCs) and their supernatant (MSC-Sp) was evaluated in vitro using a scratch assay in corneal fibroblasts. The significant decrease in the scratch area after exposure to either MSCs or MSC-Sp suggested their potential to improve corneal healing. Interestingly, the

use of MSC-Sp may provide a stem cell-derived but cell-free product that could be more easily stored and applied [42].

Regarding in vivo studies, in one case of bacterial ulcerative keratitis unresponsive to conventional therapies, a single dose of autologous peripheral blood-derived stem cells (PB-SCs) was applied both systemically (intravenously (IV), jugular vein) and in transverse facial artery. In addition, local application was performed three times a day for seven days. Three months later, the ulcer was almost unnoticed and the clinical signs of inflammation, pain and irritation disappeared [43]. Similar results were observed in another case of bacterial ulcerative keratitis and in three cases of corneal ulcers treated with IV and local administration of autologous PB-SCs [40].

2.2. Equine Recurrent Uveitis

Equine recurrent uveitis (ERU) is a spontaneous and immune-mediated disorder characterized by recurrent episodes of intraocular inflammation separated by periods of remission [44]. The exact pathophysiology of ERU is not clear, although it is thought that *Leptospira interrogans* can be implicated by initiating an infection that leads to ocular immune privilege breakdown [45]. The subsequent immune response involves cytokines and chemokines that activate helper T cells (Th), and Th17-associated cytokines seem to play a role [46]. Currently, there is no cure, and treatment is focused on preserving vision, alleviating pain, and limiting the recurrence of episodes by using mydriatics and anti-inflammatory drugs. The end stage of the disease in the majority of affected horses is blindness [44].

Mesenchymal stem cells are effective at reducing immune cell activation in vitro in many species, making them a potential therapeutic option for ERU [47]. It has been shown in several mammals, such as cats, dogs and horses, that MSCs can induce a switch from pro-inflammatory to regulatory T cell subsets when applied in immune diseases [47–49]. The same has been suggested for ERU in an in vitro assay in which lymphocytes from ERU-affected horses were co-cultured with adipose-derived MSCs (AT-MSCs) [50]. However, to the best of the authors' knowledge, there is no literature on controlled equine clinical studies employing MSCs in ERU except for a brief mention in the study of Saldinger et al., which stated satisfactory treatment of three cases (unpublished data).

Interestingly, ERU closely resembles human autoimmune uveitis regarding clinical and immune pathological features, including the same autoantigens involved and the remitting-relapsing onset of the disease. Therefore, ERU has been suggested as a reliable spontaneous model to study the histopathological changes and the inflammatory processes in uveitis [14,51]; thus, therapeutic advancements in ERU and human uveitis can be mutually beneficial.

2.3. Equine Immune Mediated Keratitis

Equine immune mediated keratitis (IMMK) is a generic term used to describe a heterogeneous group of chronic, non-ulcerative corneal opacities accompanied by intraocular inflammation. The etiopathogenesis is unknown, but seems to be related with dysregulated immune responses involving a complex cytokine cascade and amplified pro-inflammatory response [41]. Long-term topical anti-inflammatory and immunosuppressive therapy is the main management protocol, although it does not offer a definitive solution and can be challenging to maintain with low owner compliance or poor response to treatment [52].

Table 1. Ophthalmology in vivo studies using stem cells in horses. Peripheral blood stem cells (PB-SCs), phosphate-buffered saline (PBS), equine recurrent uveitis (ERU), bone marrow-derived mesenchymal stem cells (BM-MSCs), immune-mediated keratitis (IMMK), intravenous (IV).

Pathology	Study	Type of MSCs	Experimental Design	Administration Regime	Outcome	Considerations
Corneal ulcers	Spaas et al., 2011 [43]	Autologous PB-SCs	Naturally occurring pathology (one case): 20-year-old gelding, with bacterial (*Pseudomonas aeruginosa*) ulcerative keratitis, resistant for 6 months against conventional therapies and surgical intervention.	A one-time injection in the jugular vein (125×10^3 PB-SCs in 5 mL PBS) and in transverse facial artery (125×10^3 PB-MSCs in 5 mL PBS) + local application (eye drops formulation, 500×10^3 PB-SCs in 5 mL PBS) 3 times/day, 7 days.	• 2 weeks: inflammation and tear flow decreased; ulcer size reduced • 3 months: eye ulcer almost invisible and inflammation, pain and irritation disappeared. Better general condition.	Single case with no control Cells used not expanded and not fully characterized as MSCs After 7 days, eye drop application was stopped because the cell suspension appeared cloudy, probably because of cell death. Storage conditions of drop bottle between administrations not specified.
	Marfe et al., 2012 [40]	Autologous PB-SCs	Naturally occurring pathology (4 cases) • Case 1: 20-year-old gelding with bacterial (*Pseudomonas aeruginosa*) ulcerative keratitis resistant for 6 months against conventional therapies and surgical intervention • Case 2: 7-year-old mare with a corneal ulcer treated for 2 weeks and IMKK treated for a year • Case 3: 12-year-old gelding with traumatic corneal ulcer treated for 6 months • Case 4: 13-year-old gelding with ERU-derived corneal ulcer treated for 2 weeks	1–2 systemic administration (IV) + local instillation 2–3/day for 2 weeks.	Case 1: • 2 weeks: inflammation and lacrimation decreased; ulcer size reduced and stable. • 3 months: eye ulcer reduced, inflammation stable, pain and irritation disappeared. • >3 months: eye ulcer disappeared Case 2: • 1 month: no signs of relapse Case 3: • 2 weeks: ulcer significantly reduced • 1 month: deposit of melanin (scarring effects) Case 4: • 2 months: corneal ulcer completely disappeared	Low number of cases and with corneal ulcers of different origins Absence of control group, but animals unresponsive to previous treatments Cells used not expanded and not fully characterized as MSCs Number of PB-SCs administrated not stated Administration route and protocol unclear at some points

Table 1. *Cont.*

Pathology	Study	Type of MSCs	Experimental Design	Administration Regime	Outcome	Considerations
IMMK	Marfe et al., 2012 [40]	Autologous PB-SCs	Naturally occurring pathology (one case): 7-year-old mare, poorly responsive to traditional medical treatments for a year (Case 2 above)	2 systemic administration (IV) + local instillation 2/day for 2 weeks	• 1 month: no signs of relapse • 3 months: complete recovery of clinical signs	A single case with no control group Number of PB-SCs administered not stated
	Davis et al., 2019 [53]	Autologous BM-MSCs	Naturally occurring pathology (4 cases): unilateral IMMK poorly responsive to traditional medical treatments • Case 1: 9-year-old gelding with midstromal keratitis • Case 2: 12year-old mare with superficial to midstromal keratitis • Case 3: 9-year-old gelding with midstromal keratitis • Case 4: 10-year-old gelding with midstromal keratitis	Subconjunctival injection (15×10^6 MSCs in 1 mL PBS) every 3–4 weeks for 1–5 injections • Case 1: 1 single injection • Case 2: 3 injections every 3–40 weeks • Case 3: 3 injections every 3–6 weeks • Case 4: 3 injections every 3 weeks Cases 2 and 3 received 1 and 2 additional injections, respectively	• 3 weeks: improvement of clinical signs (decreased fibrosis/opacity, irregularity, and vascularization). • No relapse for average 1 year. • Case 1, 2 and 3: Resolution of fibrosis and neovascularization • Case 4: Enucleation due to disease worsening and discomfort	Low number of cases with no control group, but selected upon disease similarities and unresponsiveness to medical treatment. Variability in the administration protocol Additional topical treatments in some case in conjunction with the BM-MSCs (cyclosporine, bromfenac, diclofenac or flunixin meglumine)

Due to the immunomodulatory action of MSCs, these have been suggested as potential therapeutic tools. One case of IMMK was reported to have a positive response after two intravenous injections of autologous PB-SCs and local instillation, without signs of relapse [40]. In another study involving four horses with IMMK unresponsive to conventional treatment, subconjunctival injection of autologous BM-MSCs (1–5 injections every 3–4 weeks) was tested along with usual treatment. Three horses had a positive clinical response, as demonstrated by decreased corneal opacity, diminished neovascularization, and improvement in surface irregularity [53].

The limitations of most in vivo ophthalmologic studies include a small sample and variability in patient selection, along with lack of a control group. Nevertheless, all cases were poorly responsive to medical management and showed improvement after MSC therapy. The administration protocol, including route, number of applications, and duration of the treatment, as well as medical management before and during MSC therapy, varied significantly. Regarding the route of administration, the subconjunctival space is relatively common for ocular medications and provides high drug concentration to the cornea and anterior structures for prolonged periods [54]; therefore, it could represent a suitable approach for local MSC administration that avoids repeated manipulation of the painful eye for local instillation, which also poses the problem of prolonged storage [43]. Other delivery systems include local intra-arterial injection in order to reach the internal structures of the eye, and contact lenses seeded with MSCs have been used in other models [55]. Further research based on controlled studies and standardized protocols supported by distribution assays is needed to demonstrate the benefits of such treatments and implement its use in clinical practice.

3. Applications of Equine MSCs in Reproduction

Due to the MSC regenerative and immunomodulatory properties, these might be used to treat damaged reproductive tissues, as has been proposed in different animal models, such as rats [56]. Table 2 summarizes the in vivo reproduction-related studies discussed below.

3.1. Endometritis

Endometritis is the infection and/or inflammation of the endometrium, and constitutes the main cause of subfertility and is the third most common disease affecting horses [57]. Repeated inflammation of the endometrium can lead to endometrosis, a chronic state characterized by fibrosis with glandular alterations [58]. Both persistent breeding-induced endometritis (PBIE) and endometrosis are of pivotal importance for reproductive health in mares.

3.1.1. Equine Persistent Breeding-Induced Endometritis

Persistent breeding-induced endometritis is an acute inflammatory response of the endometrium to semen, linked to an incapability of the uterus to remove bacteria, spermatozoa, and inflammatory exudate post-breeding. This disease affects broodmares of all breeds, leading to reproductive inefficiency and important economic losses [59]. Traditional therapeutic modalities lack sufficient effectivity, which along with increasing antibiotic-resistances, has led to the development of alternative therapies such as MSCs [60], because of their engraftment and immunomodulatory abilities.

Studies in healthy mares have provided a first insight into the effects of MSCs on reproductive tissues. Autologous MSCs isolated from endometrial biopsies harvested from healthy mares were infused into both uterine horns during the early diestrus to avoid ready elimination from the uterine lumen. Endometrial mesenchymal stem cells (eMSCs) were detected in the uterine lumen up to 24 h after infusion, but they did not engraft into the endometrium. Moreover, eMSCs effectively attenuated the inflammatory response produced by the uterine infusion itself [61]. Another study showed that allogenic BM-MSCs infused into the uterus 24 h before insemination modulated the uterine

inflammatory response in healthy mares [62]. Subsequently, PBIE was induced in nine healthy mares which received intrauterine instillation of allogeneic AT-MSCs or eMSCs. The MSC administration significantly reduced inflammation regardless of the origin of the cells, but their engraftment after one month was limited, suggesting that their function in this context is preferentially exerted by paracrine mechanisms [63].

3.1.2. Endometrosis

Endometrosis is characterized by periglandular and/or stromal endometrial fibrosis, including glandular alterations within fibrotic foci. The etiology is not fully elucidated, but it seems to be age-related [64]. The endometrial changes caused by this disease are irreversible, and therapeutic management is challenging [65].

In an ex vivo model, endometrial biopsies from healthy and pathological mares were exposed to allogeneic AT-MSCs, which were able to infiltrate both the periglandular space and single glands of endometrosis-affected tissue [66]. In an in vivo study, autologous BM-MSCs were injected directly into the uterus using a catheter in mares with subfertility history and degenerated endometrium to assess safety in pathological conditions, observing no clinical alterations, histological changes, or endometrial edema [67]. These studies suggest the feasibility and safety of endometrial injection of MSCs as a new therapeutic approach for uterine disorders.

Another study reported that allogeneic AT-MSCs infused in the uterine lumen of six endometrosis-affected mares effectively homed into both the glandular and non-glandular endometrium when delivered by a simple technique, similar to that used for artificial insemination [68]. A continuation of this study showed histological improvement of endometrosis after infusing allogeneic AT-MSCs. The authors suggested that multiple mechanisms, including homing to fibrotic areas and increased epithelial cell proliferation, might mediate the anti-scarring effects observed [69]. Furthermore, amniotic membrane-derived MSCs may improve the endometrial cell replenishment when scarcity or low proliferation of endometrial cells is associated with pregnancy failure [70].

Compared to BM-MSCs, eMSCs have a higher ability to differentiate into smooth muscle [71], and display robust immunomodulatory [72] and migratory properties [73]. Moreover, eMSCs can be collected from simple endometrial biopsies, which can be safely and repeatedly obtained, thus suggesting a novel source of therapeutic cells for inflammatory conditions of the uterus [74].

3.2. Ovarian Diseases

Intra-ovarian injection of allogeneic BM-MSCs to treat ovarian dysfunction in old mares has been suggested in one study. Although there were no side effects, MSC injection was not associated with significant changes in follicle number, oocyte recovery and maturation rate, or blastocyst rate [75]. This contrasts with MSC beneficial effects observed in chemotherapy-damaged ovaries in other species [76,77], but these data should be extended to be conclusive, because to the best of the authors' knowledge, there are no further studies exploring this possibility.

3.3. Testicular Diseases

Testicular disorders are characterized by altered or suppressed activity leading to important reproductive issues in stallions and consequent economic impacts [78]. Immunomodulatory, anti-inflammatory, and anti-apoptotic effects of MSCs, as well as their ability to migrate to injured tissues, support their potential to treat testicular disorders, as suggested in an induced testicular torsion model in rats [79]. One in vivo study evaluated the intratesticular injection of allogeneic BM-MSCs in healthy stallions. The absence of clinical abnormalities and altered semen parameters suggested that this is a safe procedure [80], although further studies are required to test their therapeutic potential.

Table 2. In vivo studies using mesenchymal stem cells in equine reproduction. Endometrial mesenchymal stromal cells (eMSCs), phosphate-buffered saline (PBS), polymorphonuclear neutrophils (PMNs), bone marrow mesenchymal stem cells (BM-MSCs), anti-Müllerian hormone (AMH), autologous conditioned serum (ACS), lactate ringer (LR), intrauterine (IU), post-breeding induced endometritis (PBIE), adipose tissue MSCs (AT-MSCs), artificial insemination (AI), fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα), intracytoplasmic sperm injection (ICSI).

Pathology	Study	Type of MSCs	Experimental Design	Administration Regime	Outcome	Considerations
Safety and distribution assays in healthy mares	Rink et al., 2018 [61]	Autologous eMSCs	Intra-uterine administration of labelled eMSCs and follow-up: • Uterine cytology • Tracking of eMSCs in peripheral blood • Tracking of eMSCs in uterus (lumen and endometrial biopsies) 7 healthy cycling mares, 4–7 years old.Four of these mares were used as controls, two estrous cycles before the eMSC infusion cycle.	15×10^6 eMSCs in 1 mL or PBS infused into each uterine horn during early diestrus (day 4 after ovulation).	Mild inflammatory reaction after infusion was attenuated by eMSCs (percentage of PMNs lower in eMSC than PBS-infused mares at 6h) eMSCs detected in the uterine horn lumen for up to 24 h after infusion but did not migrate into healthy endometrium. eMSCs were not found in the peripheral blood at 6, 12, and 24 h after application.	eMSCs intra-uterine administration is safe and cells persist in the uterine lumen for up to 24 h after infusion, but do not engraft into healthy endometrium at that time.
PBIE	Ferris et al., 2014 [62]	Allogeneic BM-MSCs	Evaluate the ability of ACS, BM-MSCs or dexamethasone to modulate the inflammatory response to spermatozoa after breeding (24 h) 12 healthy mares Experiment 1: Crossover study Experiment 2: Two-way crossover study Administration of treatments, sperm challenge and follow-up: • Ultrasonographic evaluations • Lavages: PMN and cytokine analysis	Experiment 1: 6 mares treated with an IU infusion of: • 20 mL of ACS in 20 mL PBS • 20 mg of dexamethasone QS in 20 mL PBS • Control: 20 mL PBS One treatment per estrous in the same mares (every 3 weeks). Experiment 2: 6 mares treated with an IU infusion of: • 20×10^6 allogeneic BM-MSCs in 20 mL LR • 20 mL PBS One treatment per estrous in the same mares (every 3 weeks).	BM-MSC and ACS were able to modulate the uterine inflammatory response to spermatozoa in normal mares Decreased neutrophil migration into the uterine lumen in response to insemination after BM-MSC treatment may be due to increased anti-inflammatory cytokine IL-1Ra and reduced proinflammatory mediator IL-1	Healthy mares (proof of concept for PBIE-affected mares). Same mares used for different treatments. Age of mares not stated.

Table 2. *Cont.*

Pathology	Study	Type of MSCs	Experimental Design	Administration Regime	Outcome	Considerations
PBIE	Navarrete et al., 2020 [63]	Allogeneic AT-MSCs and eMSCs	Evaluate anti-inflammatory and engraftment properties of AT-MSCs and eMSCs from the same donors in vivo in mares with induced PBIE. 9 healthy mares with induced PBIE. Follow-up: • Lavages: cytokine and gene expression analysis • Uterine biopsies	2×10^7 AT-MSCs (n = 3) or eMSCs (n = 3) in 20 mL of NaCl 0.9% Control group (n = 3): 20 mL NaCl 0.9%	Both MSC types significantly reduced inflammation and showed limited engraftment, detectable after one month of infusion Decrease in IL-6 and TNFα in both MSC-treated groups over control.	Age of mares not stated. Virgin mares with no previously reported PBIE (possibility of natural PBIE resistance)
Endometrosis	Alvarenga et al., 2016 [67]	Autologous BM-MSCs	Evaluate the feasibility and safety of MSC endometrial injections 16 mares (15–24 years) with reproductive history of subfertility and endometrial degeneration Control: baseline values before endometrial injection Follow-up: • Ultrasonographic evaluations • Uterine biopsies	12 endometrial injections of 1×10^6 MSCs in 0.5 mL PBS, each one at 12 different sites, 1 cm apart from one uterine horn to another (12×10^6 MSCs in total)	Neither clinical alteration nor intrauterine fluid and endometrial edema were observed after MSC administration. No histological worsening The results suggest that the procedure is safe	Proof of concept for safety. Therapeutic effects not thoroughly assessed.
	Mambelli et al., 2013 [68] Mambelli et al., 2014 [69]	Allogeneic AT-MSCs	Evaluate the feasibility of an MSC delivery system for endometrosis-affected mares. 6 mares (6–21 years) with varying degrees of naturally occuring endometrosis Follow-up: • Uterine biopsies 7, 21 and 60 days	20×10^6 AT-MSCs in 20 mL NaCl 0.9% inoculated into uterus using a technique similar to AI Control (n = 2): 20 mL NaCl 0.9%	Migration of AT-MSCs to the uterine body and both horns. Engraftment in both glandular and periglandular spaces in three mares. AT-MSCs beneficially modulated the expression pattern of secretory proteins and promoted proliferation of glandular epithelial cells.	Small control group Different degrees of endometrosis among recruited mares

Table 2. *Cont.*

Pathology	Study	Type of MSCs	Experimental Design	Administration Regime	Outcome	Considerations
Ovarian and testicular diseases	Grady et al., 2019 [75]	Allogeneic BM-MSCs	Determine if intra-ovarian injection of BM-MSCs improves or restores ovarian function in aged mares 8 aged mares (20–29 years old) and 6 young mares (7–12 years old) Assessment (aged and young mares): • Antral follicle count • Serum AMH • Assessment (aged mares only): • Oocyte meiotic and developmental competence • Gross and histological ovarian assessment • Gene expression in ovarian tissue as assessed by RNA sequencing.	2 intraovarian injections of 10 × 10^6 BM-MSCs (different donors) in 1mL (95%FBS and 5%DMSO) in four different locations per ovary Aged mares: First injection • Group 1 (n = 3): BM-MSCs from donor A • Group 2 (n = 3): BM-MSCs from donor B • Group 3: Control (n = 2): 95%FBS and 5%DMSO Second injection: at 6 weeks from different donor Young mares: • Group 1 (n = 2): BM-MSCs from donor A • Group 2 (n = 2): BM-MSCs from donor B • Group 3 (n = 2): Control (95% FBS and 5% DMSO)	No adverse events after intra-ovarian injections were observed Oocyte recovery on follicle aspiration, oocyte maturation, and blastocyst development rates after ICSI remained unchanged in the MSC-treated aged mares, suggesting no detriment of ovarian function but neither a beneficial effect. Injection of BM-MSCs not associated with significant changes in follicle number in young mares. No significant changes in peripheral AMH concentrations in aged and young mares observed, indicating a lack of effect on growing follicles.	BM-MSCs administered immediately after thawing (viability not stated) Small size of groups

Table 2. *Cont.*

Pathology	Study	Type of MSCs	Experimental Design	Administration Regime	Outcome	Considerations
Ovarian and testicular diseases	Papa et al., 2020 [80]	Allogeneic BM-MSCs	Evaluate the effect of intratesticular injection of BM-MSCs in healthy stallions, and its outcome on seminal parameters and fertility Experiment 1: 24 stallions (3–4 years) Experiment 2: 3 stallions (3–10 years) Assessment: Experiment 1: • Testicular morphometry, thermography • Testosterone concentrations • Ultrasonography • Histopathology (after orchiectomy) Experiment 2: • Sperm collection: concentration, kinetics, plasma membrane integrity • Insemination of six healthy mares (8 ± 3.5 years old)	Experiment 1: • Intratesticular injection 10 × 10^6 BM-MSCs in PBS in 5 different points (1 mL in each point) (n = 12) • Same injections with 5 mL PBS (n = 12) Experiment 2 (n = 3): • Intratesticular injection 20 × 10^6 BM-MSCs in PBS in 5 different points (1 mL in each point)	Experiment 1: No signs of inflammation. No differences on testicular volume, parenchyma echogenicity, testicular blood flow and serum testosterone levels between treated and control at 24 h and 28 h. Experiment 2: no physical alterations or changes in sperm parameters. Satisfactory fertility rate (83%; 5/6) after AI. The results suggest that MSC intra-testicular administration would be safe and would not affect testicular function.	No sperm parameters evaluated after BM-MSCs injection in experiment 1 Absence of control group in experiment 2 Small size of group in experiment 2

4. Application of Equine MSCs in Metabolic Disorders

Equine Metabolic Syndrome

Equine metabolic syndrome (EMS) is an endocrine disorder characterized by pathological obesity, insulin dysregulation, altered hepatic function, and predisposition to developing laminitis. Obesity is the main risk factor for EMS, because adipose tissue acts as an important secretory organ producing different molecules such as pro-inflammatory cytokines with associated adverse local and systemic effects [81]. Management of EMS should include a well-balanced diet and physical activity. Some drugs such as metformin can help regulate this disorder, although there are no treatments available that can effectively resolve the EMS [82].

Therefore, MSCs have been proposed as a therapeutic strategy in different metabolic syndromes in several species, such as rodents [83–85], dogs [86] and monkeys [87]. Importantly, great attention has been paid to MSC treatment of human diabetes type 2 [88,89]. Thus, the One Medicine concept applies in both directions; knowledge from human studies is also highly valuable for veterinary medicine.

Adipose-derived MSCs from EMS-affected horses display senescent phenotype, increased apoptosis, and reduced viability and differentiation capacity, which would make autologous therapy suboptimal [90–92]. In a recent study of the same group, autologous AT-MSCs were exposed in vitro to pharmacotherapy with 5-azacytidine (AZA) and resveratrol (RES) before their clinical application in order to reverse the aged phenotype of these cells. These rejuvenated autologous AT-MSCs showed in vivo potential to improve liver metabolism in one EMS-diagnosed horse, as demonstrated by a decrease in specific liver enzymes. Nonetheless, MSC therapy was combined with conventional EMS management, which could have also contributed to this improvement [93].

5. Application of Equine MSCs in the Respiratory System

Equine Asthma

Equine asthma syndrome encompasses a spectrum of inflammatory airway diseases characterized by chronic respiratory signs ranging in severity that can significantly affect athletic performance [6]. Although its exact etiopathogenesis remains incompletely defined, immune-mediated responses are involved and lead to excessive mucus production, neutrophilic accumulation, bronchial hyperreactivity, and bronchospasm [94]. Treatment is limited to environmental management, anti-inflammatory drugs, and bronchodilator therapy. Nevertheless, the use of inhaled corticosteroids may be contraindicated in some cases, and may also be cost-prohibitive and inconvenient for some owners [6].

Studies in murine models of induced asthma have shown that MSCs may be beneficial for managing this process [32,95,96]. To date, there are no published reports on the use of MSCs in vivo to treat equine asthma. However, the use of cell derivatives has been tested. An in vitro assay showed the ability of conditioned medium and microvesicles from equine amniotic mesenchymal cells to modulate lipopolysaccharide (LPS)-stimulated equine alveolar macrophages, suggesting that these products can play a role in treating inflammatory diseases of the lung [97]. In vivo, the intra-tracheal instillation of autologous bone marrow-derived mononuclear cells in eight asthma-affected horses showed that the procedure is safe and improved clinical signs, suggesting amelioration of the asthmatic inflammatory response [98].

Moreover, equine and human asthma syndromes share several features, with the difference that the process is dominated by neutrophils in horses and by eosinophils in humans [6]. The similarities in the airway remodeling processes make equine asthma an ideal model to study the cellular and molecular pathways associated with the asthmatic airway response and its reversibility [10].

6. Application of Equine MSCs in Disorders of the Integumentary System

The main findings of in vivo studies using MSCs for integumentary-related disorders are highlighted in Table 3.

6.1. Wounds

Due to their "flight" instinct in response to frights, horses are particularly susceptible to trauma [99]. Actually, wound injuries are the most common medical condition affecting horses [100,101]. Traumatic wounds commonly occur in the distal limb, where healing is often delayed due to high skin tension, minimal vascularization, and a low-grade inflammatory response. This aberrant inflammation leads to a fibroproliferative response, resulting in a dysplastic healing with exuberant granulation tissue [102]. These particularities make successful wound management difficult, to which MSCs could contribute owing to their varied paracrine activities.

In vitro, equine MSCs promote dermal wound repair through the mobilization of dermal fibroblasts and by increasing the expression of genes involved in wound healing [103]. In an equine distal limb wound model using six horses, allogeneic umbilical cord blood-derived MSCs (UCB-MSCs) were applied after culture in normoxic or hypoxic conditions, either directly injected into wound margins or topically applied embedded in an autologous fibrin gel. Early healing was enhanced, with histology suggesting a pro-healing rather than a pro-inflammatory scenario and smaller sizes in MSC-treated wounds, with no additional advantage of hypoxic preconditioning [104]. Systemic administration of allogeneic UCB-MSCs resulted in engraftment into induced wounds on the forelimb and thorax at early stages, without clinically adverse reactions [105]. Autologous PB-SCs injected both locally and systemically in four naturally occurring chronic dermal wounds in the metatarsus, unresponsive to conventional treatments, showed positive outcomes with granulation tissue formed within four weeks, and no adverse effects were noted [106]. These results suggest MSCs as a promising tool to improve and accelerate the healing of chronic wounds.

Equine distal limb wounds display similarities to human wounds, with keloid formation in the latter being similar to exuberant granulation tissue formation in horses. Humans and equines are the only two species that spontaneously develop these fibroproliferative disorders, making the horse a suitable model for studying the pathogenesis and treatment of keloids and hypertrophic scars [99]. In both species, chronic wound management is a growing problem leading to a significant economic impact; thus, there are demands for advanced therapeutic options able to decrease healing time and minimize complications.

6.2. Decubitus Ulcers

Decubitus ulcers result from pressure, shear, and/or friction when the patient is lifted or put in decubitus, leading to tissue ischemia, cell death and necrosis [107]. Thus, the pathogenesis of this injury differs from that in traumatic wounds. Neonatal foals are prone to decubitus ulcers due to their thin skin and possible concomitant disorders, which force them into prolonged decubitus [108]. Mesenchymal stem cells play a key role in skin homeostasis and repair by promoting cell differentiation, immunomodulation, and the secretion of growth factors to drive re-epithelialization and neovascularization [109].

In a septic neonatal foal, local implantation of amniotic fluid-derived MSCs (AF-MSCs) along with platelet-rich plasma (PRP) gel effectively improved decubital ulcers earlier than in those ulcers treated with *Aloe vera* gel only [110]. In another study, several deep sore wounds, presented concomitantly in five septic foals, were treated by either allogeneic AF-MSCs embedded in a carboxymethylcellulose scaffold or by a commercial preparation of formosulfathiazole, the former leading to faster healing [111]. Additionally, the effectiveness of local application of allogeneic Wharton's jelly MSCs (WJ-MSCS) was reported in a six-month-old filly with a large non-healing skin wound in the hock [112]. Therefore, MSCs may be considered for healing skin ulcers in foals, with no side effects noticed, although data are limited to a few reports including low numbers of animals.

6.3. Laminitis

Laminitis is included as part of the integumentary system provided the distal third phalanx is enveloped by a specialized epithelial tissue, the corium, attaching the bone to the

hoof. However, it should be noted that this is a complex and multifactorial disease [113]. This disorder affecting the hooves of ungulates is of particular importance in equids, carrying a poor prognosis, with a strong economic impact and constituting a serious issue for animal welfare that may eventually require euthanasia [114]. A great variety of initiating causes can lead to the onset of the disease and can be influenced by different body systems, including inflammation, metabolic disorders, and endothelial or vascular dysfunction [113]. Whatever the triggering factors are, the release of pro-inflammatory mediators and the activation of metalloproteinases leads to the degradation of the basement membrane that may result in a complete mechanical collapse of the foot [115].

The largely incomplete knowledge of the pathogenic mechanisms involved in laminitis makes both prevention and treatment difficult [116]. Laminitis has been associated with the loss of p63+ epidermal stem cells in the hoof lamellae, suggesting limited proliferative potential of the laminitic hoof epithelium [117]. Therapy with MSCs is a promising tool to improve cell proliferation and tissue quality, as well as contribute to vascular stabilization and control the pro-inflammatory environment.

In clinical studies on chronic refractory laminitis, allogeneic UCB-MSCs were delivered directly to the affected foot via regional perfusion (digital vein). The outcome suggested a positive MSC effect on the prognosis of animals treated early, as reflected by the evolution with decreased radiologic distance between the bone and hoof wall [114,118]. Allogeneic BM-MSCs provided better results when digitally perfused compared to epidermally injected [119]. Another study on chronic laminitis administered allogeneic and autologous AT-MSCs suspended in autologous PRP by distal digital venous injection. Improvement of both hoof quality and animal mobility was reported, improving the life quality of treated horses [120].

These results encourage further exploring the use of MSCs to treat chronic laminitis, but it is important to highlight that MSC administration does not eliminate the need for routine management and appropriate hoof support.

Table 3. In vivo studies using mesenchymal stem cells for integumentary system disorders. Umbilical cord-derived stem cells (UCB-MSCs), transforming growth factor beta (TGF-β), cyclooxygenase-2 (COX-2), intravenous (IV), HypoThermosol FRS (HTS-FRS), peripheral blood stem cells (PB-SCs), phosphate-buffered saline (PBS), amniotic fluid MSCs (AF-MSCs), platelet-rich plasma (PRP), carboxymethylcellulose (CMC), Wharton's jelly MSCs (WJ-MSCs), bone marrow mesenchymal stem cells (BM-MSCs), adipose tissue MSCs (AT-MSCs).

Pathology	Study	Type MSCs	Experimental Design	Administration Regime	Outcome	Considerations
Traumatic wounds	Textor et al., 2017 [104]	Allogeneic UCB-MSCs	Six horses (3 mares, 3 geldings; 5–19 years) Induced model: 3 full-thickness cutaneous wounds surgically created on each distal forelimb Assessment and follow-up (6 weeks): • Wound surface area • Thermography • Gene expression • Histologic scoring	10–20 × 10^6 normoxic UCB-MSCs, hypoxic UCB-MSCs or control in 1 mL NaCl 0.9% were injected into wound margins or topically applied embedded in an autologous fibrin gel, 1 day after wound creation	MSC administration by either delivery method was safe and improved histologic outcomes and reduced wound area over control MSC-injected wounds were consistently smaller than gel-treated or control wounds. Hypoxic pre-conditioned MSCs did not provide a substantial advantage	Treatments applied very early and in aseptically created wounds (proof of concept for application in clinical situation)
	Mund et al., 2020 [105]	Allogeneic UCB-MSCs	Two 7-year-old mares Determine adverse reactions after IV MSC administration and assess their engraftment potential into wounds Induced model: standardized cutaneous wounds surgically created on the left lateral third metacarpus (7 wounds) and left hemi-thorax (7 wounds)	1.02 × 10^8 UCB-MSCs (fluorescently labelled) in 60 mL HTS-FRS via IV administration Control: 2 wounds were left to heal by second intention (in the same horses)	No clinically adverse effects (largest recorded dose of IV UCB-MSCs) UCB-MSCs preferentially engrafted into wounds during the acute and early remodeling phases.Results suggest no difference in homing potential between limb and thoracic wounds	Low number of animals Biopsy collection at the control sites created inflammation that may have influenced homing to the sequential control site (untreated control not included)

Table 3. *Cont.*

Pathology	Study	Type MSCs	Experimental Design	Administration Regime	Outcome	Considerations
Traumatic wounds	Spaas et al., 2013 [106]	Autologous PB-SCs	4 horses with naturally occurring traumatic wounds unresponsive to conventional therapies for at least 3 months: • Case 1 (11-year-old mare): dorsal surface of the metatarsal bone infected with *Clostridium* spp. • Case 2 (16-year-old gelding): plantar surface of the metatarsal bone infected (presence of pus) • Case 3 (26-year-old gelding): deep wound with bone exposition at the medial surface of the tibia • Case 4 (26-year-old gelding): wound presenting non-neoplastic exuberant granulation tissue on the plantar surface of the metatarsal bone. Nodular proliferative lesions recurred after resection and tended to ulcerate.	5×10^5 PB-SCs in 2 mL PBS were locally (intradermally) injected into 5–6 different locations at the wound's edges and 1.25×10^5 PB-SCs via IV administration	Granulation tissue began forming within 4 weeks of the PB-SC therapy in cases 1, 2 and 3. Crust formation was achieved within 2 months. In case 4, the granulation tissue could be easily removed without recurrence of the wound. 1 year: no wound recurrence or other adverse effects	Low number of cases of varying presentation with no control group, but unresponsive to previous treatments. Cells used not expanded and not fully characterized as MSCs. In cases with bacterial infection antibiotic, administration was continued.Outcome of each individual case is not deeply explained

Table 3. *Cont.*

Pathology	Study	Type MSCs	Experimental Design	Administration Regime	Outcome	Considerations
Decubitus ulcers	Iacono et al., 2012 [110]	Allogeneic AF-MSCs and allogeneic PRP gel	One septic neonatal foal with severe ulcers in fetlocks, carpus and right stifle	• Aloe gel every 48 h: Left hock and carpus • 10 mL PRP gel: right carpus • 5×10^6 AF-MSCs + 10 mL PRP gel (twice a week for 2 weeks): right hock	None of the wounds treated developed exuberant scar tissue Healing was faster using AF-MSCs + PRP. The ulcer treated this way resulted in a linear scar, while the other lesions produced star scars 7 months: ulcer treated with aloe gel was not fully healed	One single case Different wound locations Concomitant disease Oral antibiotic therapy ongoing during ulcers healing
	Iacono et al., 2016 [111]	Allogeneic AF-MSCs	5 hospitalized neonatal foals (10–15 days old) with a total of 9 pressure sores on the carpus (4), fetlock (2), and hock (3). Sores were divided into group 1 ($n = 6$) and group 2 ($n = 3$)	• Group 1: 5×10^5 AF-MSCs in CMC gel applied twice a week for 2 weeks • Group 2: formosulfathiazole ointment every 48 h	Sores treated with AF-MSCs in CMC gel healed quicker 30 days: no further treatments were needed	Low number of cases with no substance vehicle (CMC gel) treated control. Variable presentation of treated sores
	Lanci et al., 2019 [112]	Allogeneic WJ-MSCs	One 6-month-old filly hospitalized by the re-injury of a pressure wound on the left hock	5×10^6 WJ-MSCs in a CMC gel were applied every 4 days for 4 times Four days after the last application, no further bandages were applied and the wound was daily cleaned and treated with hydrotherapy (cold tap water 10 min/day)	No side effects and fast wound regression No evident exuberant scar. The hair grew completely without changing color80% regression rate between 8 days and 39 days No relapse	One single case with no control

Table 3. *Cont.*

Pathology	Study	Type MSCs	Experimental Design	Administration Regime	Outcome	Considerations
Laminitis	Morrison, 2011 [118]	Allogeneic UCB-MSCs	12 horses with naturally occurring chronic laminitis unresponsive to other treatments	20–25 × 10^6 UCB-MSCs in NaCl 0.9% infused by regional perfusion (digital vein) every 3–4 weeks (3 infusions in total per affected foot)	83% of horses with positive evolution by the time of publication.	Routine treatments for laminitis continued Long-term success rates still need to be determined Only clinical follow-up Absence of control group No age or breed information stated
	Dryden et al., 2013 [114]	Allogeneic UCB-MSCs and autologous BM-MSCs	30 horses with naturally occurring chronic laminitis	20–30 × 10^6 allogeneic UCB-MSCs in NaCl 0.9% infused by regional perfusion (digital vein) and subsequent injections with either 20–30 × 10^6 autologous BM-MSCs or allogeneic UCB-MSCs (4 infusions in total at 1 month intervals)	21 patients (70%): successful outcome. Decreased radiologic distance between the bone and hoof wall Cases receiving MSCs < 30 days after the onset of laminitis: success rate was 100%. Cases receiving MSCs > 90 days after the onset of laminitis: success rate was 50%. Results suggest improved prognosis in cases treated early.	Routine treatments for laminitis continued Absence of control group Only clinical and radiologic follow-up Variation in therapeutic regime among cases. No age or breed information stated
	Angelone et al., 2017 [120]	Allogeneic AT-MSCs and autologous AT-MSCs	9 horses (5 mares, 4 geldings; 10–21 years) with severe naturally occurring laminitis unresponsive to conventional therapies	15 × 10^6 allogeneic AT-MSCs in 15 mL autologous PRP infused by regional perfusion (digital vein) and subsequent injections with autologous AT-MSCs (3 infusions in total at 1 month intervals)	Clinical and radiologic signs improved All the animals returned to activity at six months from the first treatment. 12 months: 7 horses still performing activity 24 months: • 2 animals relapsed and were euthanized • 3 horses deceased (unrelated to laminitis)	Absence of control group Horses enrolled presented different laminitis stages. Only clinical and radiologic follow-up

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7. Application of Equine MSCs in Neurological Disorders

Neurological disorders affecting the brain and spinal cord can represent a therapeutic challenge, and many horses can have sequelae even after recommended treatment [121]. The neuroprotective effects of MSCs have been described in other species and involve anti-inflammatory, immunomodulatory, pro-angiogenic and trophic mechanisms [122,123], which could ameliorate the symptoms of several neurodegenerative disorders [124]. Furthermore, MSCs can trans-differentiate in vitro into neuronal lineages [125]. In vivo related studies in horses are summarized in Table 4.

7.1. Peripheral Nerve Injury

Horses suffer injury to peripheral nerves from trauma, metabolic and genetic disorders, toxins or degenerative and infectious diseases [126]. The degree of restoration of nerve function depends on the severity and chronicity of the damage, with the worst prognosis in cases where the nerve is transected [127]. There are few surgical techniques for repairing nerves, and clinical results are often poor [128]. Consequences frequently include poor performance, disability, or even death [129,130].

In an in vivo model of acute peripheral nerve injury using three horses, allogeneic BM-MSCs were implanted into the fascia surrounding the *ramus communicans* of one forelimb after a portion was transected. No evidence of nerve regeneration was observed, neither were histological differences between MSC-treated and control injuries found 45 days later [127]. Nevertheless, improvement of nerve regeneration after MSC treatment has been observed in other large animals such as sheep [131].

7.2. Wobblers Syndrome

Wobblers syndrome, also known as cervical vertebral stenotic myelopathy (CVSM) or incoordination syndrome, is characterized by ataxia and weakness, caused by the narrowing of the cervical vertebral canal and/or compression of the spinal cord. Medical treatment commonly includes steroidal and nonsteroidal anti-inflammatory drugs, along with diet and exercise restrictions. Surgery can be considered in some cases, but this option is expensive and involves significant risks [132].

An intrathecal injection could extensively deliver cells through the cerebrospinal fluid to reach the equine central nervous system. To evaluate the feasibility and safety of intrathecal transplantation of cells, autologous BM-MSCs were administered to healthy horses and no clinical alterations were developed [133]. A posterior study aimed at determining the safety of a relatively high dose of intrathecal allogeneic AT-MSCs in both healthy and CVSM-affected horses. Neurological status was not altered regardless of atlanto-occipital or lumbosacral administration. Atlanto-occipital injection is apparently distributed more efficiently through the subarachnoid space, suggesting that this approach might be more suitable for cranial spinal cord lesions. As for diseased horses, MSCs could not be found at 15 days after injection at the site of injury, so either cells did not reach the lesion site or did not persist at that time [134].

7.3. Laryngeal Hemiplegia or Left Recurrent Laryngeal Neuropathy

Recurrent laryngeal neuropathy is characterized by varying degrees of arytenoid paralysis, constituting a highly prevalent pathology of the upper airway in horses [135]. Affected horses emit abnormal respiratory sounds and may present exercise intolerance in severe cases. Although the overall success rate of laryngoplasty with or without ventriculocordectomy is elevated, post-operative complications such as a gradual loss of abduction are very common [136].

Table 4. In vivo studies using mesenchymal stem cells in equine neurological disorders. Bone marrow mesenchymal stem cells (BM-MSCs), adipose tissue MSCs (AT-MSCs), cervical vertebral compressive myelopathy (CVCM), 99m technetium-hexamethyl-propylene-amine-oxyme (99mTc-HMPAO), atlanto-occipital (AO), lumbosacral (LS), recurrent laryngeal neuropathy (RLN), muscle-derived MSCs (M-MSCs).

Pathology	Study	Type MSCs	Experimental Design	Administration Regime	Outcome	Considerations
Peripheral nerve injury	Villagrán et al., 2016 [127]	Allogeneic BM-MSCs	Induced model: 3 healthy mares (9–13 years old) with surgically created 15-mm longitudinal incision over the *ramus communicans*	10×10^6 BM-MSCs in 1 mL NaCl 0.9% or 1 mL NaCl 0.9% (control) instilled into the fascia surrounding the medial and lateral stumps Stumps of *ramus communicans* of each fore limb were harvested 45 days after treatment or control administration	No evidence of nerve regeneration No histological differences between MSC-treated and control nerve stumps No histological evidence of BM-MSCs or primitive cells (e.g., neural or Schwann-cell progenitors)	Small size Immediate treatment of aseptically created injury (proof of concept) Poor vasculature of the anatomical region may have influenced the outcome
CVCM	Barberini et al., 2018 [134]	Allogeneic AT-MSCs	Distribution and safety assessment in 6 healthy mares (6–21 years) and 3 diseased horses presenting moderate to severe neurological signs (presumedly CVCM) Assessment: • Scintigraphy • Detection of anti-AT-MSC alloantibody • Necropsy (diseased horses)	Healthy horses: 100×10^6 AT-MSCs in 5 mL NaCl 0.9% were injected either AO (n = 3) or LS (n = 3). One horse in each group received 99 mTc-HMPAO-labeled AT-MSCs. One additional horse was injected with free label as a control. Diseased horses: 100×10^6 AT-MSCs via AO	Healthy horses: AO and LS intrathecal injection of relatively high doses of AT-MSCs was well tolerated AT-MSCs apparently distributed more efficiently through the subarachnoid space after AO injection (suggested as preferred route to deliver MSCs to the cervical area) Diseased horses: AT-MSCs not found at 15 days after injection at the site of injury (either did not have time to reach the lesion site or did not survive long enough) No horses developed detectable anti-AT-MSC alloantibodies.	Low number of horses per group. Tracking and control only in one horse
RLN	Sandersen et al., 2018 [137]	AutologousM-MSCs	5 healthy mares (ages 10–22) Evaluate the feasibility and safety of administering MSCs by a peri-neural injection to the left recurrent laryngeal nerve in healthy horses by using an electrical nerve stimulator	10×10^6 M-MSCs in 1 mL cryopreservation medium directly administered into recurrent laryngeal nerve by a nerve stimulator-guided injection	Feasibility and safety of the procedure suggested by absence of functional changes upon endoscopic evaluation up to 28 days. No signs of adverse events in four out of the five mares up to 1 year after the injection	No control group M-MSCs administered immediately after thawing Composition of cryopreservation (administration) media not mentioned

To explore the feasibility of using MSCs to promote nerve function restoration, a nerve-stimulator-guided injection of muscle-derived autologous MSCs near the left recurrent laryngeal nerve was performed in five healthy horses. Laryngeal function was not affected, thus suggesting that this delivery technique is safe [137]. These findings would facilitate future studies assessing MSC effectiveness to treat this pathology, which has not yet been tested, to the best of the authors' knowledge.

8. Application of Equine MSCs in Endotoxemia

Systemic inflammatory response syndrome (SIRS) is characterized by an exaggerated inflammatory response to an aggression, which causes a series of unspecific clinical signs that can seriously compromise the patient's life. This process is of great importance in horses due to the particularities of their inflammatory response and the high incidence of pathologies such as acute abdominal syndrome, pneumonia, and metritis, which can provoke an endotoxemia or sepsis and subsequent SIRS development. These processes can lead to complications such as disseminated intravascular coagulation, vascular endothelial damage, laminitis, and multiple organ dysfunction syndrome [138]. Therefore, SIRS is associated with a significantly higher risk of death in horses presenting acute colic [139] or other disorders. There are no specific treatments for endotoxemia and SIRS other than controlling the primary cause and providing supportive therapy, mostly based on fluids and antiendotoxics [138]. The complexity and poor prognoses of these processes and the lack of effective treatments have led to the interest in using MSCs because of their immunomodulatory capacity. Actually, there are a number of studies in rodent models of endotoxemia showing promising results of the administration of MSCs, including decreased levels of circulating proinflammatory cytokines and increased survival rates [140].

In horses, and to the best of the authors' knowledge, there is only one study reporting the effects of administering MSCs in an experimental model of endotoxemia [141]. In this study, lipopolysaccharide was IV injected into six horses, and three of them immediately received 100×10^6 allogeneic BM-MSCs IV. Serial clinicopathological assessment and determination of pro-inflammatory cytokine production did not show significant differences between treated and control animals, but adverse reactions after MSC infusion were not observed either, thus suggesting that the procedure is safe; however, further studies with higher numbers of animals and studying variables such as the dosage and moment of administration are needed to elucidate the potential benefit of this therapy. Importantly, endotoxemia, sepsis, and SIRS also affect a high percentage of human patients in intensive care units, and the horse has been proposed as a model for understanding human innate immunity and shedding light onto pathology processes and therapeutics [142].

9. Conclusions and Future Perspectives

The unique properties of MSCs hold a great potential for the treatment of different equine diseases other than musculoskeletal injuries, for which current pharmacologic or surgical approaches often do not provide satisfactory results.

Several studies have explored the use of MSCs for different equine diseases beyond the locomotor system. Their application for ophthalmologic and reproductive disorders has been particularly investigated, and the treatment of wounds, asthma, and SIRS holds a special interest to develop One Medicine approaches. However, the variability among clinical case conditions, source of the MSCs used, cell isolation and culture techniques, and therapeutic protocols (MSC dose, route, number, and frequency of administrations), as well as the low sample size and lack of control groups in some of the studies, limit extracting definitive conclusions. Nevertheless, case reports are highly valuable as a proof of concept, indicating that there is potential for in further investigations. In addition, in vitro studies provide interesting preliminary insight into MSC mechanisms for each pathology, and in vivo studies in healthy animals enable safety and feasibility assessments.

More in-depth research is needed to test the safety and efficacy of these novel treatments, and future clinical trials would include a larger number of similar cases and stan-

dardized measurements of the outcomes, in order to establish specific therapeutic protocols. Furthermore, more in vitro and experimental work is needed to understand the pathways through which MSCs elicit their effects, in order to achieve their highest therapeutic potential. Therefore, researchers and clinicians should work together to develop evidence-based treatments and exploit the MSC potential by extending their use to different pathologies in both equine and human patients.

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Equine Mesenchymal Stem Cells Influence the Proliferative Response of Lymphocytes: Effect of Inflammation, Differentiation and MHC-Compatibility

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Simple Summary: Mesenchymal stem cells are investigated for therapy because of their ability to regulate the immune response to an injury. Cell therapy is increasingly important in veterinary patients such as horses, which are also valuable as a model. Therefore, what is learned in these animals can benefit both them and people. However, the patient's immune system could recognize and destroy mesenchymal stem cells, impairing effectiveness and potentially leading to adverse effects. In this study, we analysed how equine mesenchymal stem cells interact with immune cells in different scenarios. We tested the effect of inflammation and differentiation of these cells, and how they acted depending on donor–patient compatibility. As we expected, inflammation activated the regulatory ability of equine mesenchymal stem cells, but also increased the risk of immune recognition. We anticipated that, after differentiation, these cells would lose their regulatory ability and would be more easily targeted by the immune system. However, they maintained similar features after differentiating into cartilage cells. The balance between the ability of mesenchymal stem cells to stimulate and to regulate an immune response is of the utmost importance to develop safe and effective cell therapies for animals and people.

Abstract: Immunomodulation and immunogenicity are pivotal aspects for the therapeutic use of mesenchymal stem cells (MSCs). Since the horse is highly valuable as both a patient and translational model, further knowledge on equine MSC immune properties is required. This study analysed how inflammation, chondrogenic differentiation and compatibility for the major histocompatibility complex (MHC) influence the MSC immunomodulatory–immunogenicity balance. Equine MSCs in basal conditions, pro-inflammatory primed (MSC-primed) or chondrogenically differentiated (MSC-chondro) were co-cultured with either autologous or allogeneic MHC-matched/mismatched lymphocytes in immune-suppressive assays (immunomodulation) and in modified one-way mixed leukocyte reactions (immunogenicity). After co-culture, frequency and proliferation of T cell subsets and B cells were assessed by flow cytometry and interferon-ɣ(IFNɣ) secretion by ELISA. MSC-primed showed higher regulatory potential by decreasing proliferation of cytotoxic and helper T cells and B cells. However, MHC-mismatched MSC-primed can also activate lymphocytes (proliferative response and IFNɣsecretion), likely due to increased MHC-expression. MSC-chondro maintained their regulatory ability and did not increase their immunogenicity, but showed less capacity than MSC-primed to induce regulatory T cells and further stimulated B cells. Subsequent in vivo studies are needed to elucidate the complex interactions between MSCs and the recipient immune system, which is critical to develop safe and effective therapies.

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1. Introduction

Mesenchymal stem cells (MSCs) are adult multipotent stem cells that have raised interest in the field of regenerative medicine due to their unique biological properties [1]. Mesenchymal stem cells have potential for treating several pathologies, such as those affecting the musculoskeletal system. However, MSC paracrine activity, including immunomodulatory effects, have broadened their scope for inflammatory and immune-mediated pathologies. As the etiopathogenesis of some diseases is similar in both people and animals, the results obtained can be valuable for both human and veterinary patients [2]. The horse presents a remarkable value for translational medicine [3], but further research on the mechanisms of equine MSC, especially their immune properties, is needed. The immune regulatory mechanisms of MSCs mainly depend on their paracrine activity [4], which is not only linked to their therapeutic effects, but also to their ability to escape from immune recognition when administered allogenically. Actually, a highly relevant paradigm change is that MSCs are not truly immune-privileged but immune-evasive, and thus, their recognition and elimination by the immune system in the allogeneic setting should be considered [5]. Allogeneic application presents several advantages over autologous therapy, including the possibility of providing quicker and wider availability of well-characterized MSCs, and its application when autologous cells are not appropriate [6].

Therefore, MSC immunogenicity should be further investigated to develop safe and effective treatments. Cellular and humoral immune responses may be generated against allogeneic MSCs, thus limiting their effects and potentially leading to adverse reactions [7,8]. Furthermore, immune memory mechanisms could be developed and compromise the repeated administration of allogeneic MSCs in the horse [9]. In recent years, the importance of the major histocompatibility complex (MHC) matching between donor and recipient has been studied by several authors in the horse [7,10], as well as in other species [11,12]. As the therapeutic effectiveness could be compromised by the generation of antibodies specifically directed against the equine leukocyte antigen (ELA) of the donor, ELA haplotypes and compatibility should be taken into account.

In addition, it has also been reported that the MHC level expression in MSCs in basal conditions is quite dependent on the equine donor [7]. However, there are other factors that may modify the initial immune properties of MSCs, such as their exposure to an inflammatory environment and the process of differentiation. The knowledge on these factors is critical to use them for designing more effective therapeutic strategies.

Regarding inflammatory exposure, priming equine MSCs with pro-inflammatory cytokines like interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα) increases their immunomodulatory properties and may result in enhanced regulatory effects in vivo [13]. However, priming MSCs may also raise their immunogenicity because of induced expression of MHC, thus potentially limiting the allogeneic administration [14]. The balance between immunomodulation and immunogenicity induction seems to depend on the type of inflammatory exposure. For example, high doses of interferon (IFNγ) appears to increase the expression of MHC-II [7], whereas a short priming of equine MSCs with low doses of IFNγ and TNFα could maintain the balance between immunomodulatory and immunogenic factors [15]. It has also been suggested that MSCs may lose their regulatory abilities and increase their immunogenicity after differentiation [16]. The increased expression of MHC-I and/or MHC-II could result in a higher risk of allo-recognition if equine MSCs differentiate after administration or are administered pre-differentiated [17], especially if their regulatory abilities are also impaired by differentiation.

In spite of all the aforementioned, allogeneic MSC administration has been shown to be safe and effective in several conditions [10,18,19]. Therefore, it has been suggested that the balance between their immunomodulatory and immunogenic potentials would allow allogeneic MSCs to evade the immune system and elicit their actions [14]. To strengthen the development of allogeneic cell therapies, it is critical to gain knowledge into how factors such as MHC matching/mismatching, inflammation and differentiation may affect such balance in equine MSCs. To the best of authors' knowledge, there are not studies simultaneously assessing these factors on equine MSCs.

Therefore, the aim of this study was to analyse the changes elicited in vitro by equine MSCs on relevant lymphocyte subpopulations under different conditions: inflammation, differentiation, and compatibility for the MHC. For this purpose, lymphocyte proliferation assays consisting of immunosuppression assays (i.e., immunomodulatory capacity) and modified one-way mixed leukocyte reactions (MLRs) (i.e., immunogenic potential) were carried out using equine MSCs in basal conditions (MSC-naïve), pro-inflammatory primed (MSC-primed) or chondrogenically differentiated (MSC-chondro), co-cultured with either autologous or allogeneic MHC-matched/mismatched lymphocytes. The initial hypothesis was that inflammatory priming would markedly increase MSC immune regulatory potential with a slight effect on MSC immunogenicity, whereas differentiation would diminish their immunomodulatory ability and increase their immunogenicity. Regarding the MHC compatibility, it was hypothesized that MHC-matched cells would generate similar results than autologous ones, whereas the MHC-mismatched co-cultures would result in similar immunomodulation but increased immunogenicity.

2. Materials and Methods

2.1. Study Design

Three MHC homozygous horses were used as donors of bone marrow derived MSCs to study their immunomodulatory capacity and immunogenic potential in three conditions: basal (MSC-naïve), proinflammatory-primed (MSC-primed) and chondrogenically-differentiated (MSC-chondro) conditions. For this purpose, MSCs under the different conditions were co-cultured with peripheral blood lymphocytes (PBLs) autologous (n = 3) or allogeneic from MHC-matched (n = 8) and mismatched (n = 7) animals. A total of 11 animals, selected by their MHC haplotype, were involved for MSCs and PBLs collection and co-cultures were established using the combinations shown in Figure 1. Two types of co-cultures were used: immunosuppressive assays to study MSC immunomodulatory potential, and one-way modified MLR to assess MSC immunogenicity. PBLs were stained with carboxyfluorescein succinimidyl ester (CFSE) to evaluate their proliferation after each type of co-culture. In addition, at the end of the co-culture assays, PBLs were stained with a panel of antibodies to study changes in the frequency and proliferation of each lymphocyte subset by flow cytometry. The secretion of IFNγ in co-culture supernatants was assessed by ELISA as a reflection of T cell activation.

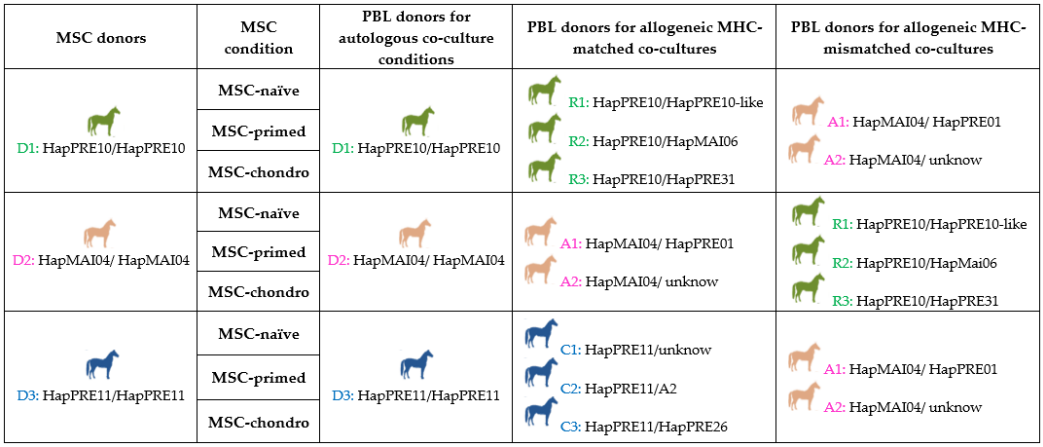


Figure 1. Study design showing mesenchymal stem cells (MSCs) and peripheral blood lymphocytes (PBLs) donors to establish autologous and allogeneic major histocompatibility complex (MHC)-matched and MHC-mismatched co-cultures, to study immunomodulatory capacity and immunogenic potential. MSCs were assayed unstimulated (MSC-naïve), primed with cytokines (MSC-primed) or chondrogenically differentiated (MSC-chondro).

2.2. Animal Selection by MHC-Haplotyping

Eleven mixed-breed horses (1 stallion, 3 geldings, 7 mares; aged 2–8 years, weight 412–493 kg) in good health status and with no previous pregnancy history were chosen based on their MHC haplotypes. Haplotypes were determined by microsatellite typing using a validated panel of 10 highly polymorphic intra-MHC regions, as previously described [9,20]. To find and select animals, a screening of 60 Purebred Spanish and mixed-breed horses from a local farm was performed. Blood was collected with informed owner's consent and methodology for DNA extraction, multiplex PCRs and fragment analysis was performed as previously reported by our group [9].

Definitive haplotypes were established for homozygous animals and the remaining animals were assigned with provisional haplotypes based on previously known ones, either reported in the bibliography [10,21], or described in a preliminary study of our group in Purebred Spanish horses [22]. Three groups of animals were selected, including one a homozygous horse for each of the haplotypes HapPRE10, HapPRE11 or HapMAI04, and two to three heterozygous animals sharing one haplotype. In each group, the homozygous horse served as a MSC donor as it was MHC-matched with the heterozygous animals in the group, but MHC-mismatched with animals in other groups, thus allowing autologous, matched and mismatched combinations (Figure 1). The premise of using homozygous individuals for matching with different heterozygous individuals is the same proposed to create haplo-banks of human induced pluripotent stem cells (iPSCs) [23,24], and also used in equine MSC studies [25]. Table 1 shows the microsatellite alleles of each haplotype identified in the different horses chosen.

All the procedures involving animals were carried out under the Project License PI 15/16 approved by the in-house Advisory Ethics Committee for Animal Research from the University of Zaragoza. The care and use of animals were performed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63. All animals were kept on paddocks of the facilities of the Animal Research Service of the University of Zaragoza, with free access to water and fed with ad libitum grass hay.

Table 1. List of major histocompatibility complex (MHC) microsatellite haplotypes identified in the horses enrolled in the study.

	MHC Class I		MHC Class III		MHC Class II						
Micros-atellite Loci	**UMNJH-38**	**COR110**	**ABGe9019**	**UMNe65**	**ABGe9030**	**EQMHC 1**	**COR112**	**COR113**	**UM011**	**COR114**	
Horses ID											**Haplotype**
D1	165	221	301	261	215	190	262	270	179	241	HapPRE10
	165	221	301	261	215	190	262	270	179	241	HapPRE10
R1	165	221	301	261	215	190	262	270	179	241	HapPRE10
	156	215	301	261	215	190	262	270	179	241	HapPRE10-like
R2	165	221	301	261	215	190	262	270	179	241	HapPRE10
	156	221	320	250	219	190	254	270	172	249	HapMAI06
R3	165	221	301	261	215	190	262	270	179	241	HapPRE10
	156	207	318	263	215	184	262	260	172	243	HapPRE31
D2	156	217	312	261	205	194	258	260	169	243	HapMAI04
	156	217	312	261	205	194	258	260	169	243	HapMAI04
A1	156	217	312	261	205	194	258	260	169	243	HapMAI04
	156	205	305	253	205	194	266	268	174	234	HapPRE01
A2	156	217	312	261	205	194	258	260	169	243	HapMAI04
	156	207	312	263	211	192	264	270	172	249	Unknown1
D3	156	221	314	259	215	190	262	272	169	255	HapPRE11
	156	221	314	259	215	190	262	272	169	255	HapPRE11
C1	156	221	314	259	215	190	262	272	169	255	HapPRE11
	156	221	312	261	205	190	262	270	180	245	Unknown2
C2	156	221	314	259	215	190	262	272	169	255	HapPRE11
	156	211	301	259	209	192	262	268	174	234	A2 *
C3	156	221	314	259	215	190	262	272	169	255	HapPRE11
	156	207	314	261	215	190	262	270	180	247	HapPRE26

MHC homozygous horses are indicated in bold. Asterisks indicate haplotypes that have been previously identified in other horse breeds [26].

2.3. Isolation and Characterization of MSCs

Equine bone marrow MSCs were obtained as previously described [15,17]. Briefly, bone marrow was harvested from the sternum of D1, D2 and D3 animals under sedation (0.04 mg/kg IV romifidine; Sedivet, Boehringer-Ingelheim, Barcelona, Spain and 0.02 mg/kg IV butorphanol; Torbugesic, Pfizer, Madrid, Spain) and local analgesia with lidocaine (Anesvet, Laboratorios Ovejero, León, Spain). Mononuclear cells were separated by density gradient centrifugation and seeded in culture medium consisting of low-glucose Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 U/mL penicillin and 10% foetal bovine serum (FBS) (all from Sigma-Aldrich, Madrid, Spain). Cells were expanded until passage three, and then cryopreserved in 10% DMSO (Sigma-Aldrich) and 90% FBS medium until subsequent experiments started.

Cells were characterized at passage three by their phenotype and tri-lineage differentiation, as previously described [15,17,27] according to minimal criteria for defining human MSCs established by the International Society for Cellular Therapy [28]. Details on the methodology used and characterization data obtained are provided in Supplementary Materials S1.

Surface expression of MHC-I and MHC-II was studied by flow cytometry in both MSC-naïve and MSC-primed, since inflammatory exposure can induce changes in MHC expression. Methodology followed and antibody suitability was previouslydescribed [13,29], and is briefly explained in Supplementary Materials S1.

Preparation of MSC Experimental Conditions: MSC-Naïve, MSC-Primed and MSC-Chondro

Cryopreserved MSCs (n = 3) were thawed and seeded at 5000 cells/cm^2 in the basal medium described above, at 37 °C and 5% CO_2 for 72 h to recover from freezing. Subsequently, MSCs were detached with 0.25% trypsin-EDTA (Sigma–Aldrich) and seeded into a 24-well plate at 100,000 cells per well for the immunomodulatory assays, and at 20,000 MSCs per well for the immunogenicity assays. For MSC-naïve and MSC-primed, plating was done 24 h prior to co-cultures to allow MSCs to attach to the well. For MSC-chondro, plating was performed with the same amount of cells, but 14 days before the co-cultures to induce differentiation.

For the MSCs-primed condition, corresponding MSCs were exposed for 12 h to 5 ng/mL of equine recombinant TNFα (R&D Systems, Barcelona, Spain) plus 5 ng/mL of equine recombinant IFNγ (R&D Systems), added to the basal culture medium described above [15], before adding PBLs.

Chondrogenic differentiation for the MSC-chondro condition was induced with the StemPro™ Chondrogenesis Differentiation Kit (Thermo Fisher, Madrid, Spain) using the micro-mass system during 14 days [30], before adding PBLs. The protocol was adapted to suit the required amount of MSCs for the ratios selected for immunomodulatory and immunogenicity assay, which were 100,000 and 20,000 MSCs per well, respectively. For each donor, two replicates of each MSC type were prepared to run each co-culture in duplicate. Wells were washed with phosphate buffered saline (PBS, Gibco, Thermo Fisher), before adding PBLs.

2.4. Co-Cultures for Lymphocyte Proliferation Assays: Immunosuppression and Modified One-Way MLR

2.4.1. Optimization of the Assays: CFSE Staining and Mitogen Stimulation

Prior to any experimental assay, the optimal concentration of CFSE (Sigma-Aldrich) and PBLs for staining were determined. Two different CFSE concentrations (2.5 μM and 5 μM) and two PBL concentrations (10 × 10^6 PBL/mL and 20 × 10^6 PBL/mL) were examined based on previously reported conditions [31]. After testing all the combinations, using 2.5 μM CFSE to stain cells at 20 × 10^6 PBL/mL were set as the most appropriate conditions. Subsequently, a gradient of concentrations of the mitogen phytohemagglutin isoform P (PHA, Sigma-Aldrich) (2.5, 5, 10, and 20 μg/mL) were tested according to previous reports [32] and assessed after 2, 3 and 4 days [33,34]. The combination of PHA concentration and time of stimulation that provided maximal proliferation was 10 μg/mL of PHA during 3 days, and as such was set for all the immunomodulatory assays.

2.4.2. Blood Collection and Isolation of PBLs

Peripheral blood lymphocytes were isolated using the carbonyl iron granulocyte depletion method, followed by density gradient centrifugation with Lymphoprep™ (Fisher Scientific, Madrid, Spain) as previously described [8,9]. Briefly, blood was collected aseptically via jugular venipuncture into sterile 60-mL syringes with 17 I.U./mL of lithium heparin (Sigma-Aldrich), and plasma was allowed to separate for 20′ at room temperature (RT).

Plasma was separately collected into conical tubes using extension sets and incubated with carbonyl iron (Sigma-Aldrich) in agitation for 30′ at 37 °C. Then, carbonyl iron was placed in the bottom of the tubes by using a magnet, and supernatant was collected and centrifuged at 310× g 5′. The cellular pellet was resuspended in PBS and overlayed on Lymphoprep™. After 690× g 15′ centrifugation (without brake), a lymphocyte layer was recovered and washed with PBS. Cells were counted in a hemocytometer chamber using Trypan Blue 0.4% as dye exclusion, and concentration was adjusted to 10 × 10^6 live cells/mL in PBS. This isolation technique has been reported to provide an enriched lymphocyte population (95–99%) [9,35].

2.4.3. Carboxyfluorescein Succinimidyl Ester Labelling and Analysis of Proliferation

Cells were labelled with 2.5 μM CFSE to measure lymphocyte proliferation by assessing CFSE dilution using flow cytometry [7,36]. After isolation, PBLs were placed in 15 mL conical tubes, centrifuged and resuspended at 20 × 10^6 PBLs/mL in RPMI 1640 medium (Thermo Fisher), supplemented with 5% FBS to minimize cell toxicity, and 2.5 μM CFSE. Cells were evenly mixed to ensure rapid and homogeneous labelling and were incubated 5′ RT in dark [31]. The reaction was blocked by adding 2 mL of ice-cold FBS 1′ RT, and then the cells were washed once with PBS and twice with PBL medium (consisting of RPMI 1640 medium supplemented with 10% FBS, 0.1 mM 2-mercaptoethanol (Sigma), 100 U/mL penicillin, and 100 μg/mL streptomycin). Cells from the same horse but stained in different tubes were not mixed but used for separate immunomodulation or immunogenicity assays, to prevent heterogeneous intensity of the CFSE staining. After the staining procedure, PBLs were counted again and adjusted to 10 × 10^6 live cells/mL in PBL medium.

2.4.4. Immunosuppression Assay

The immunomodulatory function of MSCs was determined by their ability to modulate the proliferation of mitogen-stimulated lymphocytes. As described above, corresponding MSCs were previously plated in 24-well plates at 100,000 cells per well in duplicate and prepared for each condition (MSC-naïve, MSC-primed, MSC-chondro).

CFSE-labelled lymphocytes from autologous, MHC-matched and mismatched horses were seeded at 1 × 10^6 PBL per well (1:10 ratio MSC:PBL), based on previous studies [34,37] with PBL medium containing mitogen (PHA 10 μg/mL) [32,38], using the combinations explained above under experimental design.

Appropriate controls were set along with experimental conditions in duplicate: 500,000 unlabelled (background control) and CFSE-labelled (with and without PHA, non-proliferating and proliferating controls, respectively) were seeded alone in a 96-well U-bottom. All the cultures were maintained for 3 days, after which corresponding analysis were performed, as will be detailed below.

2.4.5. Modified One-Way MLR

To assess the ability of MSCs to stimulate a proliferative response in lymphocytes, modified one-way MLRs were performed. Stimulator MSCs were previously plated at 20,000 cells per well on 24-well plates in duplicate for each condition, as described above. Autologous, MHC-matched and mismatched responder CFSE-stained PBLs were seeded at 1 × 10^6 PBL per well following the experimental design aforementioned, thus resulting in a MSC:PBL ratio of 1:50 [7,34]. Appropriate controls were set as described for immunosuppression assays (unlabelled PBLs for background signal and CFSE-stained PBLs, with and without PHA, as non-proliferating and proliferating internal controls). In addition, classic MLRs were established for each responder. Briefly, MHC-matched and mismatched PBLs were used as stimulators by treating them with 50 μg/mL mitomycin C (Sigma-Aldrich) (37 °C 30′ incubation followed by 2 washes with PBS) to inhibit proliferation [10,39]. Stimulator PBLs were plated at 500,000 PBLs/well in 96-well plates immediately before the addition of 500,000 CFSE-stained responder PBLs (ratio 1:1) to create MHC-matched and mismatched MLRs for all the PBLs tested with MSCs.

All the cultures were maintained for 5 days without media exchange, after which corresponding analyses were performed, as will be detailed below.

2.5. Analysis of Lymphocyte Proliferation and Subpopulations

After co-culture, experimental PBLs from proliferation assays (both immunosuppression and modified one-way MLRs) were collected from 24-well plates, centrifuged at 310× *g* 5′, resuspended in PBS, and split for the 2 flow multi-colour panels. The supernatants were collected and centrifuged at 500× *g* 15′ to remove any contaminating cell, and subsequently frozen at –20 °C for further ELISA analysis. Control PBLs in 96-well plates were centrifuged at 310× *g* 5′, supernatants collected as aforementioned, and cells

suspended in PBS. Experimental PBLs were then transferred to the 96-well plates along with controls for antibody staining for flow cytometry.

Two multi-colour panels of markers were designed to allow assessment of different lymphocyte subpopulations, along with the proliferation (CFSE dilution). In panel 1, anti-CD3, Pan Ig and CD21 were used to assess T and B cells. In panel 2, anti-CD8, CD4 and CD25 antibodies were used to assess cytotoxic, helper and regulatory T cells. All primary antibodies were selected based on previous reports [40–43], and used directly conjugated or in combination with appropriate secondary antibodies [44]. All the antibodies were previously titrated in order to determine the optimal dilution. Information regarding antibodies characteristics, dilution and conjugated fluorochromes is presented in Supplementary Materials S2: Tables S2.1 and S2.2.

For panel 1 staining, all control and experimental PBLs were blocked with 100 µL/well of 10% normal goat serum (Panbiotech, IBIAN, Zaragoza, Spain) in PBS, washed twice (wash buffer consisting of PBS with 3% FBS and 310× *g* 5′ spinning) and stained with primary anti-Pan Ig-cells antibody. Then, PBLs were washed twice, incubated with corresponding secondary antibody, and washed again prior to staining with anti-CD21 antibody. At this point, viability staining was performed with Ghost dye Violet 450 (Tonbo Biosciences, Bio-Rad, Barcelona, Spain) as per manufacturer's instructions and prior to fixation and permeabilization. Cells were washed twice in PBS and fixation/permeabilization were performed using Leucoperm reagents (Bio-Rad, Barcelona, Spain) according to manufacturer's indications, provided that the anti-CD3 antibody was directed against an intra-cellular epitope. Immediately after the permeabilization step, PBLs were blocked with 100 µL/well of 10% normal mouse serum in PBS, washed and stained with primary anti-CD3 antibody. After two washing steps, cells were incubated with corresponding secondary antibody. Cells were washed twice and fixed with 4% paraformaldehyde (Fisher Scientific) in PBS for 15′ 4 °C, with one washing step afterwards.

For panel 2, all PBLs were blocked in PBS containing 10% donkey serum and 10% rat serum (100 µL/well) (both from Panbiotech). After two washing steps, cells were stained simultaneously with both primary anti-CD4 and anti-CD25 antibodies, washed twice, and incubated with corresponding secondary antibodies. After two washing steps, cells were stained with primary anti-CD8 antibody. Subsequently, cells were washed twice with PBS and incubated with viability dye prior to fixing them, as described above.

All blocking steps and incubation with primary antibodies were performed at 4 °C for 30′. All the incubations with secondary antibodies were carried out at 4 °C for 20′. The volume per well of each primary or secondary antibody was of 50 µL. Two technical replicates per sample were performed and all the procedures were carried out protecting the samples from light. All samples in each panel were subjected to the same steps and were kept in 3% FBS in PBS at 4 °C in the dark up to 24 h for analysis.

Samples were analysed in a Gallios flow cytometer (Beckman Coulter, Madrid, Spain), acquiring a minimum of 10,000 events per sample. Flow cytometry data was analysed with FCS Express 7 Flow software (De Novo Software, Pasadena, CA, USA).

Unstained and secondary controls (cells stained with secondary antibodies alone) were used to assess fluorescence background and establish gates. Single colour stains were used for compensation controls and fluorescence minus one (FMO) controls were performed to assess the fluorescence spread from other channels. The population of lymphocytes was first gated in the forward and side scatter (FSC × SSC) plot, and doublets were excluded. Dead cells were subsequently excluded by incorporation of the viability staining (FL9 channel). In panel 1, live cells were gated for T cell population as CD3+ and for B cells as CD3−/pan-Ig +/CD21+ [45]. In panel 2, live cells were gated as cytotoxic and helper T cells as CD8+/CD4− and CD4+/CD8−, respectively. The subpopulation of regulatory T cells (Treg) was gated from the CD4+ cells as CD4+/CD25^{high} [46]. Due to the variability among individuals, for each animal, the percentage of each lymphocyte subpopulation was normalised over the positive control (PHA-stimulated PBLs) in the immunosuppressive assays, and over the MLR-matched control in the one-way MLR assays.

In both immunosuppressive and one-way MLR assays, proliferation was assessed for each lymphocyte subset by studying CFSE dilution (FL1 channel) in cells gated as aforementioned. Unstimulated and unstained PBLs were used to define the autofluorescence (background). Unstimulated and CFSE-labelled PBLs were used to establish the non-proliferating population, considering cells to the left (lower fluorescence intensity) as proliferating. Supplementary Material S2: Figure S2.1 represents the gating strategy.

In the immunosuppressive assays, PHA-stimulated PBLs alone from each horse were used as the positive control and their proliferation was considered as 100%. Lymphocyte proliferation after co-culture was calculated by comparing samples to paired positive control to account for inter-individual variability in PHA response.

In the one-way modified MLRs, PHA-stimulated PBLs served only as internal control to verify their proliferative ability. Proliferation after MSC exposure was normalised over that observed in MLR-matched controls.

2.6. Interferon Gamma Secretion Assay

Supernatants collected from both immunosuppression and modified one-way MLR assays were used to evaluate IFNγ production by using a commercially available ELISA kit (Equine IFN-gamma DuoSet ELISA, R&D Systems, REF: DY1586), as previously reported [33,47]. The supernatants from unstimulated and PHA-stimulated PBLs seeded alone were used as negative and positive controls, respectively, for the immunosuppression assays. For the modified one-way MLRs, the supernatants from the classical MLRs with MHC-matched or mismatched PBLs as stimulators were used as the negative and positive control, respectively. All supernatants were diluted 1:1 in reagent diluent.

All the procedures were performed as per manufacturer's instructions and concentrations determined using a standard curve, including a blank.

The standard curve was set from 31.25 pg/mL to 8000 pg/mL of IFNγ. All the samples and points of the standard curve were run in duplicate. All the colorimetric assays were analysed on a microplate reader (Biotek Synergy HT, Winooski, VA, USA) and read immediately at 450 nm with wavelength correction set to 540 nm. The duplicate readings for each standard, control, and sample were averaged, and the average zero standard optical density was extracted. The standard curve was created generating a four-parameter logistic curve-fit and the concentrations extrapolated were multiplied by the dilution factor.

2.7. Statistical Analysis

Statistical analysis was performed with GraphPad Prism 5 software (San Diego, CA, USA). Data sets were checked for normality using the Shapiro–Wilk test, and parametric or non-parametric tests were chosen accordingly. Differences in the surface expression of MHC-I and MHC-II between MSC-naïve and MSC-primed were analysed by paired *t*-test. Results from flow cytometry and ELISA were separately analysed for each assay (immunomodulation or immunogenicity). For each one, data were compared among MSC-naïve, MSC-primed and MSC-chondro, and regarding corresponding controls, in each type of co-culture (autologous, allogeneic MHC-matched or allogeneic MHC-mismatched) by using one-way ANOVA or the Kruskall–Wallis test followed by Dunn's post-hoc test. The effect of the type of co-culture was analysed by comparing the results for each type of combination (autologous, MHC-matched, MHC-mismatched) for each type of MSC (MSC-naïve, MSC-primed, MSC-chondro) using paired tests (repeated measures ANOVA or Friedman test, followed by Dunn's post-hoc test). Significance was set as $p < 0.05$ in all cases.

3. Results

3.1. Surface Expression of MHC-I and MHC-II

The expression of MHC-I was highly variable among MSCs from the three horses, both before and after priming MSCs with proinflammatory cytokines. The mean value of MHC-I expression was 19% of positive cells for MSC-naïve and 65% for MSC-primed.

Expression of MHC-II in MSC-naïve was low and similar among donors (mean of 8.5% positive cells) and experienced a similar increase in the MSCs from the three horses after priming (mean 62%). It is worth noting that MSCs from one donor (D1, green dot) showed very low expression of both MHC-I and MHC-II before priming (MSC-naïve), but these cells experienced the greatest MHC-I and II increase after priming (MSC-primed) (Figure 2).

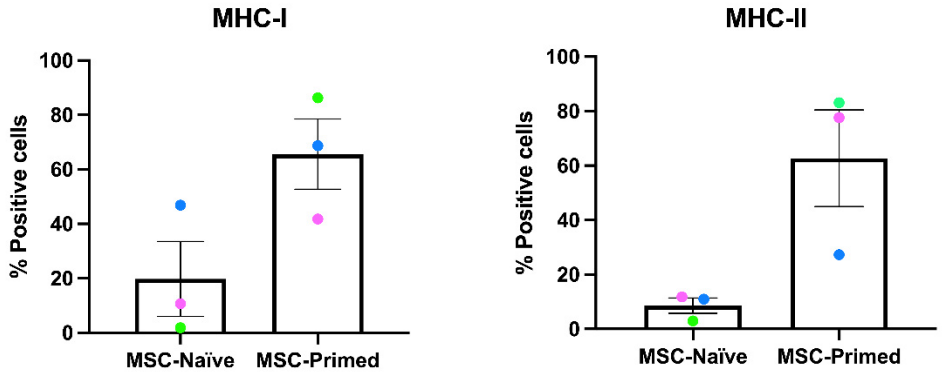


Figure 2. Mean ± S.E.M (n = 3) of the percentage of MSC before (MSC-naïve) and after being exposed to proinflammatory cytokines (MSC-primed) positive for the surface markers MHC type I and MHC-II determined by flow cytometry. Each colour dot represents the value for each donor (D1, green dot; D2, pink dot; D3, blue dot). These results corroborated the increased expression of both MHC type I and II in MSC-primed, and the inter-individual variability for MHC expression.

3.2. Changes in Lymphocyte Subpopulations and Proliferation in Immunomodulatory Assays

3.2.1. CD3+ T Lymphocytes

The percentage of CD3+ T lymphocytes remained similar under the different conditions, so neither the type of combination nor the type of MSCs seemed to produce relevant changes in the global T cell population (Figure 3A).

However, the proliferation of CD3+ T cells was reduced in the presence of all types of MSCs and for all the combinations (autologous and allogeneic MHC-matched and mismatched) compared to PHA-stimulated lymphocytes alone. Overall, MSC-primed were superior suppressing the proliferation of lymphocytes, followed by MSC-chondro and MSC-naïve. In the MHC-matched co-cultures, CD3+ T cell proliferation was significantly reduced by MSC-primed ($p < 0.05$) and by MSC-chondro ($p < 0.001$) compared to MSC-naïve, whereas in MHC-mismatched combination, the suppression was significant only between MSC-primed and MSC-naïve ($p < 0.05$). Significant differences were not observed between MSC types under autologous combinations, likely because of the lower n. Nevertheless, autologous MSCs tended to further suppress CD3+ T cell proliferation, followed by allogeneic matched and mismatched MSCs (non-significant) (Figure 4A).

3.2.2. CD8+ Cytotoxic and CD4+ Helper T Lymphocytes

When looking at specific T cell populations, CD8+ cytotoxic and CD4+ helper T cells showed opposite changes. The percentage of CD8+ T cells tended to increase in the presence of MSCs, as shown by significant differences over PBLs alone in both positive and negative controls. Specifically, the population of CD8+ T cells was significantly increased by MSC-naïve MHC-matched ($p < 0.001$) and mismatched ($p < 0.05$) compared to PHA-stimulated PBLs alone. Compared to the negative control (non-stimulated PBLs alone), matched MSC-naïve ($p < 0.001$), mismatched MSC-naïve ($p < 0.05$), matched MSC-primed ($p < 0.01$) and mismatched MSC-primed ($p < 0.05$) significantly raised the CD8+ population. There were not significant differences among combinations, but a tendency was observed with MHC-mismatched MSCs inducing higher CD8+ percentages, followed by MHC-matched and autologous MSCs. Overall, the type of MSCs (naïve, primed or chondro) also influenced the T cytotoxic population, with MSC-naïve promoting higher percentages of CD8+ cells, followed by MSC-primed and MSC-chondro. Specifically, this increase was statistically

significant between MSC-naïve and MSC-chondro for both MHC-matched ($p < 0.05$) and MHC-mismatched ($p < 0.01$) combinations. In the mismatched setting, MSC-primed also induced a significantly higher percentage of CD8+ T cells compared to MSC-chondro ($p < 0.05$) (Figure 3B).

On the contrary, the population of CD4+ T cells tended to decrease in the presence of MSCs. The percentage of helper T cells was significantly reduced over both the positive and negative controls when exposed to MHC-matched or MHC-mismatched MSC-naïve and MSC-primed ($p < 0.001$ in all cases). In spite of no significant differences detected among combinations, a tendency was observed with autologous MSCs inducing higher CD4+ percentages, followed by MHC-matched and mismatched MSCs—on the contrary to that observed for CD8+ T cells. In addition, inversely to the cytotoxic T cell population, MSC-naïve promoted lower percentages of CD4+ cells, followed by MSC-primed and MSC-chondro. Specifically, PBLs co-cultured with MSC-naïve and MSC-primed showed lower percentages of CD4+ cells than those exposed to MSC-chondro in both MHC-matched ($p < 0.05$) and mismatched ($p < 0.001$) allogeneic settings (Figure 3C).

Regarding the proliferation of these subpopulations, MSC-primed and MSC-chondro tended to suppress proliferating CD8+ T cytotoxic cells over the positive control, but MSC-naïve promoted an increase in the proliferation of CD8+ cells. These differences were statistically significant only in the MHC-matched setting ($p < 0.05$ for all the three types of MSCs). Interestingly, MSC-chondro showed the highest suppressive capacity, followed by MSC-primed. Specifically, MSC-chondro significantly suppressed CD8+ T cells proliferation compared to MSC-naïve in both MHC-matched and mismatched co-cultures ($p < 0.001$). Significant differences were not observed between MSC types under autologous combinations, as reported for CD3+ T cells. Significant differences were neither seen among combinations, but a tendency towards further suppressive capacity of autologous MSCs over allogeneic ones was noted, as well as the aforementioned for CD3+ lymphocytes (Figure 4B).

Proliferation of CD4+ T cells was suppressed in all the co-cultures with MSCs. Compared to PHA-stimulated PBLs alone, MSC-naïve and MSC-primed significantly reduced CD4+ cells proliferation in both MHC-matched and mismatched combinations ($p < 0.001$ for all conditions). MSC-chondro also showed suppressive capacity of CD4+ cells, but it was more variable, being significant only over MSC-naïve in the MHC-matched setting ($p < 0.01$). Similarly to that described for CD3+ and CD8+ cells, the effect of the type of combination was not statistically significant, but autologous MSCs tended to show further suppression (Figure 4C).

3.2.3. CD4+ $CD25^{high}$ Regulatory T Cells

In this study, Treg were gated as CD4+ $CD25^{high}$ cells. Phytohemagglutinin increased the percentage of CD4+ $CD25^{high}$, as shown in the positive control over the negative one ($p < 0.05$), and as also previously reported using a similar lectin mitogen [48]. In the presence of MSC-naïve and MSC-primed, PHA-activated PBLs further increased the percentage of CD4+ $CD25^{high}$ T cells, whereas MSC-chondro clearly tended to decrease this population. Specifically, and compared to the positive control, Treg population increased when co-cultured with MSC-naïve and MSC-primed, either MHC-matched ($p < 0.001$) or MHC-mismatched ($p < 0.05$), and decreased when exposed to MHC-matched MSC-chondro ($p < 0.001$). Similar increases were also noted over the negative control (MHC-matched MSC-naïve and primed, $p < 0.001$; MHC-mismatched MSC-naïve, $p < 0.001$; MHC-mismatched MSC-primed, $p < 0.05$). The effect of the combination followed a clear tendency in spite of no significant differences observed, with MHC-mismatched MSCs inducing higher levels of Treg cells, followed by MHC-matched and autologous MSCs. The effect of the type of MSCs was also clear, with MSC-naïve further increasing Treg cells, followed by MSC-primed. In particular, MHC-matched and mismatched MSC-naïve promoted higher percentages of Tregs over MSC-chondro ($p < 0.001$ in both cases), and MHC-mismatched MSC-primed also increased this population compared to MSC-chondro ($p < 0.01$) (Figure 3D).

Despite of the fact that MSC-naïve and MSC-primed can increase the CD4+ CD25^{high} population, all the co-cultures suppressed the proliferation of these cells compared to the positive control, but this reduction was not statistically significant in any condition. Suppression of Treg proliferation was stronger for MHC-matched MSC-chondro over MSC-naïve ($p < 0.05$), which may be related to the tendency of MSC-chondro to reduce the percentage of these cells. No clear effect of the type of combination was observed, but overall autologous MSCs appeared to be more suppressive (Figure 4D).

3.2.4. CD3− Pan-Ig+ CD21+ B Cells

B cells were gated as CD3− Pan-Ig+ CD21+ and this population tended to increase in the presence of MSCs, especially with MSC-naïve. Specifically, MHC-matched MSC-naïve promoted higher percentage of CD3− Pan-Ig+ CD21+ compared to the positive ($p < 0.001$) and negative ($p < 0.01$) controls, and also to MSC-primed ($p < 0.05$) (Figure 3E).

When looking at the proliferation of B cells, MSCs had a clear suppressive effect. Compared to PHA-activated PBLs alone (positive control), MHC-matched and mismatched MSC-naïve and MSC-primed significantly suppressed CD3− Pan-Ig+ CD21+ proliferation ($p < 0.001$ in all the four comparisons). When comparing types of MSCs, MSC-primed elicited a higher suppressive potential of this population, followed by MSC-naïve and MSC-chondro. In particular, suppression of the B cell population was further suppressed by MHC-matched MSC-primed ($p < 0.01$) and MSC-naïve ($p < 0.05$), and by MHC-mismatched MSC-primed ($p < 0.001$), compared to corresponding MSC-chondro. The type of combination did not significantly influence the suppression of B cells proliferation, but autologous MSCs tended to further reduce this proliferating population (Figure 4E).

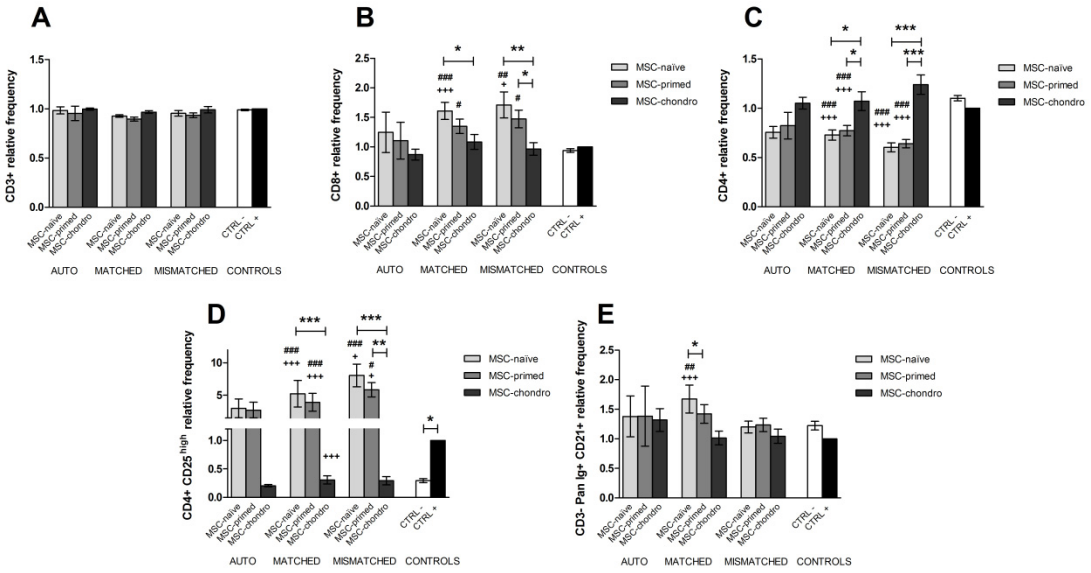


Figure 3. Mean ± SEM of the relative frequency of different lymphocyte subsets in the immunomodulatory assays: (**A**) CD3+ T lymphocytes, (**B**) CD8+ cytotoxic T cells, (**C**) CD4+ helper T cells, (**D**) CD4+ CD25^{high} regulatory T cells, (**E**) CD3−Pan-Ig+ CD21+ B cells. Phytohemagglutinin (PHA)-activated PBLs were exposed in vitro to MSC-naïve (light grey bar), MSC-primed (medium grey bar) and MSC-chondro (dark grey bar). Co-cultures of MSCs and PBLs were autologous ($n = 3$) or allogeneic, matched ($n = 8$) or mismatched ($n = 7$) for the MHC. Data from each PBL donor is normalised over the positive control (CTRL+) consisting of PHA-stimulated PBLs alone from the same donor (value 1), to account for inter-individual variability. Significant differences of each condition over the CTRL+ (black bar) are represented by a cross (+) above the corresponding bar (+ = $p < 0.05$; +++ = $p < 0.001$). Significant differences over the negative control (CTRL−; non-activated PBLs alone, white bar) are represented by hashes (#) above the corresponding bar (# = $p < 0.05$; ## = $p < 0.01$; ### = $p < 0.001$). Significant differences between experimental conditions are represented by a squared line with an asterisk (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

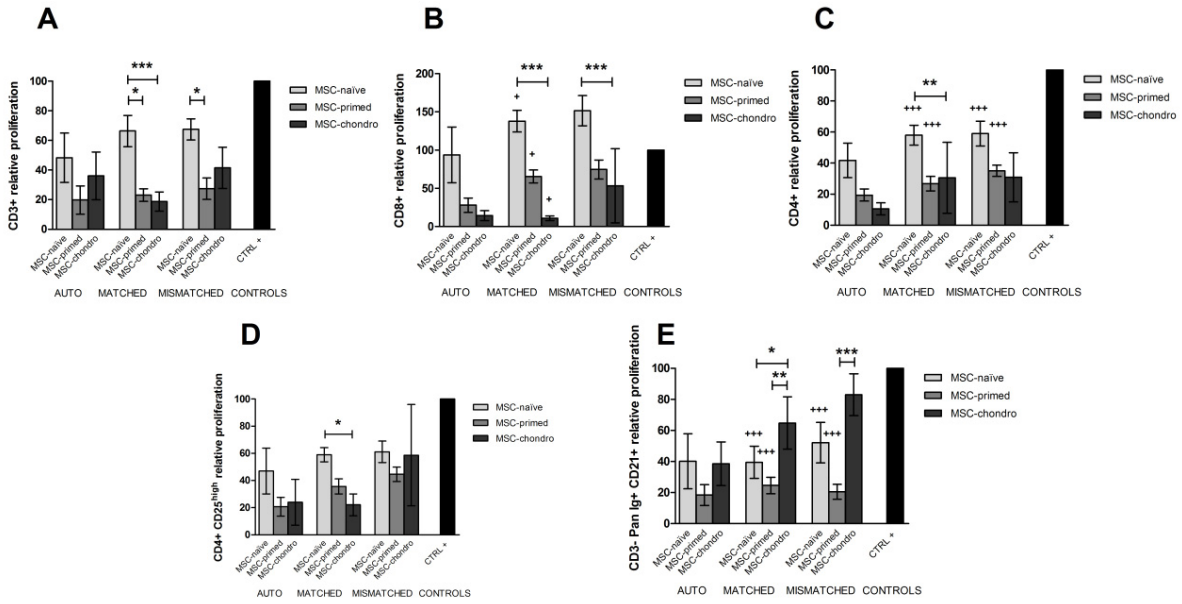


Figure 4. Mean ± SEM of the relative proliferation of different lymphocyte subsets in the immunomodulatory assays: (**A**) CD3+ T lymphocytes, (**B**) CD8+ cytotoxic T cells, (**C**) CD4+ helper T cells, (**D**) CD4+ $CD25^{high}$ regulatory T cells, (**E**) CD3−Pan-Ig+ CD21+ B cells. Phytohemagglutinin (PHA)-activated PBLs were exposed in vitro to MSC-naïve (light grey bar), MSC-primed (medium grey bar) and MSC-chondro (dark grey bar). Co-cultures of MSCs and PBLs were autologous (n = 3) or allogeneic, matched (n = 8) or mismatched (n = 7) for the MHC. Data from each PBL donor is normalised over the positive control (CTRL+) consisting of PHA-stimulated PBLs alone from the same donor (100%, maximum proliferation) to account for inter-individual variability. Significant differences of each condition over the CTRL+ (black bar) are represented by a cross (+) above the corresponding bar (+ = $p < 0.05$; +++ = $p < 0.001$). Significant differences between experimental conditions are represented by a squared line with an asterisk (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

3.3. Changes in Lymphocyte Subpopulations and Proliferation in Immunogenicity Assays

3.3.1. CD3+ T Lymphocytes

The percentage of CD3+ T cells did not significantly change in response to the different MSCs in the modified one-way MLRs, neither regarding their type (MSC-naïve, MSC-primed, MSC-chondro), nor regarding the combination (autologous, allogeneic MHC-matched or mismatched) (Figure 5A).

Compared to the negative control (MLR-matched), proliferation of T cells was not significantly induced under any condition. Actually, a lower proliferation of T cells was observed when co-cultured with MSC-naïve and MSC-primed in all the three combinations. Specifically, proliferation of T cells was significantly lower with MSC-naïve than with MSC-chondro in both autologous and MHC-mismatched settings ($p < 0.05$ in both conditions). Regarding the mismatched co-cultures, these tended to produce a higher T cell response, followed by MHC-matched and autologous combinations. Nevertheless, the difference was only significant for MSC-primed between mismatched and matched conditions ($p < 0.05$) (Figure 6A).

3.3.2. CD8+ Cytotoxic and CD4+ Helper T Lymphocytes

The percentage of CD8+ T cells did not significantly change under any condition in the one-way modified MLR assays, contrary to that observed in the immune suppression assays (Figure 5B). However, the percentage of CD4+ T cells was reduced in the presence of MSC-primed in the allogeneic MHC-matched ($p < 0.05$) and mismatched ($p < 0.01$) combinations, and after the exposure to MSC-chondro in the MHC-mismatched scenario ($p < 0.05$), compared to the MLR-matched control. Furthermore, in the mismatched co-

cultures, MSC-chondro ($p < 0.001$) and MSC-primed ($p < 0.05$) reduced the percentage of CD4+ T cells compared to the MSC-naïve. Overall, mismatched combinations tended to further reduce the percentage of helper T cells, but only MSC-chondro showed a significant reduction ($p < 0.05$) compared to the matched setting (Figure 5C).

A statistically significant increase of CD8+ T cells proliferation was neither observed compared to the MLR-matched control under any condition, but a marked tendency towards cytotoxic T cells induction was observed with MSC-primed. Actually, CD8+ T cell proliferation was higher in the presence of MSC-primed compared to MSC-naïve and MSC-chondro in the three combinations, but the difference was statistically significant only regarding MSC-chondro in the mismatched setting ($p < 0.05$) (Figure 6B). Proliferation of CD4+ T cells followed a similar pattern to CD8+ T cells: whereas no significant differences were observed between any condition and the MLR-matched control, MSC-primed tended to induce T helper proliferation in the two allogeneic settings. Specifically, proliferation of CD4+ T cells was higher after exposure to MSC-primed compared to MSC-naïve ($p < 0.05$) and to MSC-chondro in both the MHC-matched ($p < 0.05$) and mismatched ($p < 0.01$) scenarios. Significant differences among conditions were only observed for MSC-chondro, with higher proliferation in the MHC-mismatched combination compared to the MHC-matched one, but not exceeding the MLR-matched values (Figure 6C).

3.3.3. CD4+ CD25^{high} Regulatory T Cells

Overall, MSC-naïve and MSC-primed tended to increase the percentage of CD4+ CD25^{high} and MSC-chondro to reduce it, regardless of the type of combination. Specifically, MHC-mismatched MSC-primed significantly increased the CD4+ CD25^{high} population ($p < 0.05$) compared to the MLR-matched control and to both MSC-naïve ($p < 0.01$; MHC-mismatched) and MSC-chondro ($p < 0.05$; MHC-matched and mismatched) (Figure 5D).

Similarly to that observed for cytotoxic and helper T cells, Treg cells were induced to proliferate after exposure to MSC-primed regardless of the type of combination. However, the proliferation observed was not statistically different to that reported for the MLR-matched control. Nevertheless, MSC-primed significantly induced proliferation of Tregs compared to MSC-chondro in both the allogeneic MHC-matched ($p < 0.01$) and mismatched ($p < 0.001$) combinations, as also seen with CD4+ helper T cells. Furthermore, the proliferation induced by MSC-primed was significantly higher in the mismatched setting compared to the matched ($p < 0.05$) (Figure 6D).

3.3.4. CD3− Pan-Ig+ CD21+ B Cells

The frequency of the B cell population did not significantly change compared to control MLRs after exposure to different types of MSCs in autologous and allogeneic MHC-matched combination. Overall, MSC-naïve tended to further diminish the percentage of B cells. However, in the MHC-mismatched combination, MSC-chondro promoted a greater reduction, being significant compared to both MLR-matched ($p < 0.05$) and mismatched ($p < 0.01$) controls. In this setting, both MSC-chondro and MSC-naïve elicited a reduction of B cells compared to MSC-primed ($p < 0.05$ in both cases) (Figure 5E).

In the three combinations, proliferation of B cells was similar to that in the MLR-matched control for MSC-naïve and MSC-primed, whereas it was higher for MSC-chondro but not statistically significant. However, B cell proliferation elicited by MSC-chondro was significantly higher ($p < 0.05$) than that observed with MSC-primed in both allogeneic MHC-matched and mismatched settings. Among combinations, proliferation of B cells exposed to MSC-primed was higher ($p < 0.05$) in the mismatched scenario than in the matched, but did not differ from the MLR-matched control (Figure 6E).

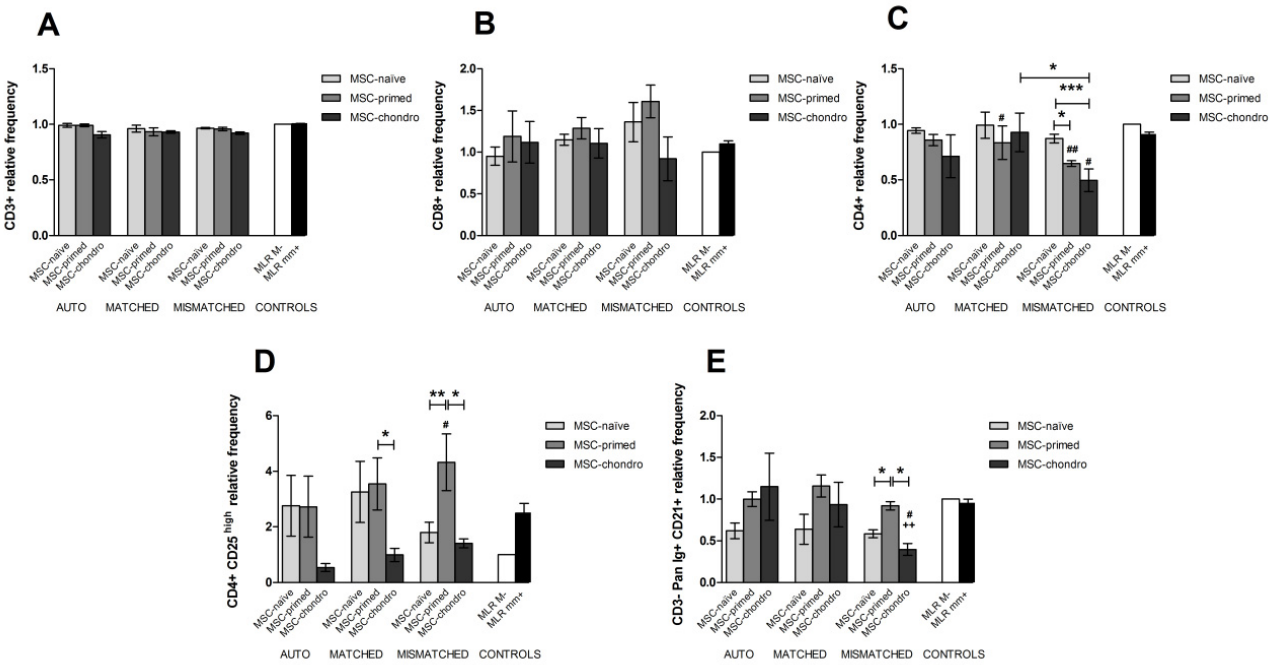


Figure 5. Mean ± SEM of the relative frequency of different lymphocyte subsets in the immunogenicity assays (one-way modified mixed lymphocyte reaction): (**A**) CD3+ T lymphocytes, (**B**) CD8+ cytotoxic T cells, (**C**) CD4+ helper T cells, (**D**) CD4+ CD25^{high} regulatory T cells, (**E**) CD3−Pan-Ig+ CD21+ B cells. Non-activated PBLs were exposed in vitro to MSC-naïve (light grey bar), MSC-primed (medium grey bar) and MSC-chondro (dark grey bar). Co-cultures of MSCs and PBLs were autologous (n = 3) or allogeneic, matched (n = 8) or mismatched (n = 7) for the MHC. Data from each PBL donor is normalised over the negative control (MLR M−, matched MLR) consisting of responder PBLs from the same donor exposed to MHC-matched stimulator PBLs (value 1), to account for inter-individual variability. Significant differences of each condition over the positive control (MLR mm+, mismatched MLR consisting of responder PBLs exposed to MHC-mismatched stimulator PBLs; black bar) are represented by a cross (+) above the corresponding bar (++ = $p < 0.01$). Significant differences over the negative control (MLR M−, white bar) are represented by hashes (#) above the corresponding bar (# = $p < 0.05$; ## = $p < 0.01$). Significant differences between experimental conditions are represented by a squared line with an asterisk (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

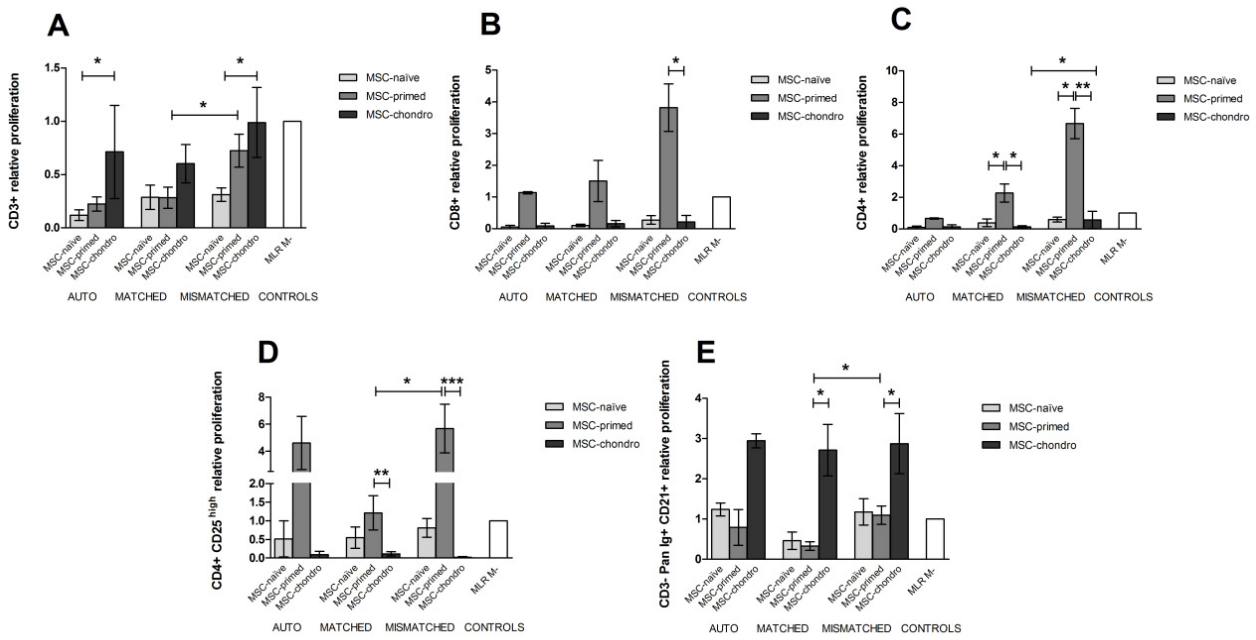


Figure 6. Mean ± SEM of the relative proliferation of different lymphocyte subsets in the immunogenicity assays (one-way modified mixed lymphocyte reaction): (**A**) CD3+ T lymphocytes, (**B**) CD8+ cytotoxic T cells, (**C**) CD4+ helper T cells, (**D**) CD4+ $CD25^{high}$ regulatory T cells, (**E**) CD3−Pan-Ig+ CD21+ B cells. Non-activated PBLs were exposed in vitro to MSC-naïve (light grey bar), MSC-primed (medium grey bar) and MSC-chondro (dark grey bar). Co-cultures of MSCs and PBLs were autologous (n = 3) or allogeneic, matched (n = 8) or mismatched (n = 7) for the MHC. Proliferation of each PBL donor is normalised over the proliferation observed in the negative control (MLR M−, matched MLR) consisting of responder PBLs from the same donor exposed to MHC-matched stimulator PBLs (value 1), to account for inter-individual variability. Significant differences over the negative control (MLR M−, white bar) were not observed. Significant differences between experimental conditions are represented by a squared line with an asterisk (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

3.4. Interferon Gamma (IFNγ) Production in Immunomodulatory and Immunogenicity Assays

The concentration of IFNγ was measured in co-culture supernatants from both immunomodulatory and immunogenicity assays as reflection of lymphocyte activation. Values of IFNγ were overall higher in the immunomodulatory assays, where PBLs were activated by PHA. Significant differences among autologous, allogeneic MHC-matched and MHC-mismatched co-cultures were not observed, neither in immunomodulatory assays nor in the immunogenicity ones, but for both types of assays, IFNγ concentration was higher in the co-cultures with MSC-primed (Figure 7).

In the immunomodulatory assay, co-cultures with MSC-naïve showed IFNγ concentrations similar or slightly higher to the positive control (PHA-stimulated PBLs alone), but significant differences were not observed. IFNγ presence in co-cultures with MSC-chondro was similar to that in the negative control (unstimulated PBLs alone). However, supernatant from MSC-primed co-cultures contained significantly higher concentrations of IFNγ than the negative control in both MHC-matched ($p < 0.01$) and MHC-mismatched ($p < 0.05$) combinations. In addition, IFNγ in these conditions also increased significantly compared to corresponding MSC-chondro co-cultures ($p < 0.001$ for MHC-matched, $p < 0.05$ for MHC-mismatched) (Figure 7A).

In the one-way MLRs (immunogenicity assays), both MSC-naïve and MSC-chondro induced IFNγ secretion in amounts similar to the negative control (MLR-matched) and lower than in the positive control (MLR-mismatched), but no significant differences over controls were observed. Presence of IFNγ in the supernatants from MSC-primed co-cultures

was higher than with the other MSC types and compared to controls, but significant differences were only observed between the MHC-matched MSC-primed co-culture and the negative control ($p < 0.05$) (Figure 7B).

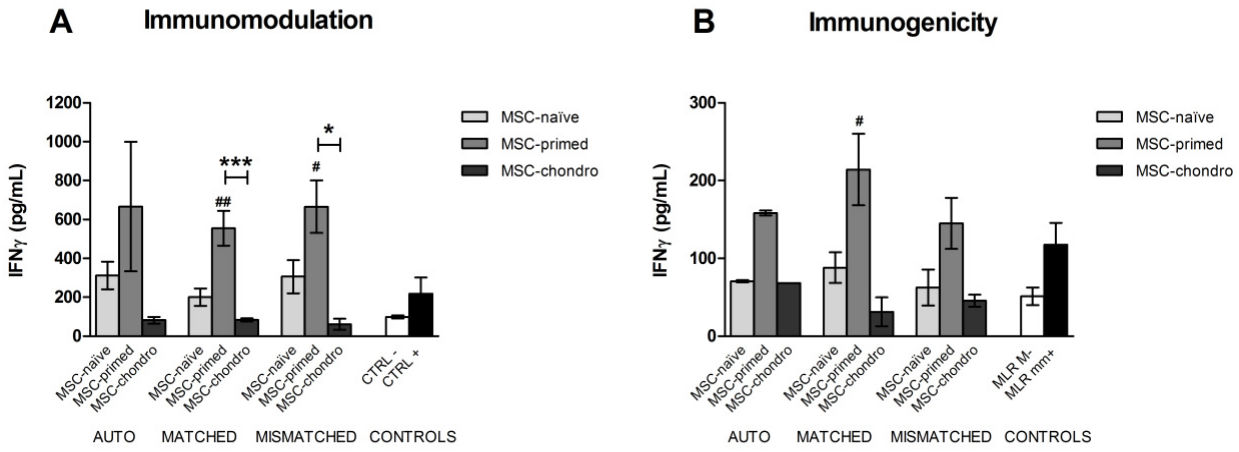


Figure 7. Mean ± SEM concentration (pg/mL) of interferon gamma (IFNɣ) in co-culture supernatants from immunomodulatory (**A**) and immunogenicity (**B**) assays. Phytohemagglutinin (PHA)-activated PBLs (**A**) or non-activated PBLs (**B**) were exposed in vitro to MSC-naïve (light grey bar), MSC-primed (medium grey bar) and MSC-chondro (dark grey bar). Co-cultures of MSCs and PBLs were autologous ($n = 3$) or allogeneic, matched ($n = 8$) or mismatched ($n = 7$) for the MHC. Significant differences of each condition over the positive controls were not observed. Significant differences over the negative controls are represented by hashes (#) above the corresponding bar (# = $p < 0.05$; ## = $p < 0.01$). Significant differences between experimental conditions are represented by squared line with asterisk (* = $p < 0.05$; *** = $p < 0.001$).

4. Discussion

To the best of authors' knowledge, this is the first study in the equine species assessing the effect of proinflammatory priming, chondrogenic differentiation, and MHC compatibility on the immunomodulatory and immunogenic potentials of MSCs. Under the conditions of this study, equine MSCs stimulated by proinflammatory cytokines (MSC-primed) were more immunosuppressive and presented mild immunogenicity compared to non-manipulated MSCs (MSC-naïve). Interestingly, equine MSCs differentiated into chondrocytes (MSC-chondro) did not lose their regulatory capacity, and neither did they significantly increase their immunogenicity. In addition, autologous MSCs were slightly more suppressive, whereas allogeneic MHC-mismatched MSCs tended to be more immunogenic, especially when they were primed with cytokines.

Previous studies have shown that both autologous and allogenic MSCs significantly suppressed the proliferation of PHA-activated T cells [32,39,49], but fewer studies have analysed the ability of MSCs to stimulate an immune response in resting lymphocytes [7,34,50]. In addition, the effects of MSCs under different conditions on specific lymphocyte subpopulations, and the way these populations change and proliferate after co-culture in vitro, has been barely studied in the horse. Overall, and in agreement with previous reports, co-culture assays conducted in this study showed that allogeneic MSCs are able to suppress activated lymphocytes. Moreover, allogeneic MSCs do not stimulate marked changes on the proliferation and frequency of different lymphocyte subpopulations.

According to our initial hypothesis, MSC-primed were more immune suppressive but also more immunogenic than MSC-naïve. However, we also hypothesized that MSC-chondro would lose their regulatory ability and would be more easily recognized by the immune cells; however, it turned out that differentiated MSCs were able to suppress the proliferation of different lymphocyte subpopulations in a similar way to MSC-primed, and did not provoke a marked proliferative response in resting lymphocytes. Therefore,

the capacity of suppressing CD3+ T cells was enhanced in both MSC-primed and MSC-chondro over MSC-naïve. Actually, when analysing the two main subpopulations of T cells, MSC-naïve were not able to suppress the proliferation of CD8+ cytotoxic T cells, indicating that the suppression in T cell proliferation elicited by these cells was exclusively due to the suppression of CD4+ helper T cells. Thus, and according to previous studies in the horse [34,51], a suppressive effect of MSC-naïve is observed in the general T cell population. However, contrary to our findings, Ranera et al. (2016) found that untreated MSCs were able to suppress the proliferation of CD8+ lymphocytes, while CD4+ cells were only slightly modified [32]. Meanwhile, both MSC-primed and MSC-chondro decreased the proliferation of both cytotoxic and helper T cells in a similar way, suggesting a further activation of their immunomodulatory properties over untreated MSCs, since cytotoxic T cells (CD8+) are primarily involved in the destruction of cells presenting foreign antigens, and T helper cells (CD4+) play an important role in establishing and maximizing the immune response [52].

Even though MSC-primed and MSC-chondro similarly suppressed the proliferation of cytotoxic and helper T cells, they differ in their behaviour regarding Treg and B cells. An increase in Tregs would be related to the immunosuppressive ability of MSCs, as these lymphocytes would help dampen the adaptive immune response and prevent rejection of foreign cells by the host [53]. In the one-way MLRs conducted in our study, relative frequency and proliferation of Treg cells were consistently and significantly increased after exposure to MSC-primed compared to MSC-chondro. In addition, MSC-naïve also induced an increase of Treg relative frequency in the three combinations, similarly to that reported by Kamm et al. (2021), which also showed an increase in Treg lymphocytes in contact with MSCs from universal blood donors and with low MHC class II expression [50]. Furthermore, for the population of B lymphocytes, which principal function is the production of antibodies against foreign antigens [52], MSC-primed showed the most marked suppressive capacity. In agreement with our results, a previous study in human MSCs [54] observed that MSC-primed inhibited the proliferation of B cells over MSCs-naïve. Inversely, for lymphocytes B, MSC-chondro were significantly less suppressive than MSC-primed.

Previous studies measuring the ability of proinflammatory primed equine MSCs to suppress the proliferation of allogeneic activated T cells have shown similar results. The study of Caffi et al. (2020) analysed the effect of pre-conditioning MSC with proinflammatory cytokines (TNFα and IFNγ) and, according to our results, reported that priming MSCs increases their inhibitory effect on lymphocyte proliferation [55]. Another study [51] concluded that MSCs primed with either polyinosinic:polycytidylic acid, lipopolysaccharide, or inflammatory macrophages, produced a similar enhancement in their ability to modulate T cell proliferation. This suppressive ability of MSC-primed would be related to paracrine immunomodulation mechanisms, which are induced by inflammation as reflected by the increase of the expression and secretion of different mediators, such as cyclooxygenase 2 (COX-2), indoleamine 2-3-dioxygenase (IDO), and interleukin 6 (IL-6) [15].

Furthermore, previous studies in other species reported that chondrogenically differentiated rat MSCs had decreased immunomodulatory potential, in disagreement with our results [16]. However, there are also reports on human chondrogenically-induced MSCs showing a regulatory profile similar to undifferentiated cells [56]. Therefore, variability among species could be expected, and thus it is important to explore this scenario in the horse. Beyond the potential inter-species variability, other possible explanations for these findings should be considered. First, the ratio between MSC-chondro and PBLs could have varied over MSC-naïve and MSC-chondro in this experiment. For MSCs-naïve and MSC-primed, the number of cells seeded to reach the appropriate ratio of MSC:PBLs can be controlled, and was chosen based on previous studies [7,32,34]. However, for MSC-chondro, because two weeks were necessary for inducing their differentiation, it is difficult to adjust the exact final number of cells. There is no consensus regarding whether MSCs proliferate or not while differentiating into chondrocytes. Some authors [57] described that proliferation of MSCs in micromasses is decreased during the first week of chondrogenic differentiation, whereas other authors [58] reported that MSCs proliferate as they

differentiate into cartilage. In this study and prior to any experimental assay, MSCs were differentiated into chondrocytes by different methods (monolayer, micromass and pellet culture). After two weeks, the extracellular matrix of chondrogenically differentiated MSCs were digested by collagenases, and the number of cells evaluated as previously described [59,60]. Based on these preliminary assays, the micromass technique was chosen as the most consistent method for differentiation under our conditions, but the effect of this variable on the final MSC:PBL ratio cannot be totally disregarded. In addition, the possible presence of undifferentiated MSCs in the MSC-chondro co-cultures could have contributed to the suppression of lymphocyte proliferation observed.

Regarding immunogenicity, and according to our initial hypothesis, MSC-primed induced a proliferative response in cytotoxic and helper T cells. However, B cells were not activated and Treg were increased. However, even though we hypothesized that MSC-chondro would be more immunogenic, they did not increase proliferation or induce changes in the cytotoxic and helper T cell populations. However, MSC-chondro induced proliferation of B cells and showed the lowest ability to stimulate Tregs. Kamm et al. (2021) showed that proliferation of B cells was not stimulated when faced with allogeneic MSCs [50], similarly to that observed with both MHC-matched and mismatched MSC-naïve and MSC-primed in this study. Regarding MSC-chondro, a previous study in rat MSCs [16] reported a significant increase in B cells when co-cultured with chondrogenically differentiated cells, similarly to our findings.

The increased immunogenicity of MSC-primed would be associated with the increase in MHC expression. The upregulation of MHC-I and MHC-II after priming MSCs observed in this study agrees with previous reports in the equine species [7,15]. A recent study in horses [50] described that the MHC expression level in MSCs largely depends on the horse donor, and these cells could be classified as MHC class II-high or low expression, which would affect their immune recognition. According to our results, the MSCs in our study would be classified as MHC-II low, but will change into the MHC-II high category after cytokine priming, with a parallel increase in their capacity to stimulate an immune response.

Studies reporting immune response against MSC-chondro have also shown increased levels of MHC surface expression compared to undifferentiated MSCs [16]. In a previous work from our group, an upregulation of MHC-I and II was observed after chondrogenic differentiation of equine MSCs [17]. However, in the current study, immunogenicity of MSC-chondro was not observed, except for the B cells response. In the equine species, there is only one study assessing the capacity of chondrogenic induced equine allogeneic peripheral blood-derived MSCs (ciMSCs) to activate lymphocytes [18]. Their results showed no cellular immune response in one-way MLRs with ciMSCs, and no immunomodulatory capacity of these cells in immunosuppressive assays either; although, it should be noted that all co-cultures were carried out from 5 days to one year and a half after treating 10 horses with ciMSCs. In addition, in their study, the MHC-haplotype of ciMSC donors and recipients was not established, and neither were lymphocyte subpopulations assessed. It should also be considered that the methodology for chondrogenic induction varies between this and our study, which might explain the lack of immunomodulatory properties seen by Van Hecke et al. (2021) [18].

In our study, MSC-chondro were thoroughly washed prior to adding PBLs but the possible effect of TGF-β3 in the differentiation media regarding their immunogenic profile should also be noted, provided that TGF-β2 can diminish MHC expression in equine MSCs [61]. These authors observed that TGF-β2-treated MHC-mismatched MSCs, which have reduced MHC-I surface expression, and induced less T cell receptor downregulation and cytotoxicity than untreated MHC-mismatched MSCs in vitro, thus suggesting that treatment with this growth factor could reduce the cell-mediated immunogenicity of MHC-mismatched MSCs in vitro.

We also hypothesized that MHC-matched MSCs would generate similar results than autologous ones, while MHC-mismatched MSCs would present similar regulatory ca-

pacity but increased immunogenicity. Even though we observed similar results in both immunomodulatory and immunogenicity assays for the three types of combinations, it should be noted that the autologous group tended to further supress the proliferation of all lymphocyte subpopulations, followed by the allogeneic MHC-matched group and the MHC-mismatched one. Furthermore, MHC-mismatched MSCs tended to induce higher lymphocyte proliferation, but this effect was only obvious with MSC-primed.

Ranera et al. (2016) studied the immunomodulatory ability of MSCs co-cultured with activated PBLs of autologous and MHC-mismatched horses, and showed that both of them exhibited a similar capacity to significantly reduce lymphocyte proliferation in vitro [32]. However, in this study MHC-matched co-cultures were not analysed and only MSC-naïve were tested. Another study [34] showed that both autologous and allogeneic MSCs decreased proliferation of activated lymphocytes. Both autologous and allogeneic MSCs appeared to be equally immunosuppressive, and no difference was noted between them, but only CD3+ T cells were analysed. For the general T cell population, our study also showed similar results between combinations for MSCs-naïve, but further tendencies were observed when assessing lymphocyte subpopulations and MSCs under different conditions. Moreover, in the study of Colbath et al. (2017), MHC haplotypes of horses were not established and they reported a low expression of MHC-II in MSCs, which remained low after the co-cultures [34].

To assess the ability of MSCs to activate lymphocytes, Colbath et al. (2017) also co-cultured autologous or allogenic MSCs with inactivated PBLs, and concluded that equine allogeneic MSCs were not inherently more immunogenic in terms of T cell activation, as they only induced a mild lymphocyte proliferation in vitro, similar to that observed with autologous MSCs [34]. In our study, a higher immunogenic response in cytotoxic and helper T lymphocytes were observed in MHC-mismatched co-cultures when MSCs were primed, in agreement with [50], which observed increased proliferation of CD8+ and CD4+ T cells after co-culture with MHC-mismatched MSCs showing MHC-II high expression. Nevertheless, Kamm et al. (2021) concluded that differences between autologous and allogeneic MSCs were minimal regarding their effect on the activation of lymphocytes [50]. On the contrary, another in vitro study where MHC-haplotypes were controlled showed that MHC-mismatched MSCs induced lymphocyte activation [7]. Their results were in agreement with our findings, as Schnabel et al. (2014) revealed that MHC-mismatched MHC class II-positive MSCs caused a significant increase in the proliferation of T cells compared to MHC-matched MSCs and to MHC-mismatched MHC class II-negative, concluding that MHC-mismatched MSCs induced greater lymphocyte activation in vitro, particularly when MHC-II expression was high, as it was observed with MSC-primed in our study [7].

It should also be considered that, in the autologous group of our study, only three horses were involved. This *n* was lower than in the allogeneic groups and could help explain why in most autologous conditions no significant differences appeared among MSC types, even though they followed the same trend as allogeneic MHC-matched and mismatched groups. However, this setup was chosen to use the same MSCs across all the experiments, avoiding the involvement of more animals and the increase of inter-individual variability.

Finally, IFNγ was determined in co-culture supernatants as this proinflammatory cytokine is related to cell-mediated immunity produced by T lymphocytes. The principal function of IFNγ is to stimulate both innate and adaptive immune responses [62], so its secretion indicates expansion of CD8+ or CD4+ effector and memory cells against donor MSCs [5]. However, other authors report that IFNγ production stimulates the regulatory ability of MSCs, thus subsequently reducing the proliferation of immune cells [63]. In our study, overall lower secretion of IFNγ was observed in the immunogenicity assays compared to the immunomodulatory ones, but it must be considered that in the latter, the lymphocytes were mitogen-activated and thus produce higher concentrations of IFNγ [34,47,49]. In both immunomodulatory and immunogenicity assays of our study, lymphocytes co-cultured with MSC-chondro showed the lowest IFNγ secretion, and lymphocytes co-cultured with MSC-primed resulted in the highest secretion.

In the immunomodulatory assays, and contrary to that previously reported [39,49], a significant reduction in IFNγ secretion over the positive control was not observed after co-culture of activated lymphocytes with the different types of MSCs and in the different combinations. Actually, IFNγ secretion increased in the presence of MSC-primed in both immunomodulatory and immunogenicity co-cultures. This finding could reflect a further activation of lymphocytes in the presence of MSC-primed, as seen by increased proliferation of CD8+ and CD4+ T cells in the one-way MLRs of this study and, according to their increased MHC expression, also observed in previous studies [55]. However, the possible presence of residual exogenous IFNγ from the priming process prior to co-culturing MSCs with lymphocytes should also be considered, even though cells were washed with PBS.

At the same time, MSC-naïve and MSC-chondro did not significantly reduce IFNγ secretion by activated lymphocytes, but neither stimulated their secretion over the controls in resting ones. Secretion of IFNγ by lymphocytes in the presence MSC-chondro agrees with observations discussed above regarding suppression of CD8+ and CD4+ T cell proliferation in immunomodulatory assays and lack of their induction in one-way MLRs, further pointing at the ability of equine MSCs to maintain their regulatory properties and not increase their immunogenicity after chondrogenic differentiation.

Finally, and in addition to the limitations discussed along this section concerning the lower *n* of the autologous group, the challenges of MSC-chondro co-cultures and possible interferences of molecules in the culture media, few other points should also be noted. First, for one of the MSC donors (D2), it was only possible to find two other MHC-matched horses (A1, A2). The heterogeneity reported in ELA haplotypes [20,21] likely prevented finding a third matched animal to complete the group, even after a large screening. Nevertheless, the *n* of this study is similar to that in related reports [6,35], since the complexity of randomly finding compatible animals makes working with larger study groups difficult. Secondly, MHC haplotypes were determined as previously described but, since familiar information of horses was not available, only homozygous animals could be assigned as definitive [20,21]. Third, using mitogens such as PHA is a simple and effective method for stimulating PBLs in immune suppression assays, and has been widely reported [39,47], but does not completely resemble the in vivo activation. It should also be considered that in vitro assays provide valuable information to guide in vivo research, but the complexity of the interactions with the immune system cannot be completely reproduced.

5. Conclusions

This study reports how inflammation, differentiation, and compatibility for the MHC can influence the immunological properties of equine MSCs. Priming MSCs with proinflammatory cytokines activates their regulatory potential, as seen by decreased proliferation of cytotoxic and helper T cells, and B cells. However, inflammation can also increase the immune recognition of these cells through induction of MHC expression, thus making the allogeneic MHC-mismatched MSCs more likely to be targeted by the immune system. Importantly, equine MSCs do not lose their regulatory ability, and neither increase their immunogenicity after chondrogenic differentiation, but have reduced capacity to stimulate Treg cells and can stimulate the proliferation of B cells. Even though lymphocyte proliferation assays are important tools to assess both the immunomodulatory and immune evasive properties of MSCs, and thus exploring their therapeutic potential and immunogenicity, in vivo studies are needed to fully comprehend the complexity of the interactions of MSCs with the recipient immune system to develop safe and effective cell therapies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12080984/s1, Supplementary Material S1: Table S1.1. Primers used for gene expression analysis by RT-qPCR; Figure S1.1. Gene expression RT-qPCR; Figure S1.2. Surface markers expression; Figure S1.3. Results from the differentiation assays. Supplementary Material S2: Table S2.1. Antibodies used in panel 1 for flow cytometry assays; Table S2.2. Antibodies used in panel 2 for flow cytometry assays; Figure S2.1. Flow cytometry gating strategy.

Author Contributions: All authors (A.C., A.R., F.J.V., A.V., E.B., S.F., P.Z., C.R. and L.B.) substantially contributed to the conception and design of the study. L.B., A.C. and E.B. were responsible for acquisition of data. L.B., A.C., F.J.V. and C.R. were responsible for analysis and interpretation of data. A.R., A.V., S.F. and F.J.V. provided technical support. A.R., P.Z., F.J.V. and C.R. provided important conceptual guidance. P.Z. and C.R. were responsible for obtaining funding. L.B. and A.C drafted the article and all authors (A.R., F.J.V., A.V., E.B., S.F., P.Z. and C.R.) revised the manuscript for important intellectual content. All authors (A.C., A.R., F.J.V., A.V., E.B., S.F., P.Z., C.R. and L.B.) approved the final submitted version of the article and agree to be accountable for all aspects of the work in ensuring that accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors have read and agreed to the published version of the manuscript.

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The immunomodulation–immunogenicity balance of equine Mesenchymal Stem Cells (MSCs) is differentially affected by the immune cell response depending on inflammatory licensing and major histocompatibility complex (MHC) compatibility

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The immunomodulatory properties of equine mesenchymal stem cells (MSCs) are important for their therapeutic potential and for their facilitating role in their escape from immune recognition, which may also be influenced by donor–recipient major histocompatibility complex (MHC) matching/mismatching and MHC expression level. Factors such as inflammation can modify the balance between regulatory and immunogenic profiles of equine MSCs, but little is known about how the exposure to the immune system can affect these properties in equine MSCs. In this study, we analyzed the gene expression and secretion of molecules related to the immunomodulation and immunogenicity of equine MSCs, either non-manipulated (MSC-naive) or stimulated by pro-inflammatory cytokines (MSC-primed), before and after their exposure to autologous or allogeneic MHC-matched/-mismatched lymphocytes, either activated or resting. Cytokine priming induced the immunomodulatory profile of MSCs at the baseline (MSCs cultured alone), and the exposure to activated lymphocytes further increased the expression of *interleukin 6 (IL6)*, *cyclooxygenase 2*, and *inducible nitric oxide synthase*, and IL6 secretion. Activated lymphocytes were also able to upregulate the regulatory profile of MSC-naive to levels comparable to cytokine priming. On the contrary, resting lymphocytes did not upregulate the immunomodulatory profile of equine MSCs, but interestingly, MSC-primed exposed to MHC-mismatched lymphocytes showed the highest expression and secretion of these mediators, which may be potentially linked to the activation of

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lymphocytes upon recognition of foreign MHC molecules. Cytokine priming alone did not upregulate the immunogenic genes, but MSC-primed exposed to activated or resting lymphocytes increased their *MHC-I* and *MHC-II* expression, regardless of the MHC-compatibility. The upregulation of immunogenic markers including *CD40* in the MHC-mismatched co-culture might have activated lymphocytes, which, at the same time, could have promoted the immune regulatory profile aforementioned. In conclusion, activated lymphocytes are able to induce the equine MSC regulatory profile, and their effects seem to be additive to the priming action. Importantly, our results suggest that the lymphocyte response against MHC-mismatched MSC-primed would promote further activation of their immunomodulatory ability, which eventually might help them evade this reaction. Further studies are needed to clarify how these findings might have clinical implications *in vivo*, which will help developing safer and more effective therapies.

KEYWORDS

mesenchymal stem cells, horse, allogeneic therapy, haplotype, co-culture, immune response, gene expression, mediator secretion

Introduction

Mesenchymal stem cells (MSCs) are of great interest to treat several pathologies, including musculoskeletal injuries such as those affecting the horse, which is a species of remarkable value as both patient and translational models (1, 2). The regulatory and immunomodulatory properties of MSCs are currently considered their main therapeutic mechanism and involve both direct cell-to-cell contact and contact-independent paracrine signaling, *via* the expression of adhesion molecules like vascular cell adhesion molecule 1 (*VCAM1*) and the secretion of molecules such as interleukin 6 (IL6) and prostaglandin E2 (PGE_2) (3), respectively (4). Since the immunomodulatory properties of equine MSCs might have profound therapeutic implications in the treatment of many inflammatory-mediated processes in the horse, a growing number of studies have focused on analyzing such immune properties (4). To elucidate possible pathways for immunosuppression exerted by equine MSCs, and how these are influenced by different factors, it is critical to study the expression and secretion of mediators implied in their paracrine mechanisms, including the enzymes producing these molecules, such as cyclooxygenase 2 (*COX2*), indoleamine 2,3-dioxygenase 1 (*IDO1*), or inducible nitric oxide synthase 2 (*iNOS2*).

Furthermore, the MSC immunomodulatory activity is not only important for its therapeutic mechanism but also for its facilitating role in its escape from immune recognition when administered allogenically. Allogeneic application presents several advantages over autologous therapy as it increases the availability of thoroughly characterized cells for therapy, particularly when autologous cells are not suitable because of genetic or metabolic diseases, or in aged patients (5). However, MSCs are no longer considered truly immune-privileged but are considered immune-evasive, so their recognition and elimination by the immune system after their allogeneic administration should be considered (6). Allogeneic MSCs may be rejected due to the expression of foreign antigens on their surface, which may raise both cellular and humoral immune responses against the cells (7) and even lead to immune memory mechanisms that could prevent effective and safe repeated administration of allogeneic MSCs in the horse (8).

The surface expression of major histocompatibility complex (MHC) class I and II antigens on equine MSCs facilitates their immune recognition by lymphocytes, and antibodies can be generated specifically directed against the equine leukocyte antigen (ELA) of the donor, potentially compromising the

Abbreviations: AT, adipose tissue; B2M, beta-2-microglobulin; BM, bone marrow; CB, umbilical cord blood; CT, umbilical cord tissue; COX2, cyclooxygenase 2; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; ELA, equine leukocyte antigen; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IDO1, indoleamine 2,3-dioxygenase 1; IFNγ, interferon gamma; IL6, interleukin 6; iNOS1, inducible nitric oxide synthase 1; iNOS2, inducible nitric oxide synthase 2; iPSCs, induced pluripotent stem cells; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MSC, mesenchymal stem cell; NK, natural killer; PBL, peripheral blood lymphocyte; PBS, phosphate-buffered saline; PGE_2, prostaglandin E2; PHA, phytohemagglutinin; RT-qPCR, real-time quantitative polymerase chain reaction; TNFα, tumor necrosis factor alpha; VCAM1, vascular cell adhesion molecule 1.

therapeutic effectiveness of the cells. Therefore, MHC matching between the donor and recipient is receiving increasing attention in the last few years, and ELA haplotypes have been taken into account in equine studies (9, 10), as well as in other species (11, 12). It should be noted that the MHC haplotype is a factor intrinsic to each individual and as such cannot be modified. Furthermore, it has also been reported that the MHC level expression in MSCs in basal conditions is quite dependent on the equine donor (9). The knowledge on these factors is critical to design better therapeutic strategies, including donor selection. As a matter of fact, some researchers are exploring a possible link between low MHC antigen expression and universal blood types to select equine donors whose MSCs would defer immune recognition (7).

Nevertheless, there are other factors that may modify the inherent immune properties of MSCs, such as their exposure to an inflammatory environment. Priming MSCs with pro-inflammatory cytokines like interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) could increase their immunomodulatory properties and may result in enhanced regulatory effects *in vivo* (13). However, priming MSCs may also raise their immunogenicity by inducing the expression of MHCs, thus potentially limiting their allogeneic administration (14). These changes in MSC immune properties upon inflammatory exposure are influenced by the type and duration of priming. For example, while priming with high concentration of IFNγ can increase *MHC-II* gene expression in equine MSCs (9), priming with low doses of IFNγ and TNFα for a short period upregulated several immune regulatory-related genes without significantly increasing the expression of immunogenic markers (15). However, while a significant advance has been made on how different cytokines and ligands may influence the immune properties of equine MSCs, the effects of an immune response environment on MSCs have been less explored.

To develop allogeneic cell therapies is critical to gain knowledge of how factors such as MHC matching/mismatching and inflammation may affect the balance between the immunomodulatory and immunogenic potentials of equine MSCs. Such immune properties can be assessed by evaluating the proliferation of lymphocytes exposed to MSCs in immunosuppression assays or in modified one-way mixed lymphocyte reactions (MLRs) (5). Our group recently reported the effects of equine MSCs on different lymphocyte subpopulations after their *in vitro* co-culture with autologous or allogeneic MHC-matched/-mismatched MSCs, either unstimulated or primed with pro-inflammatory cytokines (16). These *in vitro* assays provide important information on the changes experienced by lymphocytes after contacting with MSCs, contributing to understand the immune response *in vivo*. However, little is known about how MSCs behave when they are exposed to lymphocytes, either if these are already activated during a disease or if they become activated in response to MSCs.

To better understand the fate of MSCs when they enter into contact with the immune system, this study aimed at analyzing the changes in the gene expression and secretion of molecules related to the immune regulatory and immunogenic profiles of equine MSCs after being exposed to activated or resting lymphocytes. Our specific goals were to evaluate the influence of inflammation and compatibility for the MHC in different *in vitro* co-culture settings. For these purposes, equine MSCs in basal conditions (MSC-naive) or pro-inflammatory primed (MSC-primed) were co-cultured with autologous or allogeneic MHC-matched/-mismatched lymphocytes in both immunosuppression assays (activated lymphocytes) and in modified one-way MLRs (resting lymphocytes). Subsequently, MSC gene expression and secretion of different molecules related to their immunomodulatory–immunogenicity balance were assessed.

Our initial hypothesis was that exposure of equine MSCs to lymphocytes would result in increased gene expression and secretion of immunomodulatory molecules accompanied by a slight upregulation of their immunogenic profile. We hypothesized that these changes would be more marked in MSC-primed, particularly after contacting with activated lymphocytes. Regarding the MHC compatibility, it was hypothesized that MSCs exposed to MHC-matched lymphocytes would display a profile similar to that in the autologous setting, whereas the MHC-mismatched co-cultures would result in similar immunomodulation but increased immunogenicity of the equine MSCs.

Materials and methods

Study design

Equine bone marrow (BM)-derived MSCs were obtained from three MHC homozygous donors and were assayed in both basal conditions (MSC-naive) and after pro-inflammatory priming (MSC-primed). MSC-naive and MSC-primed from each donor were co-cultured with peripheral blood lymphocytes (PBLs), either autologous ($n = 3$) or allogeneic from MHC-matched ($n = 8$) and -mismatched ($n = 7$) animals. These PBLs were obtained from the three MSC donors (autologous setting) and from eight horses selected by their MHC haplotype to establish different allogeneic matched and mismatched combinations, as shown in Figure 1A. A total of two types of co-cultures were used: immunosuppressive assays, where PBLs were previously activated, and modified one-way MLR, where resting PBLs were used. After each type of co-culture, PBLs were removed and used in a separate study (16), MSCs were harvested to analyze their gene expression, and supernatants collected to assess their secretion. The gene expression of different molecules related to the immunomodulatory (*VCAM1*, *COX2*, *IDO1*, *iNOS2*, *IL6*) and immunogenic (*MHC-I*, *MHC-II*, *CD40*,

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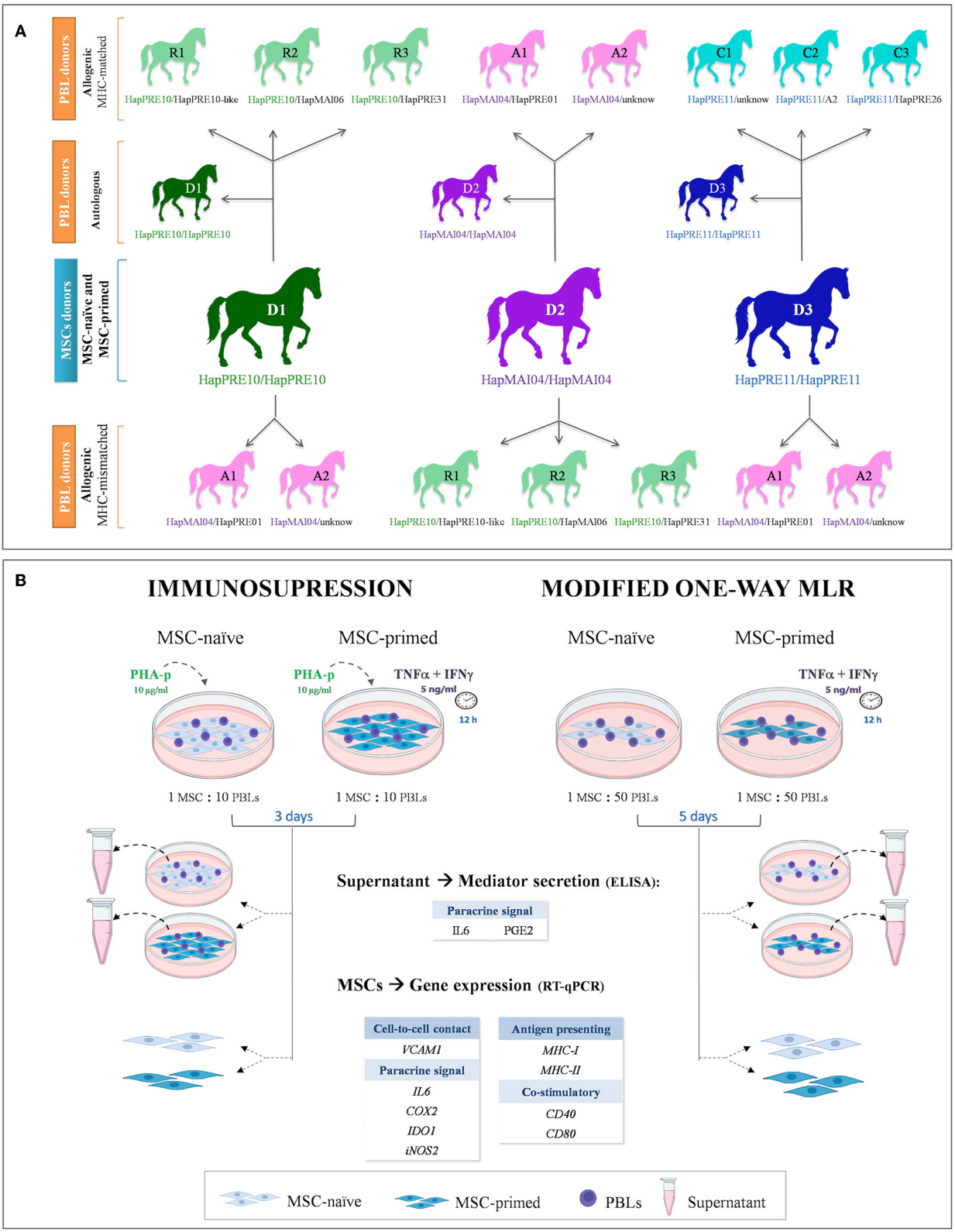


FIGURE 1
Study design. **(A)** Equine donors of mesenchymal stem cells (MSCs) and peripheral blood lymphocytes (PBLs) are presented with their ELA haplotypes and the combinations to establish autologous and allogeneic major histocompatibility complex (MHC)-matched and MHC-mismatched co-cultures. **(B)** Equine MSCs were assayed with unstimulated (MSC-naive) and primed with cytokines (MSC-primed)

(Continued)

FIGURE 1 (Continued)
in both immunosuppression and modified one-way mixed lymphocyte reaction (MLR) assays, using PBLs activated by phytohemagglutinin isoform P (PHA-p) or resting lymphocytes, respectively. After co-culture, supernatant and MSCs were collected to evaluate, respectively, the secretion and gene expression of different mediators involved in the immunomodulatory capacity and immunogenic potential of these cells.

CD80) profiles of MSCs were evaluated by real-time quantitative polymerase chain reaction (RT-qPCR), and secretion of IL6 and PGE_2 was determined by ELISA in the supernatants (Figure 1B).

Animal selection by MHC haplotyping

In total, 11 mixed-breed horses (one stallion, three geldings, seven mares; aged 2–8 years, weight 412–493 kg) in good health status and with no previous pregnancy history were chosen based on their MHC haplotypes. To find and select animals, a screening of 60 Purebred Spanish and mixed-breed horses from a local farm was performed. Haplotypes were determined by microsatellite typing using a validated panel of 10 highly polymorphic intra-MHC regions, as previously described (8, 17). Blood was collected after owner's informed consent, and methodology for DNA extraction, multiplex PCRs, and fragment analysis was performed, as previously reported by our group (8).

Definitive haplotypes were established for homozygous animals, and the remaining animals were assigned with provisional haplotypes based on previously known ones, which are either reported in the literature (10, 18) or described in a preliminary study of our group in Purebred Spanish horses (19). Overall, three groups of animals were selected, with each group including one homozygous horse of the haplotype HapPRE10, HapPRE11, or HapMAI04, and two or three heterozygous animals sharing one haplotype with the donor. Thus, the homozygous horse in each group served as the MSC donor as it was MHC-matched with the heterozygous animals. To establish MHC-mismatched combinations, PBLs from the heterozygous animals in other groups with different haplotypes were used (Figure 1A). Selecting homozygous individuals as MSC donors allows matching them with different heterozygous individuals. This strategy has been proposed to create haplo-banks of human-induced pluripotent stem cells (iPSCs) (20, 21) and has also been used in equine MSC studies (22). Supplementary Table 1 shows the microsatellite alleles of each haplotype identified in the horses involved in this study.

All procedures involving animals were carried out under the Project License PI 15/16 approved by the in-house Advisory Ethics Committee for Animal Research from the University of Zaragoza. The care and use of animals were performed in according with the Spanish Policy for Animal Protection RD53/2013, which is in line with the European Union Directive 2010/63 on the protection of animals used for scientific purposes. All animals were kept on paddocks of the facilities of the Animal Research Service of the University of Zaragoza, with free access to water and grass hay.

Isolation, characterization, and culture of MSCs

Equine bone marrow MSCs were obtained and characterized, as previously described (15) as part of a previous study of our group (16). In brief, bone marrow was harvested from the sternum of D1, D2, and D3 animals under sedation (0.04 mg/kg IV romifidine, Sedivet, Boehringer Ingelheim, and 0.02 mg/kg IV butorphanol, Torbugesic, Pfizer) and local analgesia with lidocaine (Anesvet, Laboratorios Ovejero). Mononuclear cells were separated by density gradient centrifugation and seeded in the culture medium consisting of low-glucose Dulbecco's modified Eagle medium supplemented with 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 U/mL penicillin, and 10% fetal bovine serum (FBS) (all from Sigma-Aldrich). The cells were expanded until passage three and characterized by their phenotype and tri-lineage differentiation. Characterization data of the MSC lines used in this study were previously published by our group (16). Subsequently, the MSCs were cryopreserved in 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and 90% FBS medium until the experiments started.

Prior to co-cultures, the cryopreserved MSCs ($n = 3$) were thawed and seeded at 5,000 cells/cm^2 in the basal medium, as described above, at 37°C and 5% CO_2 for 72 h to recover from freezing. At 24 h prior to co-culture, the MSCs were detached with 0.25% trypsin–EDTA (Sigma–Aldrich) and seeded into a 24-well plate at 100,000 cells per well for the immunosuppression assays, and at 20,000 MSCs per well for modified one-way MLR assays.

For the MSC-primed condition, the basal media described above was supplemented with 5 ng/mL of TNFa (R&D Systems) plus 5 ng/mL of IFNg (R&D Systems) and corresponding MSCs were exposed for 12 h to this media, as described earlier (15), before adding PBLs.

Blood collection and isolation of PBLs

Peripheral blood lymphocytes were isolated using the carbonyl iron granulocyte depletion method, followed by density gradient centrifugation with Lymphoprep™, as previously described (8, 23). In brief, blood was collected aseptically *via* a jugular venipuncture into sterile 60-mL syringes with 17 IU/mL of lithium heparin (Sigma-Aldrich), and plasma was allowed to separate for 20 min at room

temperature (RT). Plasma was separately collected into conical tubes using extension sets and incubated with carbonyl iron (Sigma-Aldrich) in agitation for 30 min at 37°C. Then, carbonyl iron was placed at the bottom of the tubes by using a magnet, and supernatant was collected and centrifuged at 310× g for 5 min. The cellular pellet was resuspended in PBS and overlayed on Lymphoprep^{TM}. After centrifugation at 690× g for 15 min (without brake), the lymphocyte layer was recovered and washed with PBS. The cells were counted in a hemocytometer chamber using 0.4% trypan blue as dye exclusion. This isolation technique has been reported to provide an enriched lymphocyte population (95–99%) (8, 24).

Co-cultures of MSCs with lymphocytes: Immunosuppression assays and modified one-way MLR

Co-culture of MSCs with activated lymphocytes: Immunosuppression assay

To simulate the environment of an immune response, MSCs were exposed to activated lymphocytes by conducting immunosuppression assays. As described before, corresponding MSCs were previously plated in a 24-well plate at 100,000 cells per well in duplicate and prepared for each condition (MSC-naive and MSC-primed). Lymphocytes from autologous, MHC-matched and -mismatched horses were seeded at 1 × 10^6 PBLs per well (1:10 ratio MSC:PBL), based on previous studies (25, 26) according to the combinations presented in Figure 1. The PBL medium used for co-culture consisted of RPMI 1640 (Thermo Fisher) supplemented with 10% FBS, 0.1 mM 2-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Sigma-Aldrich). The PBL medium was supplemented with 10 µg/mL of the mitogen phytohemagglutinin isoform P (PHA, Sigma-Aldrich) (27, 28) to activate lymphocyte proliferation.

MSC-naive and MSC-primed were cultured alone in the same conditions to provide baseline measurements for both gene expression and molecule secretion. Appropriate controls were set along with experimental conditions in duplicate. Lymphocytes from all animals, either PHA-activated or unstimulated, were cultured alone as positive and negative controls, respectively, to account for their possible contribution to the secretion of molecules measured by ELISA. All the experimental co-cultures and controls were maintained for 3 days, after which corresponding analyses were performed, as will be detailed in the following text.

Modified one-way MLR

Modified one-way MLRs were performed by co-culturing MSCs with resting (unstimulated) lymphocytes. This setting aims at reflecting what would happen to MSCs if these are recognized by the immune system and raise a response that can simultaneously change the MSC profile. Stimulator MSCs, either naive or primed, were previously plated at 20,000 cells per well on 24-well plates in duplicate for each condition, as described previously. Autologous, MHC-matched and -mismatched responder PBLs were seeded at 1 × 10^6 PBL per well according to the combinations depicted in Figure 1, thus resulting in an MSC/PBL ratio of 1:50 (9, 26).

Positive and negative controls were set by establishing, respectively, matched and mismatched classic MLRs using responder PBLs from each donor. In brief, MHC-matched or -mismatched PBLs were used as stimulators by treating them with 50 µg/mL mitomycin C (Sigma-Aldrich) (37°C 30 min incubation, followed by two washes with PBS at 310 × g 5 min) to inhibit proliferation (10, 29). Stimulator PBLs and responder PBLs were cultured at a ratio of 1:1. The supernatant from the MHC-matched and -mismatched MLRs was used to account for potential contribution of lymphocytes to the secretion of the analyzed molecules. All the co-cultures and controls were maintained for 5 days without media exchange, and corresponding analyses were performed subsequently, as detailed in the following text.

Analysis of expression of genes involved in equine immune response (RT-qPCR)

The expression level of genes coding for immunosuppression- and immunogenicity-related molecules was evaluated in MSC-naive and MSC-primed cultured alone (baseline) and after being co-cultured with autologous, MHC-matched or -mismatched PBLs in both immunosuppression and modified one-way MLR. After the co-cultures, PBLs were removed, and MSCs were washed with PBS and frozen at −80°C until mRNA was extracted. MSCs cultured alone for baseline were processed in the same way.

Isolation of mRNA and complementary DNA (cDNA) synthesis were performed using the Cells-to-cDNA II kit (Ambion) according to the manufacturer's instructions, and RT-qPCRs were performed and monitored with a QuantStudio 3 Real-Time PCR System (Applied Biosystems). All reactions were carried out in a total volume of 10 µL with 2 µL of cDNA as the template and Fast SYBR Green Master Mix (Applied Biosystems). Amplification was performed in

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TABLE 1 Primers used for gene expression analysis by RT-qPCR.

Gene	Accession number	Primer sequence (5′–3′)	Amplicon size (bp)
	Housekeeping		
GAPDH	NM_001163856	F: GGCAAGTTCCATGGCACAGT	128
		R: CACAACATATTCAGCACCAGCAT	
B2M	NM_001082502	F: TCGTCCTGCTCGGGCTACT	102
		R: ATTCTCTGCTGGGTGACGTGA	
	Immunomodulation-related molecules		
	Molecules related with cell-to-cell contact mechanism		
VCAM1	NM_001101650	F: TCTATGCTACGCTCTGGCTACG	127
		R: TTGATGGTCTCCCCGATGA	
	Molecules related with paracrine signaling mechanism		
COX2	AB041771	F: GTTTGCATTTTTTGCCCAGC	103
		R: ACTTAAATCCACCCCGTGACC	
IDO1	XM_014736538.2	F: TCATGACTACGTGGACCCAAAA	104
		R: CGCCTTCATAGAGCAGACCTTC	
iNOS2	AY027883	F: CCAACAATGGCAACATCAGGT	85
		R: TGAGCATTCCAGATCCGGA	
IL6	EU438770	F: AACAGCAAGGAGGTACTGGCA	95
		R: CAGGTCTCCTGATTGAACCCA	
	Immunogenic markers: *Antigen presenting-related molecules*		
MHC-I	AB525081	F: CGTGAGCATCATTGTTGGC	92
		R: TCCCTCTTTTTTCACCTGAGG	
MHC-II	NM_001142816	F: AGCGGCGAGTTGAACCTACAGT	172
		R: CGGATCAGACCTGTGGAGATGA	
	Antigen-presenting-related molecules: *Co-Stimulatory molecules*		
CD40	AY514017	F: ACAAATACTGCGACCCCAACC	114
		R: TTTCACAGGCATCGCTGGA	
CD80	XM_005601958.3	F: CAGGAAAGTTGGCTCTGACCA	135
		R: TCTCCATTGTGATCCTGGCTC	

GenBank accession numbers of the sequences used for primers design. Primers (F: forward and R: reverse) and length of the amplicon in base pair (bp). Genes were grouped in agreement with the functions and implications of encoded molecules. *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *B2M*, beta-2-microglobulin; *VCAM1*, vascular cell adhesion molecule 1; *COX2*, cyclooxygenase 2; *IDO1*, indoleamine 2 3-dioxygenase 1; *iNOS2*, inducible nitric oxide synthase 2; *IL6*, interleukin 6; *MHC-I*, major complex of histocompatibility I; *MHC-II*, major complex of histocompatibility II; *CD40*, cluster of differentiation 40; *CD80*, cluster of differentiation 80.

triplicate for each sample as follows: 20 s at 95°C for initial activation, followed by 40 cycles consisting of 3 s at 95°C and 30 s at 60°C, and a dissociation curve protocol run after every PCR.

The levels of gene expression were determined by using the comparative ΔΔCt method. As a reference sample, values from MSC-naive cultured alone (baseline) from each donor were used, unless otherwise stated. The normalization factor was calculated as the geometric mean of the quantity of two housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and beta-2-microglobulin (*B2M*). Genes were analyzed, and corresponding primer sequences were previously designed by our group (15) and are presented in Table 1, grouped according to their function.

Assessment of interleukin 6 and prostaglandin E2 secretion

Supernatants collected from MSC-PBL co-cultures were used to evaluate PGE_2 and IL6 production by using commercially available ELISA kits, as previously reported (26, 29–31). The secretion of these molecules was assessed at the baseline (MSC-naive and MSC-primed cultured alone) and after exposure to the different types of PBLs in both immunosuppression and modified one-way MLR assays. The supernatants from unstimulated and PHA-stimulated PBLs seeded alone were used as negative and positive controls, respectively, for the immunosuppression assays. For the modified one-way MLR assays, the supernatants from the classical MLRs with MHC-matched or -mismatched

PBLs as stimulators were used as negative and positive controls, respectively.

After all co-cultures, PBLs were collected, centrifuged at 310 × g for 5 min, and the supernatants recovered. Supernatants were recovered in the same way from controls consisting of PBLs alone and from classic MLRs, as well as from MSC-naive and MSC-primed cultured alone. All the supernatants were centrifuged at 500 × g for 15 min to remove any contaminating cell and subsequently frozen at −20°C for further ELISA. All the procedures were performed as per the manufacturer's instructions and concentrations determined using a standard curve, including a blank.

For PGE_2 analysis (Prostaglandin E2 Parameter Assay Kit, R&D Systems, Ref: KGE004B), control supernatants were diluted 1:10, baseline supernatants were diluted 1:2, and supernatants from co-cultures were diluted 1:50 in the reagent diluent. The standard curve was established from 39 to 5,000 pg/mL. For IL6 analysis (Equine IL-6 DuoSet ELISA, R&D Systems, REF: DY1886), baseline and control supernatants were diluted 1:1 in the reagent diluent, and supernatants from co-cultures were not diluted. The standard curve was set from 62.5 to 16,000 pg/mL. All the samples and points of the standard curve were run in duplicate. All the colorimetric assays were analyzed on a microplate reader SPECTROstar Nano (BMG LABTECH) and read immediately at 450 nm with wavelength correction set to 540 nm. The duplicate readings for each standard, control, and sample were averaged, and the average zero standard optical density was extracted. The standard curve was created generating a four-parameter logistic curve fit, and the concentrations extrapolated were multiplied by the dilution factor. Samples with values beyond the limit of detection were excluded from the analysis.

Statistical analysis

Statistical analysis was performed by GraphPad Prism 9.2 software (San Diego, CA, USA). Results of RT-qPCR and ELISA were separately analyzed for each type of co-culture and not directly compared as the experimental conditions were different. Analytical statistics were performed to check differences in mRNA relative expression or molecule secretion depending on the different study variables. The independent variables were "group" (three categories: autologous, allogenic MHC-matched, and allogenic MHC-mismatched co-cultures) and "cell type" (two categories: MSC-naive and MSC-primed). The existence of outlier samples was evaluated with the Grubbs test (alpha = 0.05). For comparisons between three or more groups, normality and homoscedasticity were evaluated by using the Shapiro–Wilk test and the Levene test, respectively. When data followed a normal distribution and had homogeneous variances, the parametric test ANOVA was used, followed by Tukey's comparisons test as a *post-hoc*. In normally distributed data with unequal variances, Welch's *t*-test was used. In non-normal data, the non-parametric Kruskal–Wallis test was used, followed by Dunn's test as a *post hoc*. The effect of the type of co-culture was analyzed by comparing the results for each type of combination (autologous, MHC-matched, and MHC-mismatched) for each type of MSC (MSC-naive and MSC-primed) using parametric or non-parametric paired tests. The significance level was set at $p < 0.05$ for all analyses.

Results

The expression level of different genes and the secretion of molecules were evaluated in MSCs from three donors under different conditions: MSC-naive and MSC-primed cultured alone (baseline) and after being co-cultured with autologous, and MHC-matched or mismatched PHA-activated PBLs (immunosuppression assays) or resting PBLs (modified one-way MLR assays). The expression level of five genes involved in the immunomodulatory properties of equine MSCs was analyzed (*IL6*, *COX2*, *IDO1*, *iNOS2*, and *VCAM1)*, along with the secretion of IL6 and PGE_2. Also, to account for the effect of the different conditions on MSC immunogenicity, four genes coding for antigen-presenting-related molecules were assessed: *MHC-I*, *MHC-II*, *CD80*, and *CD40*.

For gene expression, data are presented as relative expression (fold change) over corresponding baseline MSC-naive, unless otherwise stated. Baseline values are the same for both types of assays (immunosuppression and modified one-way MLR). For molecule secretion, the supernatants from lymphocytes cultured alone in resembling conditions were used as controls. The IL6 and PGE_2 concentration detected in the lymphocyte controls for the immunosuppression assay (unstimulated, CTL–; PHA-activated, CTL+) and for the modified one-way MLR assays (classic MLRs) was very low and significantly different from that measured in the co-culture supernatants, confirming that MSCs were the major contributors to IL6 and PGE_2 secretion in the co-cultures. The details on these significant differences can be found in Supplementary Figure 1. Note that the scale in Y axes of the graphs presenting results of gene expression and molecule secretion in immunosuppression and modified one-way MLR assays is different to better show the values.

Gene expression and secretion of mediators related to equine MSC immunomodulation

At the baseline (MSCs cultured alone), cytokine priming induced a significant upregulation of all the immunomodulatory genes studied: *IL6* ($p < 0.05$; Figures 2A,B), *COX2* ($p < 0.05$; Figures 3A,B), *IDO1* ($p < 0.01$; Figures 4A,B), and *VCAM1*

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($p < 0.01$; Figures 4E,F). The expression of *iNOS2* was not detected in MSC-naive at the baseline, but it was activated in MSC-primed. Therefore, the expression of *iNOS2* is presented as relative expression (fold change) over baseline MSC-primed (cultured alone), instead of MSC-naive. Following the same trend, the stimulation with cytokines induced IL6 and PGE_2 secretion in MSC-primed at the baseline, but significant differences could not be found over MSC-naive as these molecules were only detected in MSC-naive from one donor and at very low concentration (Figures 2C,D, 3C,D).

Effect of activated lymphocytes on the equine MSC immunomodulatory profile

The exposure of MSC-naive to activated PBLs upregulated the expression of different immunomodulatory genes compared with the baseline, regardless of the compatibility scenario (autologous or allogeneic MHC-matched/-mismatched). Specifically, MSC-naive exposed to activated lymphocytes significantly upregulated *IL6* ($p < 0.0001$ in all conditions; Figure 2A), *COX2* ($p < 0.001$ in all conditions; Figure 3A), and *IDO1* (autologous, $p < 0.01$; matched, $p < 0.001$; mismatched, $p < 0.001$; Figure 4A). *iNOS* and *VCAM1* expression also increased compared with the baseline, but these changes were not statistically significant (Figures 4C,E).

MSC-primed exposed to activated lymphocytes showed higher expression than MSC-naive for *IL-6* (matched and mismatched co-cultures, $p < 0.05$; Figure 2A) and *COX2* (matched co-culture, $p < 0.05$; Figure 3A). However, compared with the baseline (MSC-primed alone), the levels of *IL-6* were not further increased by the presence of activated lymphocytes (Figure 2A). On the other hand, *COX2* did experience a significant upregulation compared with the baseline in autologous and MHC-matched co-cultures ($p < 0.05$ for both conditions; Figure 3A). *iNOS2* was also overexpressed after exposure of MSC-primed to activated lymphocytes, but the differences compared with the baseline were not statistically significant (Figure 4C). In contrast to *IL6*, *COX2*, and *iNOS2*, *IDO1* and *VCAM1* were markedly downregulated in MSC-primed exposed to all the types of activated lymphocytes: autologous (*IDO1*, $p < 0.01$; *VCAM1*, $p < 0.05$), matched (*IDO1*, $p < 0.001$; *VCAM1*, $p < 0.01$), and mismatched (*IDO1*, $p < 0.01$; *VCAM1*, $p < 0.01$; Figures 4A,E). Interestingly, there was a clear trend for *VCAM1* downregulation depending on the type of activated lymphocytes, with the mismatched co-culture leading to the greatest reduction ($p < 0.05$ compared with matched, $p < 0.01$ compared with autologous; Figure 4E). Nevertheless, both *IDO1* and *VCAM1* remained higher in MSC-primed than in MSC-naive. This difference was significant for *IDO1* in the mismatched co-cultures ($p < 0.05$), which was also higher than that for the matched co-cultures ($p < 0.05$; Figure 4A) and for *VCAM1* in both allogeneic matched and mismatched co-cultures ($p < 0.05$ for both conditions; Figure 4E). Finally, differences in the expression of *IL6* and *COX2* were not found between MSCs exposed to autologous, matched and mismatched co-cultures, neither for MSC-naive nor for MSC-primed (Figures 2A, 3A).

In agreement with gene expression changes, the secretion of IL6 and PGE_2 increased compared with the baseline when MSCs were exposed to activated lymphocytes, suggesting that this environment activates MSC immunomodulatory potential. MSC-primed tended to produce more IL-6 and PGE_2 than MSC-naive, but significant differences were not observed as in gene expression. The highest concentrations of these molecules were found in the autologous co-cultures, which produced significantly more IL6 (MSC-naive, $p < 0.01$; Figure 2C) and PGE_2 (MSC-naive and MSC-primed, $p < 0.05$) than the baseline and the matched co-culture (PGE_2, MSC-naive and MSC-primed, $p < 0.05$; Figure 3C).

Effect of resting lymphocytes on equine MSC immunomodulatory profile

In contrast to that observed after the exposure to activated lymphocytes, equine MSCs co-cultured with resting lymphocytes showed a downregulation of their immunomodulatory profile. MSC-naive notably downregulated the expression of *IL6* ($p < 0.05$ in all conditions; Figure 2B) and *COX2* (non-significant; Figure 3B), while *IDO1* expression remained low in all the three types of co-cultures (Figure 4B). The expression of *iNOS2* was not detected in MSC-naive exposed to autologous resting lymphocytes and was low in the matched and mismatched co-cultures (Figure 4D). On the contrary, *VCAM1* was upregulated in MSC-naive after being in contact with resting lymphocytes, but these changes were not statistically significant (Figure 4F). In terms of secretion, IL6 was not significantly induced in MSC-naive by resting lymphocytes, and only a slightly higher amount of this molecule was detected after the co-cultures (Figure 2D). In contrast to that observed for *COX2* gene expression, the exposure of MSC-naïve to resting lymphocytes led to an increased PGE_2 secretion compared with the baseline, although, in general, at lower levels than their exposure to activated lymphocytes. Specifically, this increase was statistically significant when MSC-naïve were exposed to resting mismatched lymphocytes ($p < 0.05$; Figure 3D).

Overall, the expression of immunomodulatory genes remained higher in MSC-primed than in MSC-naïve in all the co-cultures with resting lymphocytes (*IL-6*, matched and mismatched, $p < 0.05$, Figure 2B; *COX2*, non-significant, Figure 3B; *IDO1*, mismatched, $p < 0.05$, Figure 4B; *iNOS2*, matched and mismatched, $p < 0.05$, Figure 4D; *VCAM1*, matched and mismatched, $p < 0.05$, Figure 4F). Nevertheless, and similarly to MSC-naive, MSC-primed co-cultured with autologous and matched resting lymphocytes showed a reduced expression of *IL6* ($p < 0.05$ and $p < 0.01$, respectively; Figure 2B), *COX2* (non-significant; Figure 3B), and *IDO1*

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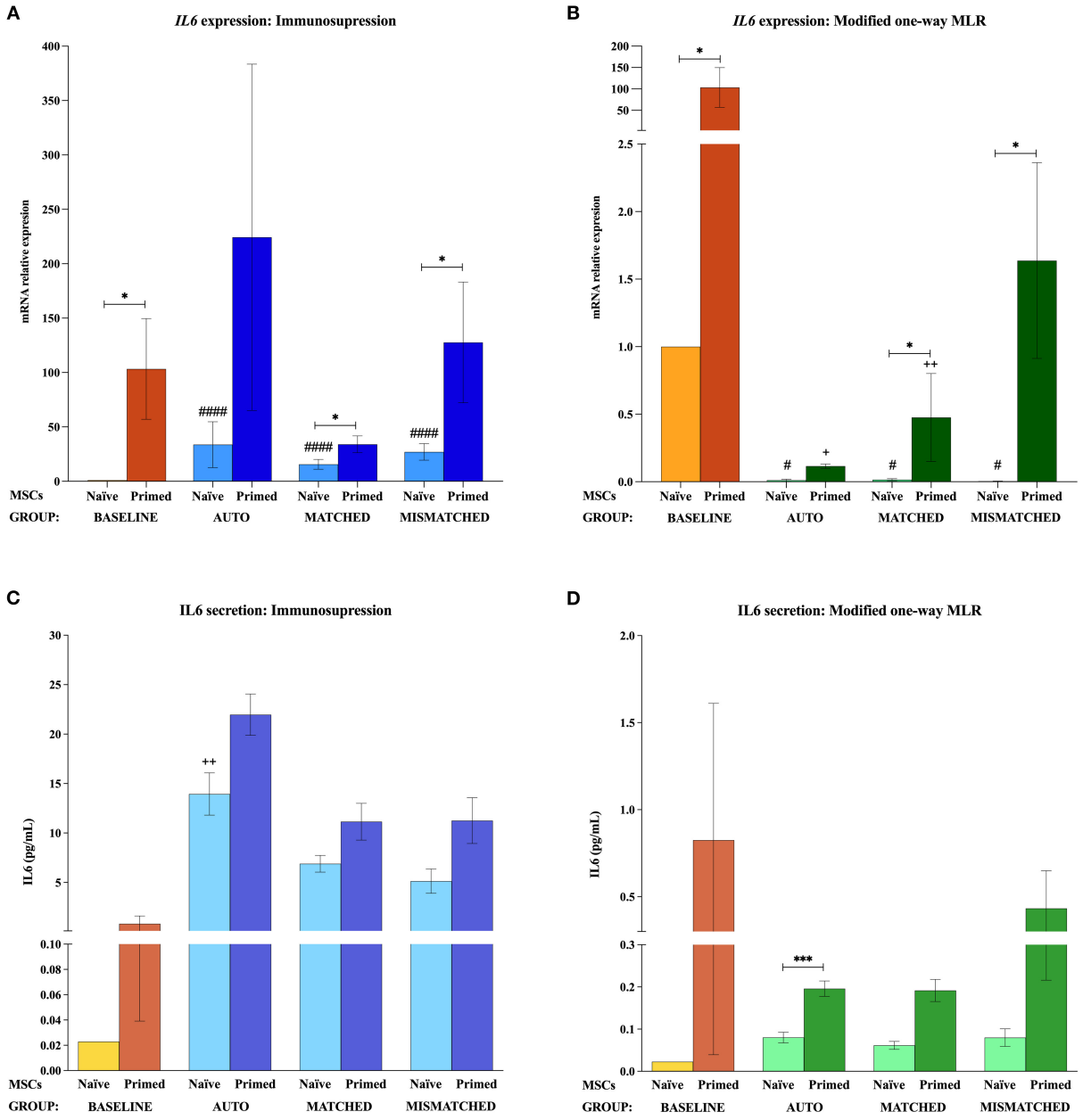


FIGURE 2
Interleukin 6 (IL6) gene expression and secretion by equine mesenchymal stem cells (MSCs) in the different scenarios. **(A)** *IL6* mRNA relative expression and **(C)** IL6 secretion (pg/mL) before (baseline; orange bars) and after equine MSC-naive (light blue bars) and MSC-primed (dark blue bars) were exposed *in vitro* to phytohemagglutinin (PHA)-activated peripheral blood lymphocytes (PBLs) (immunosuppression assays). **(B)** *IL6* mRNA relative expression and **(D)** IL6 secretion before (baseline; orange bars) and after MSC-naive (light green bars) and MSC-primed (dark green bars) were exposed *in vitro* to resting PBLs [modified one-way mixed lymphocyte reaction (MLR) assays]. Co-cultures of MSCs and PBLs were autologous ($n = 3$), allogeneic, matched ($n = 8$), or mismatched ($n = 7$) for the major histocompatibility complex. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using baseline MSC-naive as reference sample (light orange bar, value 1). Concentration of IL6 in the supernatant from the different conditions is represented as mean ± S.E.M (pg/mL). Significant differences of each condition compared with the baseline MSC-naive (light orange bar) are represented by hashes (#) above the corresponding bar ($^{\#}p < 0.05$; $^{\#\#\#\#}p < 0.0001$). Significant differences compared with the baseline MSC-primed (dark orange bar) are represented by a cross (+) above the corresponding bar ($^{+}p < 0.05$; $^{++}p < 0.01$). Significant differences between experimental conditions are represented by a squared line with an asterisk ($^{*}p < 0.05$; $^{***}p < 0.001$).

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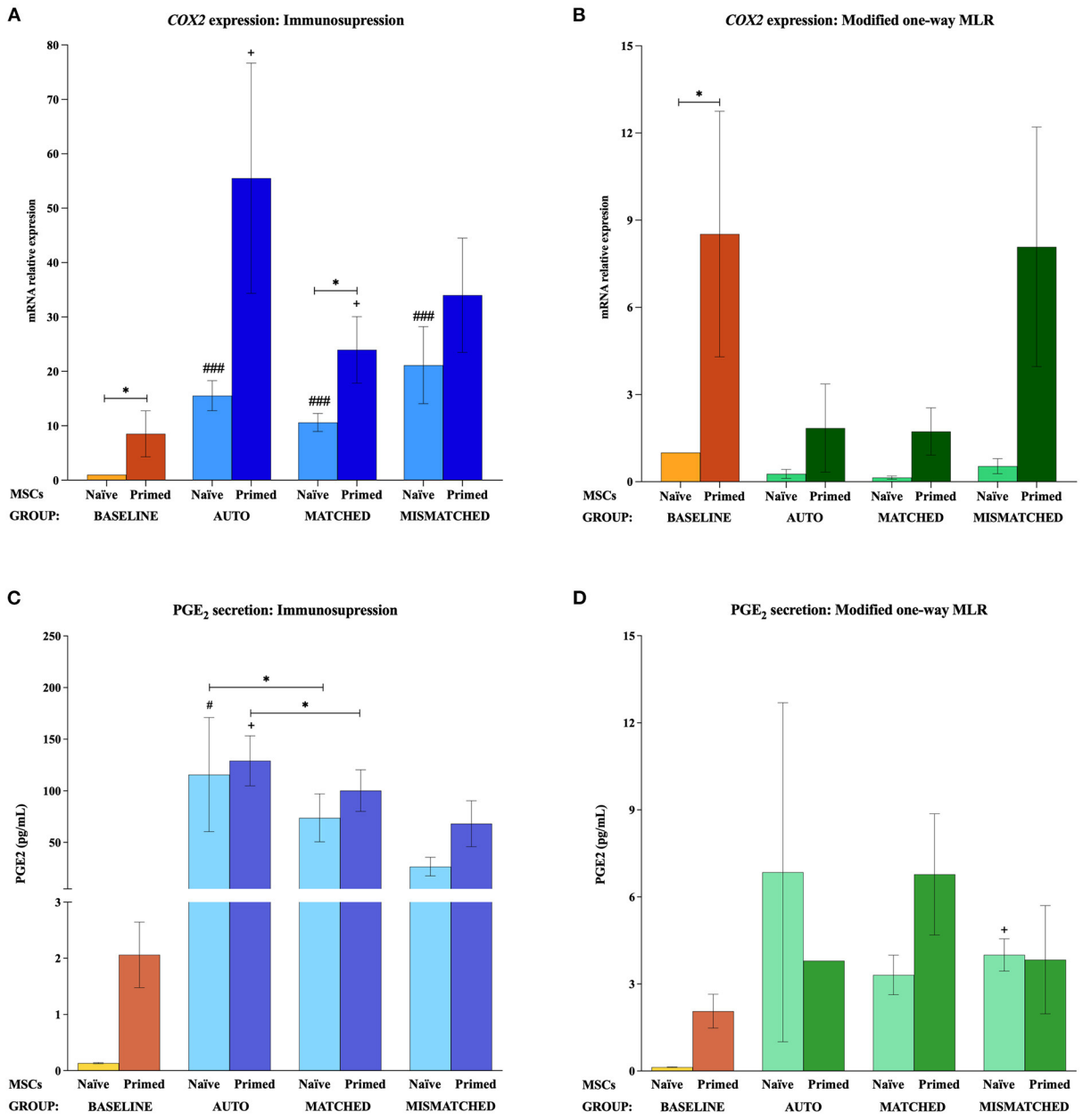


FIGURE 3
Cyclooxygenase 2 (COX2) gene expression and prostaglandin E2 (PGE_2) secretion by equine mesenchymal stem cells (MSCs) in the different scenarios. **(A)** *COX2* mRNA relative expression and **(C)** PGE_2 secretion (pg/mL) before (baseline; orange bars) and after equine MSC-naive (light blue bar) and MSC-primed (dark blue bar) were exposed *in vitro* to phytohemagglutinin (PHA)-activated peripheral blood lymphocytes (PBLs) (immunosuppression assays). **(B)** *COX2* mRNA relative expression and **(D)** PGE_2 secretion before (baseline; orange bars) and after MSC-naive (light green bar) and MSC-primed (dark green bar) were exposed *in vitro* to resting PBLs [modified one-way mixed lymphocyte reaction (MLR) assays]. Co-cultures of MSCs and PBLs were autologous ($n = 3$) or allogeneic, matched ($n = 8$), or mismatched ($n = 7$) for the major histocompatibility complex. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using baseline MSC-naive as reference sample (light orange bar, value 1). Concentration of PGE_2 in the supernatant from the different conditions is represented as mean ± S.E.M (pg/mL). Significant differences of each condition compared with the baseline MSC-naive (light orange bar) are represented by hashes (#) above the corresponding bar ($^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$). Significant differences compared with the baseline MSC-primed (dark orange bar) are represented by a cross (+) above the corresponding bar ($^{+}p < 0.05$). Significant differences between experimental conditions are represented by a squared line with an asterisk ($^{*}p < 0.05$).

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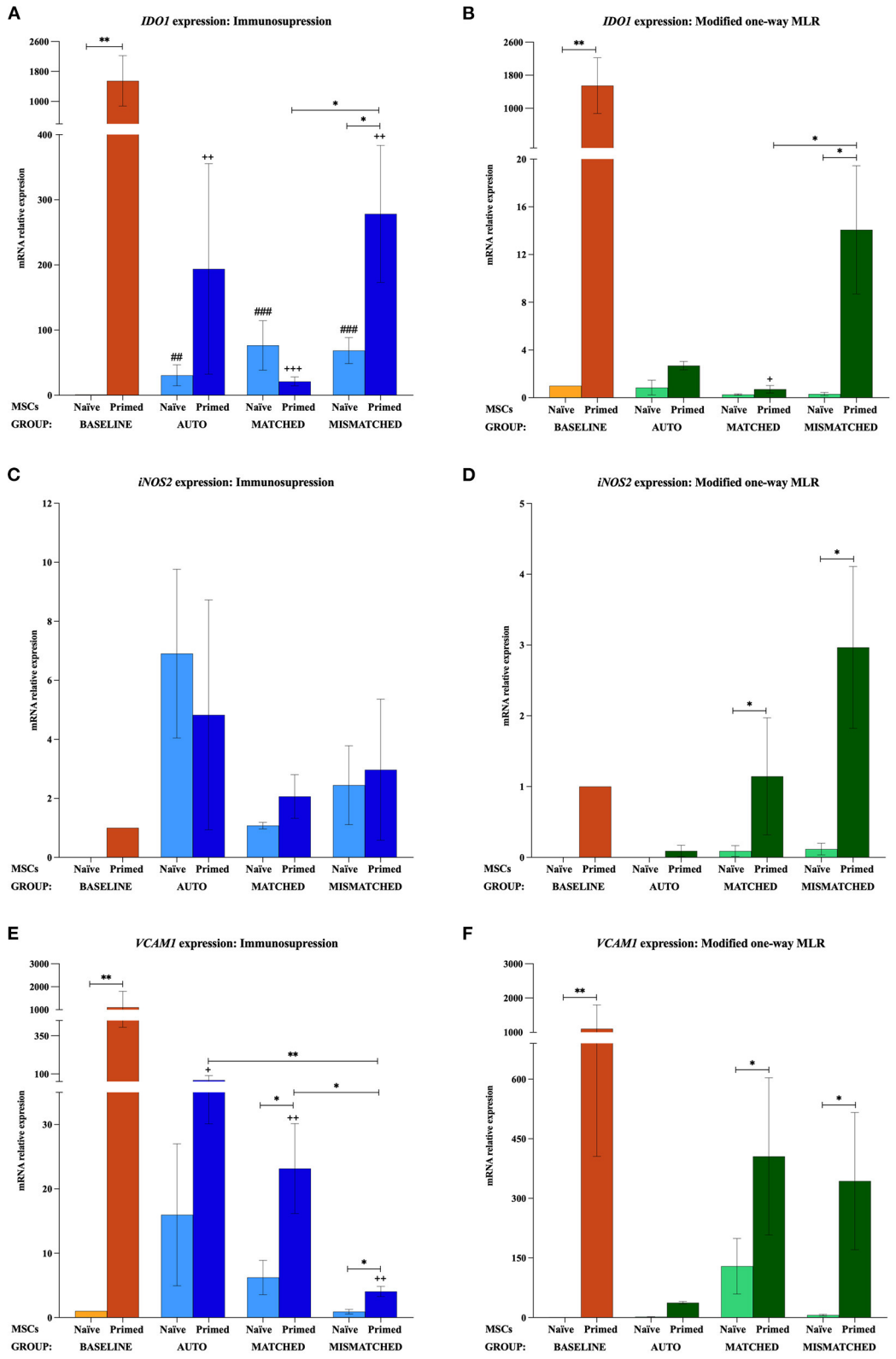


FIGURE 4
Changes in indoleamine 2,3-dioxygenase 1 (*IDO1*), inducible nitric oxide synthase 2 (*iNOS2*), and vascular cell adhesion molecule 1 (*VCAM1*) expression by equine mesenchymal stem cells (MSCs) in the different scenarios **(A)** *IDO1*, **(C)** *iNOS2*, and **(E)** *VCAM1* mRNA relative expression before (baseline; orange bars) and after equine MSC-naive (light blue bars) and MSC-primed (dark blue bars) were exposed *in vitro* to

(Continued)

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FIGURE 4 (Continued)
phytohemagglutinin (PHA)-activated peripheral blood lymphocytes (PBLs) (immunosuppression assays). **(B)** *IDO1*, **(D)** *iNOS2*, and **(F)** *VCAM1* mRNA relative expression before (baseline; orange bars) and after MSC-naive (light green bars) and MSC-primed (dark green bars) were exposed *in vitro* to resting PBLs [modified one-way mixed lymphocyte reaction (MLR) assays]. Co-cultures of MSCs and PBLs were autologous ($n = 3$) or allogeneic, matched ($n = 8$), or mismatched ($n = 7$) for the major histocompatibility complex. Changes in *IDO1* and *VCAM1* expression are represented as mean ± S.E.M of the relative mRNA expression, using baseline MSC-naive as reference sample (light orange bar, value 1). Baseline MSC-primed are used as reference sample (dark orange bar, value 1) for *iNOS2* since no expression of this gene was detected in baseline MSC-naive. Significant differences of each condition compared with the baseline MSC-naive (light orange bar) are represented by hashes (#) above the corresponding bar ($^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$). Significant differences compared with the baseline MSC-primed (dark orange bar) are represented by a cross (+) above the corresponding bar ($^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$). Significant differences between experimental conditions are represented by a squared line with an asterisk ($^{*}p < 0.05$; $^{**}p < 0.01$).

(matched co-culture, $p < 0.05$; Figure 4B). Accordingly, co-cultured MSC-primed also decreased their secretion of IL6 compared with the baseline but still secreted higher concentrations of this molecule than MSC-naïve ($p < 0.001$ in autologous settings; Figure 2D). Interestingly however, MSC-primed co-cultured with mismatched resting lymphocytes showed the highest expression of *IL6*, *COX2*, *IDO1*, and *iNOS2* among all the co-cultures, even though the differences were not statistically significant, except for *IDO1* (compared with matched co-culture, $p < 0.05$; Figure 4B). Furthermore, the same was observed in terms of IL6 secretion, with the highest concentration of this molecule being produced by MSC-primed exposed to mismatched lymphocytes (Figure 2D), although the difference was not statistically significant. Even though this was also the case for *COX2* gene expression, the same was not replicated at the level of PGE_2 secretion, and the production of this molecule did not follow a clear trend (Figure 3D).

Gene expression of markers related to equine MSC immunogenicity

The analysis of *MHC-I* could only be carried out with the MSCs from two of the donors (D2 and D3) since the MSC-naive and MSC-primed from the other donor (D1) did not express *MHC-I* neither at the baseline (MSCs cultured alone) nor after their exposure to autologous or allogeneic MHC-matched lymphocytes, regardless of these being activated or resting. Interestingly, *MHC-I* gene expression was detected in the MSCs of this donor after these were co-cultured with allogeneic MHC-mismatched lymphocytes, either activated or resting. However, the lack of reference values to establish the relative expression of *MHC-I* prevented to include the data from the D1 donor in the analysis. Even though data from only two donors were used for *MHC-I*, a consistent trend on its expression could be observed, which was very similar to that for *MHC-II* and *CD40*.

The gene expression of *MHC-I*, *MHC-II*, *CD40*, and *CD80* was not increased by the priming at the baseline (MSCs cultured alone). *MHC-I* and *MHC-II* were only upregulated when MSCs were both primed and exposed to lymphocytes, regardless of these being activated or resting, autologous or allogeneic MHC-matched/-mismatched (Figure 5). Activated lymphocytes also induced *CD40* upregulation in MSC-primed but only if mismatched, while resting lymphocytes produced this increase in all the three types of co-cultures. On the contrary, activated lymphocytes tended to downregulate *CD80* expression in MSCs, and resting lymphocytes tended to increase it but only in MSC-naive (Figure 6).

Effect of activated lymphocytes on equine MSC immunogenic profile

When MSC-primed were exposed to activated matched or mismatched lymphocytes, *MHC-I* was significantly overexpressed compared with MSC-naive ($p < 0.05$ for both conditions; Figure 5A), and *MHC-II* increased significantly compared with the baseline ($p < 0.05$ for both conditions; Figure 5C). *MHC-II* overexpression in MSC-primed was also significant compared with MSC-naive in the MHC-matched co-cultures ($p < 0.05$). However, *CD40* was upregulated only in MSC-primed exposed to activated mismatched lymphocytes, and this increase was statistically significant compared with the baseline, the corresponding MSC-naive, and the MSC-primed exposed to autologous and matched activated lymphocytes ($p < 0.05$ for all conditions; Figure 6A). On the other hand, activated lymphocytes led to a reduction of *CD80* expression in MSC-naive in matched and mismatched co-cultures compared with the baseline, the corresponding MSC-primed, and the autologous co-culture ($p < 0.05$ for all conditions; Figure 6C).

Effect of resting lymphocytes on equine MSC immunogenic profile

The exposure to resting lymphocytes upregulated *MHC-I*, *MHC-II*, and *CD40* in MSC-primed compared with MSC-naive, while *CD80* was increased in MSC-naive compared with MSC-primed. Specifically, *MHC-I* increased in MSC-primed in both matched and mismatched co-cultures ($p < 0.05$ for both conditions), this upregulation being significantly higher in the mismatched co-culture than the matched ($p < 0.05$) and the baseline ($p < 0.05$; Figure 5B). In line with this, *MHC-II* and *CD40* increased compared with

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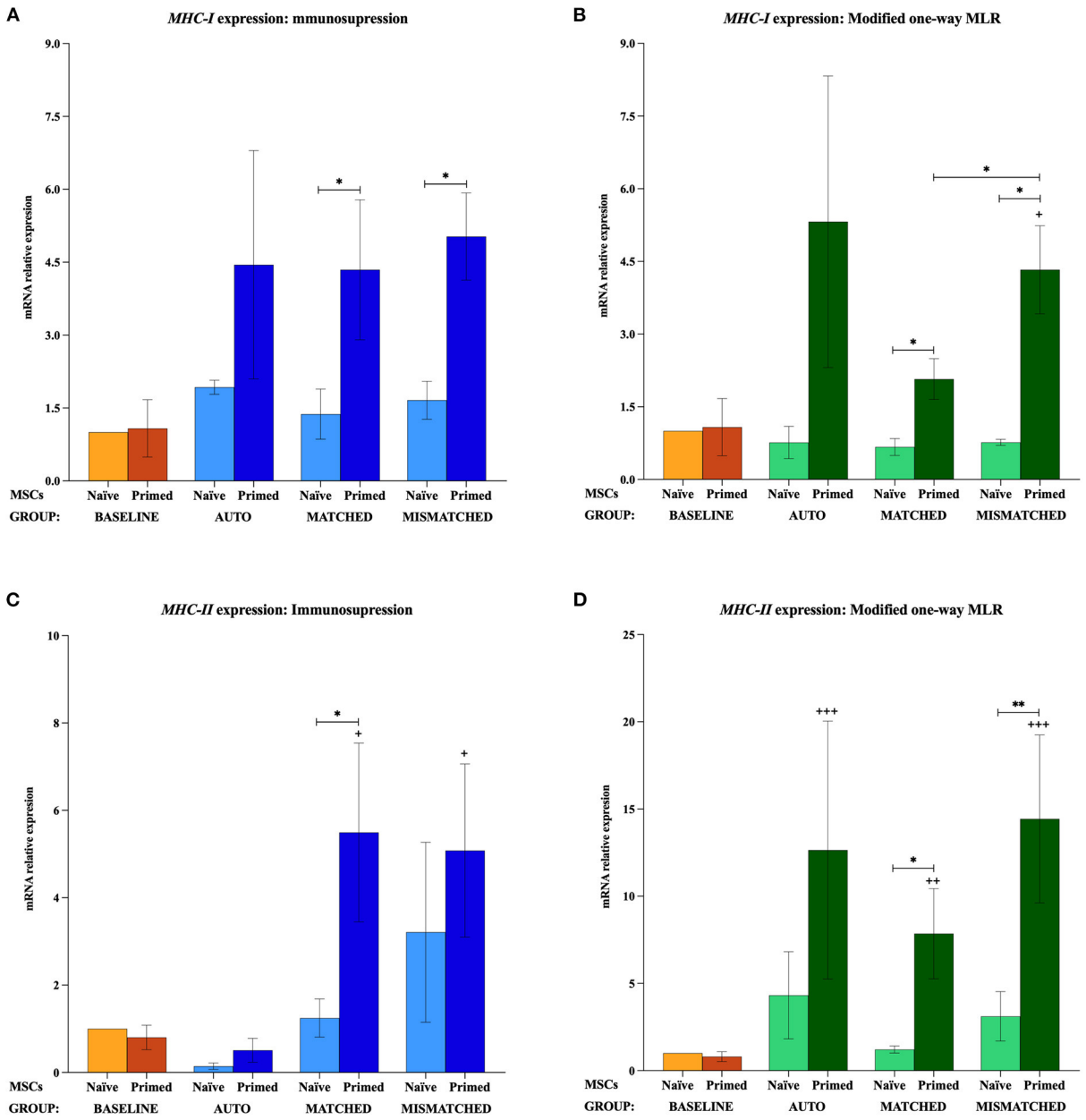


FIGURE 5
Changes in major histocompatibility complex (*MHC*)-*I* and MHC-*II* expression by equine mesenchymal stem cells (MSCs) in the different scenarios. **(A)** *MHC-I* and **(C)** *MHC-II* mRNA relative expression before (baseline; orange bars) and after equine MSC-naive (light blue bars) and MSC-primed (dark blue bars) were exposed *in vitro* to phytohemagglutinin (PHA)-activated peripheral blood lymphocytes (PBLs) (immunosuppression assays). **(B)** *MHC-I* and **(D)** *MHC-II* mRNA relative expression before (baseline; orange bars) and after MSC-naive (light green bars) and MSC-primed (dark green bars) were exposed *in vitro* to resting PBLs [modified one-way mixed lymphocyte reaction (MLR) assays]. Co-cultures of MSCs and PBLs were autologous ($n = 3$) or allogeneic, matched ($n = 8$), or mismatched ($n = 7$) for the MHC. Data from the D1 donor could not be included in the *MHC-I* analysis because of the lack of reference values since this gene was not expressed at the baseline. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using baseline MSC-naive as reference sample (light orange bar, value 1). Significant differences compared with the baseline MSC-primed (dark orange bar) are represented by a cross (+) above the corresponding bar ($^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$). Significant differences between experimental conditions are represented by a squared line with an asterisk ($^{*}p < 0.05$; $^{**}p < 0.01$).

the baseline in MSC-primed exposed to all the three types of co-cultures (*MHC-II:* $p < 0.001$ autologous, $p < 0.01$ matched, $p < 0.001$ mismatched; *CD40:* $p < 0.05$ autologous, $p < 0.05$ matched, $p < 0.001$ mismatched) (Figures 5D, 6B). Moreover, *MHC-II* was significantly overexpressed compared with MSC-naive in the allogeneic matched and

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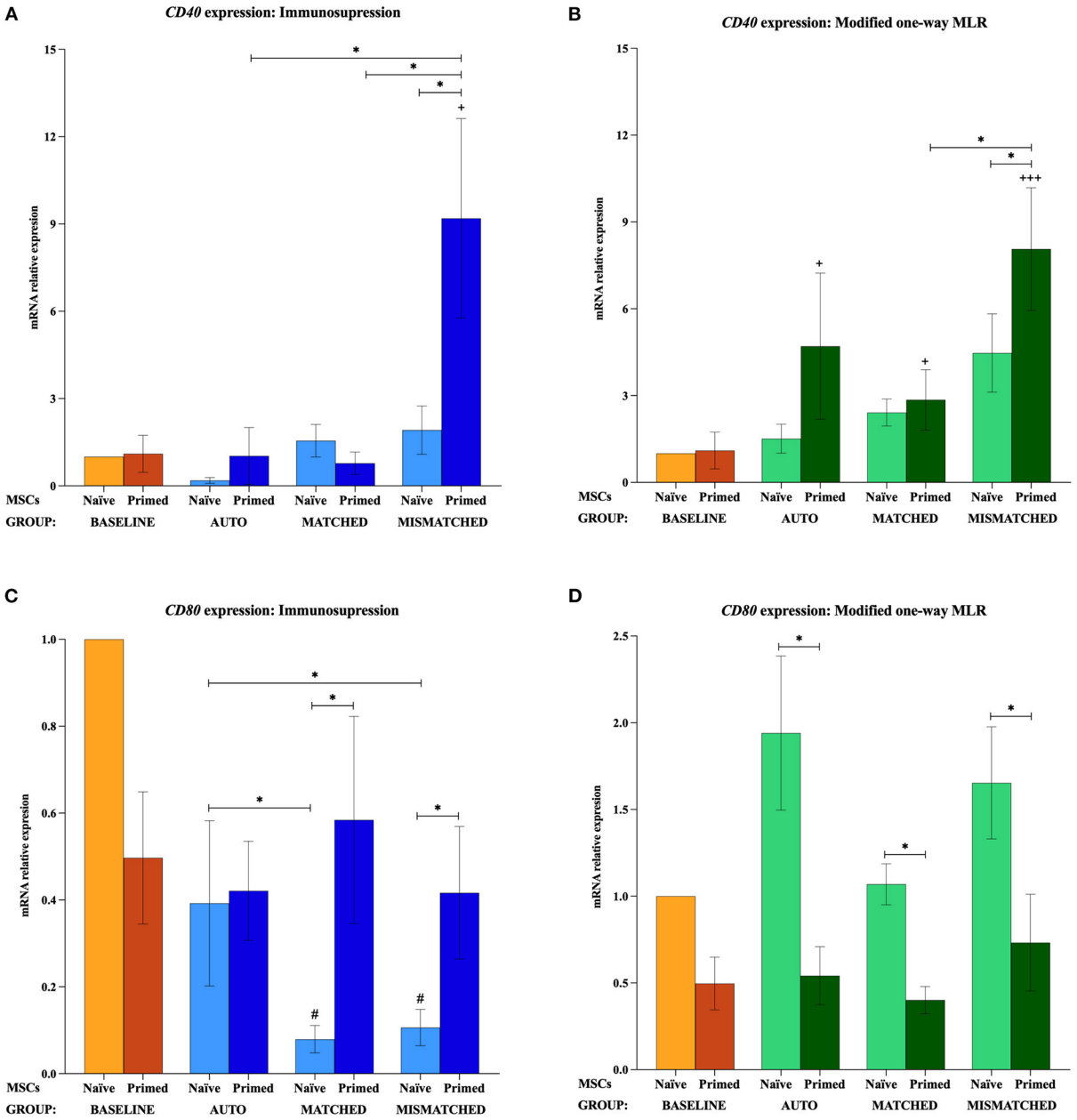


FIGURE 6
Changes in costimulatory molecules *CD40* and *CD80* expression by equine mesenchymal stem cells (MSCs) in the different scenarios. **(A)** *CD40* and **(C)** *CD80* mRNA relative expression before (baseline; orange bars) and after equine MSC-naive (light blue bars) and MSC-primed (dark blue bars) were exposed *in vitro* to phytohemagglutinin (PHA)-activated peripheral blood lymphocytes (PBLs) (immunosuppression assays). **(B)** *CD40* and **(D)** *CD80* mRNA relative expression before (baseline; orange bars) and after MSC-naive (light green bars) and MSC-primed (dark green bars) were exposed *in vitro* to resting PBLs [modified one-way mixed lymphocyte reaction (MLR) assays]. Co-cultures of MSCs and PBLs were autologous ($n = 3$) or allogeneic, matched ($n = 8$), or mismatched ($n = 7$) for the major histocompatibility complex. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using baseline MSC-naive as reference sample (light orange bar, value 1). Significant differences in each condition compared with the baseline MSC-naive (light orange bar) are represented by hashes (#) above the corresponding bar ($^{\#}p < 0.05$). Significant differences compared with the baseline MSC-primed (dark orange bar) are represented by a cross (+) above the corresponding bar ($^{+}p < 0.05$; $^{+++}p < 0.001$). Significant differences between experimental conditions are represented by a squared line with an asterisk ($^{*}p < 0.05$).

mismatched co-cultures ($p < 0.05$ matched, $p < 0.01$ mismatched). Similarly, the highest *CD40* expression was detected in MSC-primed exposed to mismatched resting lymphocytes, which was significantly increased compared with the corresponding MSC-naive ($p < 0.05$) and the matched co-culture ($p < 0.05$; Figure 6B). In contrast to these

findings, the expression of *CD80* in MSCs exposed to resting lymphocytes was higher in MSC-naive than in MSC-primed in all the three co-cultures ($p < 0.05$ for all conditions; Figure 6D).

Discussion

The role of the immunomodulation and immunogenicity of equine MSCs seems to be key for their therapeutic actions and to evade the immune system in the allogeneic administration. The environment that is encountered by MSCs greatly influences their immune properties, and the changes experienced by these cells in response can either benefit their potency (i.e., increased regulatory capacity) or compromise their effectiveness and safety (i.e., immune targeting and elimination) (6). Therefore, the knowledge on how MSCs respond to different stimuli is key to optimize cell therapies for veterinary and human patients. Although several studies have explored the effects of licensing MSCs with different cytokines and ligands, as well as the changes elicited by these MSCs on different populations of immune cells (9, 32), little is known about how immune cells lead to changes in equine MSCs that may have therapeutic implications. To the best of authors' knowledge, this is the first report on the changes experienced by equine MSCs in their immunomodulatory and immunogenic profiles upon exposure to cytokine priming and/or lymphocytes in different combinations (activated/resting; autologous/MHC-matched/MHC-mismatched). According to our initial hypothesis, co-culture of equine MSCs with activated lymphocytes resulted in an increased gene expression and secretion of immunomodulatory molecules, especially in MSC-primed. However, resting lymphocytes did not elicit remarkable changes, except when MSCs were previously primed and MHC-mismatched. As we hypothesized, a moderate activation of the equine MSC immunogenic profile was also observed, more markedly for MSC-primed but similar between activated and resting lymphocytes. We also hypothesized that MHC-mismatched MSCs would display similar immunomodulation but increased immunogenicity, which we indeed observed mostly for MSC-primed. Interestingly, the increased expression of immunogenic markers seemed to be accompanied by a further activation of the regulatory profile, which might equilibrate the balance between both properties in equine MSCs.

Prior to engaging into further discussion of our results, it is important to bear in mind the limitations of this study. First, the sample size in the experiments presented is limited due to the implications of working with a large species such as the horse (2), and it is particularly small for the baseline measurements and the autologous co-cultures ($n = 3$). Related to this, even though all horses enrolled in this study had similar characteristics (age, origin, breed, weight), the baseline gene expression of their MSCs and their response to the different conditions considerably varied. Thus, the inter-individual variability among the different donors can also be considered as a limitation and may have prevented to observe further significant differences. To account for this variability, the values of each donor were normalized compared with their corresponding baseline values. In addition, the difficulty in finding MHC-homozygote MSC donors that can be paired with PBL donors should be considered, taking the high variability of ELA haplotypes into account (10, 17). Another limitation is that not all of the gene expression results could be compared to the secretion of the molecule or its surface expression. There are some contradictory reports on iNOS2 and IDO1 activity in the equine MSC supernatant (26). For instance, Cassano et al. (33) described that the increased gene expression of *IDO1* by equine MSCs upon IFNγ stimulation is not enough to be translated into an actual increase in *IDO1* activity. Therefore, we decided to assess the gene expression of these enzymes and focus on IL6 and PGE_2 to conduct ELISA as the role of these mediators has been more consistently reported in equine MSCs (34). The surface expression of the other molecules could not be assessed partly because of the lack of appropriate antibodies for the equine species (35) and partly because of the insufficient number of MSCs to conduct both RT-qPCR and flow cytometry. Therefore, gene expression was prioritized in this study as it has been widely used in the equine MSC literature and can provide relevant information on the changes experienced by these cells (7, 36).

Several authors agree that PGE_2 is the primary mediator responsible for inhibition of lymphocyte proliferation by equine MSCs from different sources, including BM, adipose tissue (AT), umbilical cord blood (CB), and umbilical cord tissue (CT) (4, 33). PGE_2 is produced by COX2, so both the concentration of the soluble molecule and the gene expression of the enzyme are usually assessed in equine studies (26, 27, 30). The secretion of IL6 may not be mainly involved in the inhibition of T-cell proliferation by equine MSCs (30), but studies in other species agree that IL6 may contribute to a more efficient immunosuppression of B lymphocytes (37, 38). iNOS2 is mainly involved in the immune regulatory effects of rodent MSCs, while MSCs from other mammalian species (e.g., monkey, pig, dog, cattle, and human) preferentially use IDO1 (38–41). Equine studies have reported different results regarding iNOS2 participation. Carrade et al. (4) described that NO production by equine MSCs varies from different tissue sources, and subsequent studies have shown that iNOS2 inhibition does not change the inhibitory effect of equine MSCs on lymphocyte proliferation (26, 30, 42). IDO1 activity does not seem to be involved in the capacity of equine MSCs to inhibit allogeneic lymphocyte proliferation (26) but may participate in maintaining this suppressive effect (42). In addition to soluble mediators, cell–cell interactions between MSCs and lymphocytes *via* adhesion molecules may increase the effectiveness of the MSC immunomodulation (43). Indeed, some authors agree that *VCAM1* is only expressed upon direct close contact between

MSCs and lymphocytes, so this molecule may play a key role in the immunosuppressive functions of MSCs (37).

The immunomodulatory actions of MSCs are closely related not only with their therapeutic mechanisms but also with their ability to evade the immune system, in which the expression of immunogenic molecules also plays a role. Expression of MHC-I by MSCs may result in their immune recognition and elimination since cytotoxic T cells attack foreign cells bearing MHC-I receptors that are bound to an alloantigen (44). On the other hand, natural killer (NK) cells can attack cells lacking MHC-I on their surface (45), so the expression of MHC-I, although weak, protects MSCs from NK cell-mediated elimination (46). In addition, expression of MHC-II can also lead to MSC targeting, so its lack confers these cells the ability to escape immune recognition by CD4 helper cells (46). The cell surface expression of MHC-I and II on equine MSCs vary from one donor to another and even among MSC samples (47, 48), so a recent study proposed to classify equine MSCs as MHC class II-high or -low (7). According to this, MSCs from the three donors in this study would be classified as MHC-II low but would be considered MHC-II high after cytokine priming (16). In addition to MHC complexes, other costimulatory molecules are involved in the antigenic presentation and may have an impact on the immune recognition of MSCs. CD40 plays an important role in allograft rejection (49, 50), and its expression in human MSCs may contribute to an effective activation of T cells (51). Furthermore, the coupling ligand of CD80 to the CD28 receptor is the first signaling pathway necessary for T-cell costimulation and enhances T-cell proliferation and cytokine secretion (51). Equine MSCs from peripheral blood and CB-MSCs showed a moderate to strong expression of these costimulatory molecules (35); however, their role in the immune properties of equine MSCs is largely unknown.

All the molecules and/or genes assessed in the current study have been reported to change its expression and/or secretion in response to an inflammatory environment. Specifically, TNFα and IFNγ are considered inductors of immunomodulatory mediators by MSCs from different species and sources, including the horse (38, 42, 50, 52). In this study, the gene expression and secretion of immunomodulatory molecules were induced after the priming in MSCs, whereas the immunogenic markers were not upregulated in this condition; that is, the cytokine exposure alone could activate the immune regulatory profile of equine BM-MSCs without affecting their immunogenic profile. Similarly to our findings, TNFα and IFNγ have been reported to upregulate *VCAM1* in murine MSCs, and this priming rendered MSCs more adhesive to CD8+ T cells, CD4+ T cells, and CD3+ T cells (53). Other reports also observed an upregulation of the expression and secretion of *IL6* after cytokine priming of equine MSCs (15, 42, 54), as well as of the gene expression of *COX2* and secretion of PGE_2 after exposure to TNFα and IFNγ (5, 36, 42). Priming with IFNγ also resulted in significant induction of *iNOS2* and *IDO1* expression in equine MSCs in previous works (36, 42), whereas other studies did not detect its expression after priming (33). Interestingly, MSCs from the three donors in this study showed no expression of *iNOS2* at the baseline unless MSCs were primed, suggesting that some regulatory factors are only expressed upon activation, and this licensing may depend on the type and degree of stimulation.

Even though there are several reports on the effect of cytokine priming on equine MSCs, there is limited information on the changes in the immune profile of these cells after being challenged by lymphocytes. This would more closely resemble the *in vivo* environment that MSCs encounter after administration, in which the immune system may already be activated (inflammation at the injury site, immune-mediated disease) or may be stimulated in response to the MSCs (immunogenic recognition). In our study, when MSC-naive were exposed to activated lymphocytes, the gene expression of *IL6*, *COX2*, and *iNOS2* was upregulated, and the secretion of IL6 and PGE_2 increased to levels similar to or even higher than those after cytokine priming alone. Moreover, in MSC-primed, the baseline expression and secretion of the same mediators were further increased after the co-culture with activated lymphocytes. The consensus on the role of PGE_2 and IL6 in equine MSC immunomodulation is quite broad, so their upregulation upon cytokine priming and/or exposure to activated lymphocytes observed in this study agrees with previous knowledge (30, 36). However, as aforementioned, there is controversy on the participation of *IDO1* and *iNOS2* in the regulatory mechanisms of equine MSCs. Whereas, the dynamics of *iNOS2* gene expression followed the same trend as *IL6* and *COX2* in this study, the expression of *IDO1* decreased in MSC-primed co-cultured with activated lymphocytes. Some studies did not detect IDO1 activity in the supernatants of co-cultures with equine MSCs and PBMCs, either PHA activated or not, suggesting that this pathway may not be functionally active and that equine MSCs failed to produce IDO1 in the presence of stimulated T cells (4, 26). Along with our findings, it may be suggested that the induction of this molecule may depend on the type of stimuli and may require further stimulation to remain active. Similarly to that observed for *IDO1*, *VCAM1* was also downregulated in MSC-primed exposed to activated lymphocytes, which seemed to be influenced by the type of co-culture. While we do not have a clear hypothesis for this observation, it may also be related with a potentially different regulatory ability of equine MSCs in different contexts. Overall, these findings show that activated lymphocytes constitute an environment able to stimulate equine MSCs, so these could be similarly licensed *in vivo*. Furthermore, if MSCs are already primed by cytokines, activated lymphocytes can further contribute to the upregulation of modulatory factors, suggesting that the effect of both stimuli might be additive.

On the contrary, when MSCs were exposed to resting lymphocytes, IL6 secretion and the majority of the immunomodulatory genes were downregulated in both

MSC-naive and MSC-primed. *COX2* gene expression followed the same pattern of downregulation, but PGE_2 secretion differed from this tendency. Even though the deviation observed in the PGE_2 concentration prevents outlining a clear trend, higher levels of PGE_2 were overall observed in MSCs exposed to resting lymphocytes than in MSCs alone. The regulation on eicosanoid pathways is complex and happens at different levels (55), and the data of this study do not allow establishing a definitive explanation for this discrepancy between gene expression and molecule secretion. We have two potential hypotheses for this observation: First, an initial activation of *COX2* could have happened followed by a downregulation, but PGE_2 secreted upon activation could still be present in the supernatant. Second, a regulatory loop could have taken place in which PGE_2 in the medium would have downregulated the expression of *COX2*. In spite of this discrepancy, the general pattern was toward downregulation of the equine MSC modulatory profile, which may be due to the lack of further activation exerted by resting lymphocytes. Thus, the baseline expression of regulatory markers will be reduced after 5 days if MSCs are no longer stimulated. Similarly, a previous report from our group found that the overexpression of these markers induced by cytokine priming diminishes after 7 days in MSCs cultured alone (15). Therefore, it can be hypothesized that resting lymphocytes did not promote or maintain the licensing of equine MSCs, indirectly reflecting their own lack of activation.

Interestingly, the higher regulatory profile in MSC-primed exposed to resting MHC-mismatched lymphocytes may be related to the activation of the latter upon encountering mismatched MSCs as these also presented increased expression of *MHC I*, *MHC-II*, and *CD40* in this condition. The overexpression of these immunogenic markers may facilitate the allo-recognition of MSCs by lymphocytes, which response might activate the regulatory profile of MSCs, and this could facilitate their immune escape. The increased expression of *MHC-I* and *MHC-II* after priming equine MSCs has been previously reported to different extent (9, 15, 36). Similarly, previous studies reported an increase in *MHC-II* expression after equine MSCs were exposed to conditioned media from PBMCs (26). The overexpression of *MHC-I* and *MHC-II* in MSC-primed alone was not observed in this study, but these markers were induced after exposure of MSC-primed to both activated and resting lymphocytes, regardless of the MHC compatibility. It has also been reported that the costimulatory molecule *CD40* could be upregulated on MSCs under inflammatory conditions in different species. In human studies, ∼50% of AT-MSCs expressed *CD40* (50), and cytokine priming enhanced the inhibitory function of MSCs derived from tonsils when expression of *IDO1* and *CD40* increased (49). However, in the conditions of this study, *CD40* was not modified by priming by exposure to activated lymphocytes, separately, and *CD40* was only overexpressed upon simultaneous priming and co-culture with mismatched activated lymphocytes. Previous studies reported that cytokine priming of human MSCs did not increase *CD80* expression as it happens with other immune markers; furthermore, *CD80* expression could be downregulated in MSCs after priming (56, 57). Similarly, in this study, *CD80* expression was reduced upon priming, and its expression after co-culture with activated lymphocytes remained low. Curiously, *CD80* expression was higher in MSC-naive after exposure to resting lymphocytes, differing from the tendencies observed for other genes. Taken together, these findings suggest that different simultaneous stimuli are needed to induce the immunogenic profile of equine MSCs, while a single stimulus would be able to induce their immune regulatory potential.

Even though these observations were not directly correlated with functional implications in this work, it is worth discussing how these changes might translate into immune suppression and immune recognition mechanisms. Previous studies have found that the ability of pro-inflammatory primed equine MSCs to suppress the proliferation of allogeneic activated T cells is enhanced, but these primed cells could also lead more easily to immune activation (32, 42), which would agree with the expression patterns seen in our study. Furthermore, a previous work from our group found that equine MSCs, either naive or primed, were able to change the frequency and proliferation of different subsets of equine activated or resting lymphocytes (16).

In the immunosuppressive assays (activated PBLs) of the current study, the increase in regulatory gene expression and secretion by MSC-primed would agree with our previous results in which the capacity of suppressing CD3+ T cells, CD4+ T cells, CD8+ T cells, and B cells was enhanced in MSC-primed compared with MSC-naive (16). Specifically, PGE_2 can downregulate the proliferation of cytotoxic T cells and B-cell activation (58) and is considered the main factor conferring the ability of equine MSCs to suppress lymphocyte proliferation (26, 30, 42). Even though the role of IL6 in equine MSC immunomodulation needs further elucidation, it has been seen in other species that this molecule is involved in the suppression of B lymphocytes (37, 38). Therefore, the higher *IL6* expression and secretion by MSC-primed might be related with the stronger suppression of B cells in immunosuppression assays and the lack of induction of B cells in modified one-way MLR assays, as observed in a previous study (16). Furthermore, the changes in the profile of equine MSCs may also be implicated in their ability to induce changes in the subpopulation of T reg cells. Human BM-MSCs are known to promote immune suppression by inducing the production of T reg, which would downregulate the proliferation of CD8+ cytotoxic T cells (59). Specifically, PGE_2 secreted by human MSCs induces CD4+ T-cell differentiation into Tregs (39), and it has also been observed in other species that the overexpression of *COX2* prevents the downregulation in the number of CD4+CD25+ Treg cells (58). While this is not well established in horses, in our previous work, the presence of equine MSC-naive and MSC-primed increased the percentage of CD4+ $CD25^{high}$ T cells in a population

of activated lymphocytes (16), which might be related with the overexpression of *COX2* and increased secretion of PGE_2 observed in the present work. Even though a direct relation cannot be established as these are found in separate studies, it is worth mentioning that the conditions in which equine MSCs showed higher *COX2* expression and PGE_2 secretion in the current study were comparable to those that displayed the highest suppressive capacity for CD8+ T cells (16).

By conducting modified one-way MLR assays with resting lymphocytes, we previously described that MSC-primed induced a proliferative response in cytotoxic and helper T cells, and this immunogenic response was more marked when the lymphocytes were MHC-mismatched with the MSCs. Similarly, MHC-mismatched MSC-primed can induce the proliferation of resting CD8+ cytotoxic cells, suggesting their immune recognition, but this condition also activates the T reg cells, which may counter the activation of the first ones (16). Interestingly, in the present study, we observed an induction of the regulatory profile precisely in MSC-primed exposed to resting mismatched lymphocytes, which may be a licensing effect conducted by the activation of the immune cells.

Regarding changes linked to the different MHC matching between equine MSCs and lymphocytes, we overall observed higher expression and/or secretion of regulatory molecules in MSCs in the autologous co-cultures with activated lymphocytes, followed by allogeneic MHC-matched and lastly by MHC-mismatched co-cultures. This observation might relate with a trend previously found for autologous MSCs to further elicit immune suppression of PHA-stimulated PBLs, followed by MHC-matched and mismatched MSCs (16). These are general patterns that cannot be directly compared, and this tendency is not reflected as significant differences among co-cultures for all the mediators assessed; however, it is particularly well-represented by *VCAM1*. Actually, it has been reported that the higher the expression level of *VCAM1*, the greater the MSC inhibitory capacity (53).

The results of this study show that an inflammatory environment can induce a regulatory profile in equine MSCs but can also increase their immunogenic expression. Similar findings have been previously reported, but the novelty of this study is to shed light on the effect of different conditions by directly comparing several scenarios. First, both cytokine priming and activated lymphocytes are able to induce the regulatory profile of equine MSCs separately, but the changes experienced by equine MSCs are different. Furthermore, the action of both stimuli appears to be additive, especially for immunogenic markers. Second, when MSCs have been primed and are specifically exposed to MHC-mismatched lymphocytes, their regulatory profile is further increased. This has been particularly noted when the co-culture was caried out with resting lymphocytes, where MSC-primed also increased their expression of the immunogenic markers *MHC-I*, *MHC-II*, and *CD40*. We hypothesize that such upregulation may facilitate the allo-recognition of foreign MSCs, and thus, the activation of lymphocytes could induce the regulatory profile of MSCs, which, at the same time, would facilitate their immune escape. This potential explanation is in line with the concept of immunomodulation–immunogenicity balance (6, 14), according to which MSCs are able to evade the immune response by equilibrating their capacities to suppress and to activate it. In conclusion, these findings highlight the plasticity of MSCs to respond to stimuli of different nature and degree, and the key role of the balance between their immune regulatory and immunogenic properties. This study also underscores the complexity of the interactions between MSCs and the immune system, giving clues on how these cells may behave once they are administered in the patients, which also can shed light on the mixed results usually obtained in *in vivo* studies. Although the actual clinical impact of these findings remains to be further explored, this information can facilitate the development of *in vivo* studies to further understand the immune properties of equine MSCs, which are key in the path toward safer and more effective cell therapies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by Advisory Ethics Committee for Animal Research from the University of Zaragoza (Project License PI 15/16).

Author contributions

LB, AC, EB, and IG were responsible for acquisition of data. AC, LB, IG, FV, and CR were responsible for analysis and interpretation of data. AR, AV, EB, MG-M, and FV provided technical support. AR, FV, and CR provided important conceptual guidance. CR was responsible for obtaining funding. LB and AC drafted the manuscript. FV, AR, AV, EB, MG-M, IG, and CR revised the manuscript for important intellectual content. All authors substantially contributed to the conception, design of the study, approved the final submitted version of the manuscript, and agree to be accountable for all aspects of the work in ensuring that accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.957153/full#supplementary-material

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MANUSCRITO IV

Local immune response induced by allogenic equine mesenchymal stem cells (MSCs) and their gene expression changes after repeated administration to MHC-matched and MHC-mismatched recipients

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Abstract. The allogenic administration of equine mesenchymal stem cells (MSCs) has numerous advantages over autologous therapy. However, to fully understand their interactions with the recipient's immune system *in vivo*, additional knowledge is required. These interactions can be influenced by factors such as donor-receptor major histocompatibility complex (MHC) matching and mismatching and MHC expression levels, which can change under different conditions. To evaluate the immunomodulatory and immunogenic expression profiles of equine MSCs and the local immune response they induce, basal (MSC-naïve), pro-inflammatory primed (MSC-primed) and chondrogenically differentiated (MSC-chondro) MSCs were placed in alginate scaffolds and subcutaneously implanted in autologous, MHC-matched/mismatched horses. Scaffolds were recovered at different time-points and the procedure was repeated to assess the effect of a second administration. Results showed that MSC-chondro exhibited the highest immunomodulatory profile, and their administration in MHC-mismatched horses did not increase their immunogenic profile. In contrast, MSC-naïve administration in MHC-mismatched horses led to a higher local immune response and a lower immunomodulatory profile. Furthermore, MSC-primed administration resulted in an early and significant recruitment of lymphocytes, accompanied by a decrease in the expression of immunomodulation-related genes and an increase in immunogenic-related genes. The MHC haplotype, the inflammatory exposure and the chondrogenic differentiation of equine MSCs affect their immune profile and the immune response developed after administration *in vivo*. Further investigation is necessary to understand the interactions between equine MSCs and the immune system in order to optimize cell therapies.

Keywords: allogenic, major histocompatibility complex, immune response, alginate, gene expression, histology.

1 Introduction

In recent years, due to the limited repair capacity of the tissues involved in musculoskeletal injuries such as tendons, ligaments and articular cartilage, advanced biological therapies have gained special relevance (Ribitsch et al. 2021), among which the application of stem cells stands out. Given the importance of horses in different sports disciplines, the treatment of pathologies that affect their musculoskeletal system and cause a decrease in performance is a particularly relevant challenge for the veterinary profession. The main objective of these treatments is to improve the repair of these tissues and make their functionality as close as possible to the original one, not achieved with conventional treatments (Ortved 2018). Furthermore, the horse is a specie with remarkable value both as patient and as translational model (Ribitsch et al. 2020; Cequier et al. 2021).

Numerous studies have shown that for the repair of these musculoskeletal tissues, the administration of both autologous and allogeneic mesenchymal stem cells (MSCs) is of great utility, obtaining promising results (Colbath et al. 2020b). Their regulatory and immunomodulatory properties are currently considered as their main therapeutic mechanism, involving both direct cell-to-cell contact and contact-independent paracrine signaling, via the expression of adhesion molecules like vascular cell adhesion molecule 1 (*VCAM1*) and the secretion of molecules such as interleukin-6 (IL6) and prostaglandin E2 (PGE2) (Hillmann et al. 2019), respectively (Carrade et al. 2012).

However, MSCs may be rejected after allogeneic administration as MSCs are currently considered immunoevasive, but no longer immunoprivileged (Ankrum et al. 2014). This new label is based on the ability of MSCs to regulate the immune response and also that they would be less immunogenic than other cells because of their expression of the major histocompatibility complex (MHC), being variable between animals, tissues of origin and environment of MSCs exposure (De Schauwer et al. 2014).

The use of allogeneic cell therapy would be a great advantage for its application in the equine clinic due to the possibility of cell banking, which would allow the availability of well-characterized MSCs for the administration of treatment at the appropriate time. In addition, the status of the recipient would not affect the quality of the MSCs (Berglund et al. 2017; Colbath et al. 2020a).

Different studies have shown how exposure of MSCs to an inflammatory environment can increase their immunomodulatory properties by increasing the expression of these molecules. In the case of *IL6*, cyclooxygenase 2 (*COX2*), indoleamine 2,3-dioxygenase 1 (*IDO1*), inducible nitric oxide synthase (*iNOS2*) and *VCAM1* their expression increases after exposure of MSCs to pro-inflammatory cytokines such as interferon γ (IFNγ) and tumor necrosis factor-alpha (TNFα) (Barrachina et al., 2017; Caffi et al., 2020).

Regarding immunogenicity, both *in vitro* and *in vivo* studies have shown that MSCs expressing high levels of *MHC-II* stimulate the generation of an immune response (Rowland et al. 2018; Kamm et al. 2021). In contrast, when expression is low, MSCs may go unnoticed and fail to induce immune response in the recipient (Schnabel et al. 2014; Broeckx

et al. 2019). Recently, the importance of compatibility for MHC between donor and recipient has been appreciated and several studies describe the generation of immunological memory against MHC molecules, which may have an impact on repeated administration of MSCs in different species (Berglund et al. 2017). In the equine species, repeated administration of MHC-mismatched allogeneic MSCs has been observed to result in an adaptive immune response, local inflammation and reduced therapeutic action of MSCs (Rowland et al. 2021). Also in the equine species, moderate to strong expression of the co-stimulatory molecules *CD40* and *CD80*, highly related to the immunogenic profile and immune rejection, has been observed depending on the tissue of origin (De Schauwer et al. 2014).

Although the inflammatory environment entails an increase in the immunomodulatory properties of MSCs, it also increases the expression of *MHC-I* and *MHC-II* (Barrachina et al. 2017). However, the increase of the immunomodulatory properties of MSCs stimulated with pro-inflammatory cytokines could help them to regulate the inflammation and elicit a lower humoral response more effectively after performing a second administration (Barrachina et al. 2020). On the other hand, the expression of these molecules after differentiation of MSCs to chondrocyte is being increasingly studied with the aim of being able to administer this type of MSCs in joint pathologies. According to previous studies (Ryan et al. 2014), after differentiation of equine MSCs to chondrocyte, MHC expression and B lymphocyte proliferation increase, and also their immunomodulatory capacities are reduced with respect to undifferentiated MSCs. However, in recent *in vitro* and *in vivo* studies in the equine species, no such increase in immunogenicity was observed after chondrogenic differentiation of MSCs when co-cultured with allogeneic MHC-mismatched lymphocytes, although their immunomodulatory properties are still under discussion (Van Hecke et al. 2021; Cequier et al. 2022a).

At the clinical level, it is desirable that the immunomodulatory properties outweigh the immunogenic potential of MSCs. However, the exact mechanisms to achieve this is not clearly defined and further research in this field is needed to elucidate which factors and in what way can modify this balance, as an essential step to maximize the therapeutic yield.

In horses, molecules influencing this balance have been studied *in vitro* both at the level of gene expression and secretion by MSCs (Cassano et al. 2018a; Caffi et al. 2020). However, these molecules have not been studied *in vivo* after administration of equine MSCs due to the difficulty of recovering the cells once administered to the animal. The use of hydrogels such as alginate has been used in different studies to encapsulate cells, as if they were a synthetic extracellular matrix (Ho et al. 2022), and is a good option for encapsulating MSCs in them so that they can be retrieved for further detailed studies. Mammalian cells cannot interact with the polysaccharides in the alginate, which is ideal for keeping the hydrogel intact and the MSCs inside, but also allows nutrients and metabolites to pass from the organism to the cells inside and vice versa, which is ideal for studying the interaction between the animal's local immune response and the MSCs response (Watts et al. 2013).

The main objective of this study was to evaluate the immunomodulatory and immunogenic profile of equine MSCs as well as to analyze the local immune response of the recipient after repeated *in vivo* administration. Three different types of MSCs were studied; unstimulated MSCs (MSC-naïve), MSCs stimulated with pro-inflammatory cytokines (MSC-primed) and MSCs differentiated to chondrocytes (MSC-chondro) also considering the compatibility between donor and recipient for MHC.

The experimental design consisted of the analysis of gene expression of different molecules related to immunomodulation and immunogenicity of MSCs after *in vivo* administration in autologous (control), MHC-matched and MHC-mismatched recipients. In addition, the local immune response developed by each recipient to these MSCs was also assessed. For this purpose, MSCs were encapsulated in alginate gels and three subcutaneous implants were placed in the neck boards of different horses. Scaffolds were retrieved at different times and, one month after removal of the last one, the same procedure was repeated for each animal in the contralateral neck boards.

The initial hypothesis was that MSC-primed would present higher expression of immunomodulatory and immunogenic molecules compared to MSC-naïve after *in vivo* administration, based on previous studies (Cassano et al. 2018b; Caffi et al. 2020; Cequier et al. 2022b) . On the other hand, for MSC-chondro, the expression of immunomodulatory molecules would be lower and immunogenic molecules would be higher than those of MSC-naïve (Barrachina et al. 2018b; MacDonald y Barrett 2020). In addition, gene expression of immunomodulatory molecules would be higher in the MHC-matched group compared to the MHC-mismatched one, while immunogenic molecules would be higher in the MHC-mismatched group. These findings would be reflected by an increased local receptor response inducing a greater foreign body reaction in the case of MSC-chondro, although MSC-primed MSCs could attenuate this reaction due to their immunomodulatory properties. In addition, it was also hypothesized that more foreign body reaction would be observed in the case of MHC-mismatched than MHC-matched and autologous horses.

2 Material and methods

2.1 Study design

The horses that participated in the study were selected according to their MHC haplotype with the aim of conforming groups of equine MHC-matched and MHC-mismatched combinations. Ten intra-MHC microsatellite regions were analyzed by multiplex PCR according to previously described literature (Cequier et al. 2022a). In total, 20 horses aged 2-12 years were selected for the study: 12 neutered males, 7 females and 1 stallion.

First, 3 horses homozygous for specific MHC haplotypes (HapPRE10, HapPRE11 and HapMAI04) were used as BM-MSC donors. The remaining horses were selected so that two groups of allogeneic recipients could be formed: MHC-matched and MHC-mismatched with donors. In the MHC-matched group, 8 heterozygous animals sharing one haplotype with one of the donors were included (2 HapMAI04, 3 HapPRE10 and 3 HapPRE11 heterozygotes). For the MHC-mismatched group, 9 horses carrying haplotypes different from HapMAI04, HapPRE10 and 3 HapPRE11 were selected. Both

groups were further divided into three subgroups of 2-3 horses each. Each subgroup received one type of MSCs: MSC-naïve, MSC-primed or MSC-chondro. In addition, each homozygous donor (n = 3) acted as autologous control, and 1 separate animal as sham operated control (**Fig. 1**).

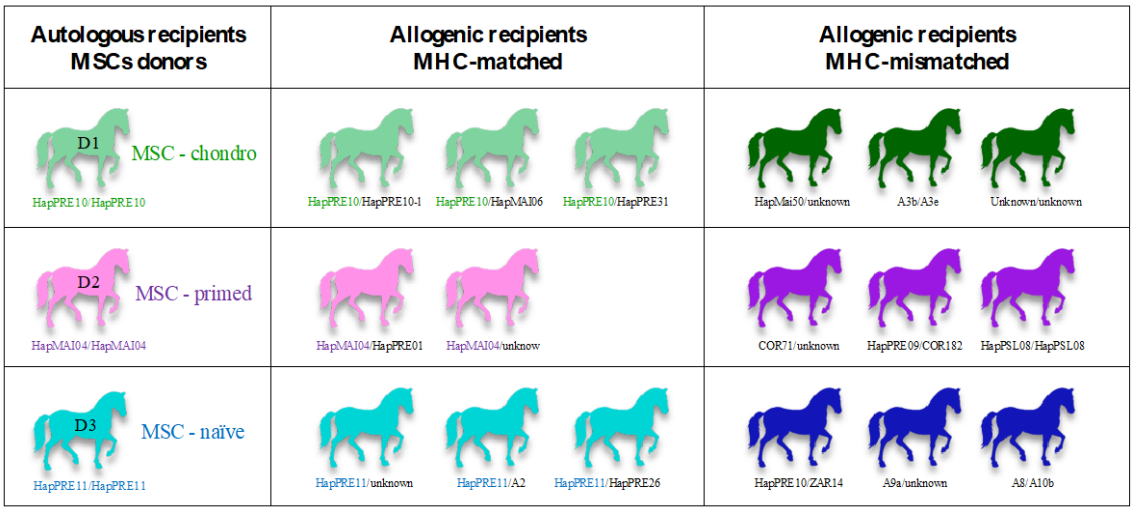


Fig. 1 Study design. Groups of horses (autologous, allogenic MHC-matched and allogenic MHC-mismatched) and types of MSCs received (MSC-chondro, MSC-primed and MSC-naïve). Below each horse its MHC haplotype is indicated.

Each animal was implanted with 3 subcutaneous alginate scaffolds on neck board, each containing 5×10^6 MSCs of the same type (MSC-naïve, MSC-primed and MSC-chondro). Acellular scaffolds were implanted in the sham control group. Each of the three scaffolds was removed at a different time-point (1, 3 and 6 weeks post implantation). Histological analysis and gene expression of molecules related to the immunoregulatory and immunogenicity profiles of the different MSCs from these animals were evaluated. One month after the removal of the last scaffold, it was verified that all the clinical parameters were normalized and each animal was re-exposed to the same type of BM-MSCs (MSC-naïve, MSC-primed and MSC-chondro; MHC-matched or MHC-mismatched) using the contralateral side of the neck, to study the potential development of immune memory mechanisms under each condition.

All procedures involving animals were approved by the Ethical Advisory Committee for Animal Experimentation of the University of Zaragoza (PI-15/16). All animals were kept on paddocks of the facilities of the Animal Research Service of the University of Zaragoza, with free access to water and grass hay.

2.2 Isolation and culture of MSCs

Mesenchymal stem cells were obtained from the sternum bone marrow of the 3 donors and were isolated, expanded and cultured at a temperature of 37 °C, 5% CO_2 and 90% relative humidity as previously described (Cequier et al. 2022b). The MSCs medium used was Dulbecco's Modified Eagle's Medium low glucose (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/mL streptomycin and 100

U/mL penicillin (Sigma–Aldrich). All MSCs used were previously characterized (Cequier et al. 2022a).

2.3 Preparation of alginate scaffolds

Alginate (3%) (ultrapure low-viscosity 67% guluronate, UPLVG; NovaMatrix, FMC Corporation) was prepared in phosphate buffered saline (PBS) by stirring for 1 hour at 55 ºC until a homogenous mixture was achieved. Alginate was sterilized at 120 °C and 0.15 Mpa and stored at 4 °C (Lin et al. 2015; Yu et al. 2017).

After detaching, MSCs were resuspended at 5×10^6 MSCs/ml in PBS and mixed 1:1 with 3% alginate for a final concentration of 1.5% alginate in total volume of 2 mL/implant (Watts et al. 2013). Scaffold polymerization was achieved as described earlier (de Vries-van Melle et al. 2014; Leijs et al. 2017; Santos et al. 2018) with some modifications. For each implant, 2 mL of the mix of cells and alginate were transferred with a syringe into a concave custom-made device for immediate immersion into a sterile 102 mM $CaCl_2$ (Sigma-Aldrich) solution for 5 min until polymerization was apparent. Then, 1 mL sterile 102 mM $CaCl_2$ solution was added to the bottom of 12-well plates, scaffolds were transferred to wells, and an additional 1 mL sterile 102 mM $CaCl_2$ solution was added on the top to completely cover the constructs. Acellular implants were constructed following the same methodology but with PBS instead of MSCs. All scaffolds were incubated 30 min, 37 °C and 5% CO_2, after which remaining $CaCl_2$ solution was removed, and constructs were washed 3 times with PBS.

Prior to any *in vivo* assay, different parameters were assessed, and certain techniques were optimized which the authors consider should be explained. Preliminary tests on the viability of MSCs and chondrocyte differentiation once encapsulated, as well as the pilot test to optimize implant recovery and its subsequent study are described in **Resource 1** (**Annex VI**).

2.3.1 MSCs preparation: MSC-naïve, MSC-primed and MSC-chondro

MSC-naïve and MSC-primed scaffolds were maintained in MSCs culture medium described above for 48 hours. For MSC-primed scaffolds, 12 hours before placement, 5 ng/mL of each pro-inflammatory cytokines TNFα and IFNγ (R&D Systems) were added as described earlier (Barrachina et al. 2018a). For MSC-chondro, chondrogenesis was induced according to the methodology explained in **Resource 1** (**Annex VI**) for 21 days.

All scaffolds (MSC-naïve, MSC-primed and MSC-chondro), 24 hours before placement, were washed twice with PBS and the FBS in the culture medium was replaced with autologous serum from each recipient so that the MSCs were free of xenogeneic antigens that could interfere with the immune response (Pezzanite et al. 2015). Subsequently, just before the intervention, scaffolds were again washed three times with PBS. In addition, an extra scaffold was previously prepared from each donor that was frozen at -80 °C for later use as a reference sample for gene expression analysis.

2.4 Placement and removal of the scaffolds

Three alginate scaffolds with 5 × 10^6 MSCs per implant were placed subcutaneously in each horse. Scaffolds were placed along the neck tables, from cranial to caudal, leaving approximately 10 cm between them. The technique used was the subcutaneous pocket technique (Bellas et al. 2015), detailed below. Each implant was removed at a different time: from cranial to caudal at 1 (T1), 3 (T2) and 6 (T3) weeks (**Fig. 2**).

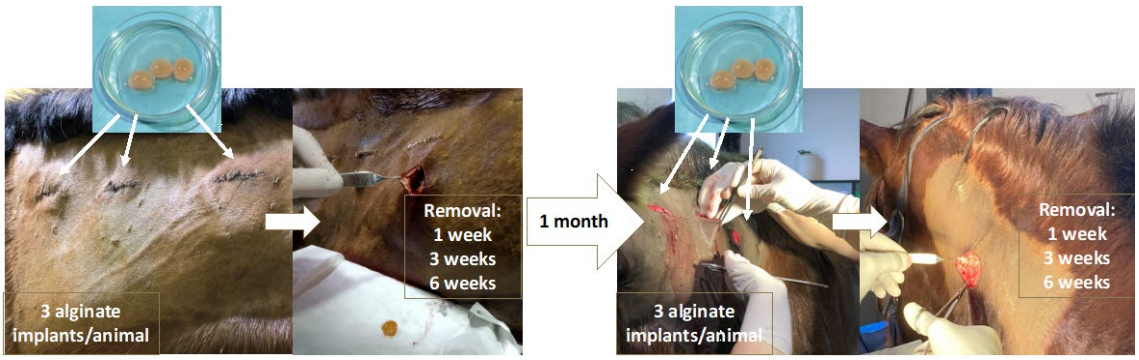


Fig. 2 Representation of the subcutaneous placement and removal of the scaffolds at different times.

One month after the last implant (T3) was removed, the same procedure was repeated in the contralateral neck tables and each animal was re-exposed to the same type of MSCs (MSC-naïve, MSC-primed and MSC-chondro; MHC-matched or MHC-mismatched) to study the possible development of different types of immune memory mechanisms under different conditions. Three new implants of the same type and donor as firstly received were placed subcutaneously in each animal and were also removed after 1 (T4), 3 (T5) and 6 (T6) weeks.

To carry out the surgical intervention, which was performed on station, a sedation based on an α-2 agonist (detomidine 0.01 mg/kg, Sedaquick, Fatro) and an opioid (butorphanol 0.02 mg/kg, Torbugesic, Pfizer) was administered. In addition, they were given a single dose of IV flunixin meglumine as premedication (1.1 mg/kg, Niglumine, Calier). While the appropriate degree of sedation was achieved, the surgical field was prepared. This preparation consisted of shaving a wide rectangle along the neck boards, and cleaning and disinfecting it with 5% chlorhexidine and 96° alcohol. This last step was performed for 5 min to achieve good aseptic conditions and, lastly, Sterillium® was applied.

Once the horse was under adequate sedation, the procedure was started, maintaining sterility at all times. First, a local anesthetic (lidocaine 5%, Braun) was injected into the incision area. It was massaged to spread along the region and three horizontal incisions of approximately 2 cm in length were made with the scalpel at the top and about 10 cm apart. Subsequently, with the dissecting scissors, the subcutaneous tissue was dissected in the entire area below the incision, leaving a kind of square pockets of about 1.5x1.5 cm with an opening in the upper part: subcutaneous pocket technique. The implants were then placed inside the subcutaneous pockets and the upper incision was sutured in two planes. First a continuous suture of the subcutaneous tissue was made (2/0 USP polyglyconate) and then to suture the skin simple staples and stitches (2/0 USP polyglyconate)

were placed. The skin staples and stitches were removed 7-10 days after surgery. In addition, staples were also placed on the ventral, caudal and rostral edges of the pockets to delimit the square and prevent the implants from migrating through the neck.

After the intervention and during the following three days, all horses were clinically monitored. Also, during those three days they were maintained on antibiotic coverage (IM procaine penicillin at 15 mg/kg once daily, Procapen, Livisto). All implant sites were ultrasonographically evaluated before implant surgery, immediately later implantation and prior to each implant removal (**Resource 2**, **Annex VI**).

For the extraction, which was carried out according to the mentioned times, the same procedure of preparation of the surgical field was followed as in the placement of the implants. To remove each implant, an incision was made at the rostral edge of each pocket about 3 cm long and the subcutaneous tissue was carefully dissected. The incision was closed as aforementioned. Following surgery for implant removal, the horses were also monitored clinically in the following days.

2.5 Assessment of local cell-mediated immune response over time after scaffolds placement

Each scaffold, once removed, was divided into two pieces to assess the cellular immune response locally. One piece of the implant was used for the histological assessment and the remaining portion of the implant was used to study the gene expression of molecules related to immunomodulation and immunogenicity of MSCs after immune system exposure *in vivo*. The implants for performing gene expression were kept at -80 °C until mRNA isolation.

2.5.1 Gene expression analysis

Gene expression of molecules related to the immunoregulatory and immunogenic capacity of MSCs under different conditions was carried out by RT-qPCR. A Direct-zol RNA Miniprep Kit (Zymo Research, Cat# R2052) was used to isolate mRNA from each scaffold, after disruption and homogenization of frozen scaffold with the TissueLyser LT instrument (Qiagen). Prior to retrotranscription, mRNA concentration of each sample was measured with the NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher) and purity was assessed using 260/280 and 260/230 absorbance ratios. Complementary DNA (cDNA) synthesis were performed from 100 ng of total RNA using qScriptTM cDNA SuperMix (Quanta Biosciences), according to the manufacturer's instructions. Resulting complementary cDNA was diluted 1:10 and gene expression was assessed by quantitative real-time PCR. The primers used for their detection were previously designed by the group (Barrachina et al. 2018a) and are detailed in **Table 1**.

Table 1. Primers used in RT-qPCR to analyze gene expression. Genes are grouped according to the functions and implications of the encoded molecules.

Gene	Accession number	Primer sequence (5' – 3')	Amplicon size
Housekeeping			
GAPDH	NM_001163856	F: GGCAAGTTCCATGGCACAGT R: CACAACATATTCAGCACCAGCAT	128
B2M	NM_001082502	F: TCGTCCTGCTCGGGCTACT R: ATTCTCTGCTGGGTGACGTGA	102
Immunomodulation related molecules			
Molecules related with cell-to-cell contact mechanism			
VCAM1	NM_001101650	F: TCTATGCTACGCTCTGGCTACG R: TTGATGGTCTCCCCGATGA	127
Molecules related with paracrine signaling mechanism			
COX2	AB041771	F: GTTTGCATTTTTTGCCCAGC R: ACTTAAATCCACCCCGTGACC	103
IDO1	XM_01736538.2	F: TCATGACTACGTGGACCCAAAA R: CGCCTTCATAGAGCAGACCTTC	104
iNOS2	AY027883	F: CCAACAATGGCAACATCAGGT R: TGAGCATTCCAGATCCGGA	85
IL6	EU438770	F: AACAGCAAGGAGGTACTGGCA R: CAGGTCTCCTGATTGAACCCA	95
Immunogenic markers			
MHC-I	AB525081	F: CGTGAGCATCATTGTTGGC R: TCCCTCTTTTTTCACCTGAGG	92
MHC-II	NM_001142816	F: AGCGGCGAGTTGAACCTACAGT R: CGGATCAGACCTGTGGAGATGA	172
CD40	AY514017	F: ACAAATACTGCGACCCCAACC R: TTTCACAGGCATCGCTGGA	114
CD80	XM_005601958.3	F: CAGGAAAGTTGGCTCTGACCA R: TCTCCATTGTGATCCTGGCTC	135

All RT-qPCR reactions were performed and monitored with a QuantStudio3 Real Time PCR System device (Applied Biosystems, USA). For each sample, 5 µL of Fast SYBER Green Master Mix (Applied Biosystems), 0.15 µL of each primer pair and 2.7 µL of H20 were used for a final volume of 8 µL. The 8 µL of the mix was pipetted into each well and 2 µL of cDNA from the corresponding samples was added to each well. In addition, for each gene, a negative control (NTC, no template control) was included in triplicate, in which 2 µL of nuclease-free H20 was pipetted in place of cDNA to check for nonspecific amplification. Amplification was performed in triplicate for each sample as follows: 20 s at 95 °C for initial activation, followed by 40 cycles consisting of 3 s at 95 °C and 30 s at 60 °C and a dissociation curve protocol run after every PCR. Gene expression levels were obtained using the comparative ΔΔCt method. Normalization factor was calculated as the geometric mean of the quantity of two housekeeping genes, *GAPDH* and *B_2M*. The control implant from each donor that was frozen at the beginning was used as a reference sample to calculate the relative expression in the experimental implants. The value of the control implants was 1 (2^0=1).

2.5.2 Histological analysis

In order to assess the recipient immune response, scaffolds were fixed in 4% paraformaldehyde (PFA) in PBS (Olderøy et al. 2014; Reppel et al. 2015; Xu et al. 2015; Zhang et al. 2015) overnight at 4 ºC. In horses where an increase in subcutaneous tissue was observed, these fibrous capsules were dissected and analyzed in parallel to the scaffolds. All preparations were embedded in paraffin, cut into 5-mm sections and the sections were hydrated with increasing gradients of alcohols and stained with H&E. All sections were rinsed with distilled water, dehydrated with decreasing amounts of alcohol, and mounted (Barrachina et al. 2018b).Three preparations from each sample were assessed by a blinded evaluator who assigned a score to each one using a score system ranging from 0 (minimal response) to 3 (maximum response) according to the presence of lymphocytes, eosinophils, polymorphonuclear cells (PMN), histiocytes and multinucleated cells around alginate scaffolds and capsules. In addition, the capsules were also assessed for vascular proliferation, fibroblast proliferation, fibrosis, hemorrhage, amount of scaffold, synovial metaplasia, hemosiderin, fat, fat necrosis and muscular fascia (**Resource 3, Annex VI**) (Aguiar et al. 2015). The score of each horse was normalized over that observed in acellular controls. Entire H&E sections were scanned using a 3D Scan Pannoramic Histech scanner (3D Histech P-1000 scanner), at 40x resolution, obtaining whole slide images. Images were analyzed using SlideViewer 2.6 software (3D Histech kft.), which provides a detailed morphometric analysis with precise measurements of different histological parameters at high resolution.

2.6 Statistical analysis

Statistical processing of the different variables was performed with the IBM SPSS Statistics version 26 statistical package. Analytical statistical tests were used to test for differences in the relative mRNA expression of each gene and each histological parameter as a function of the different study variables. The variables were "group" (two categories: MHC-matched and MHC-mismatched), "cell type" (three categories: MSC-naïve, MSC-primed and MSC-chondro) and "time" (6 categories: T1, T2, T3, T4, T5 and T6). The existence of outlier samples was evaluated with the Grubbs test (alpha = 0.05). The expression of each gene and the score of each parameter was analyzed individually, establishing it as a dependent variable, and each of the other variables as a factor to study the differences as follows (1) differences between cell types at each time within each group of receptors, (2) differences between groups of receptors for each cell type at each time, (3) differences over time within each group of receptors for each cell type. For the different analyses, non-parametric tests were used after assessing normality and homoscedasticity using the Shapiro-Wilk test and Levene's test, respectively. For variables with more than three groups, Kruskal-Wallis or Friedman tests followed by Dunn's test were used as post hoc, for independent (1) or related (3) samples, respectively. The Mann-Whitney U test was used for comparisons between two groups (2). The significance level was set at $p < 0.05$ for all analyses. GraphPad Prism 9.2 was used for graphical representation.

3 Results

The results presented in this study focus on the most relevant and significant findings that may have implications for future MSCs therapies. Detailed results are shown for those genes and histological parameters for which significant differences were observed. No substantial changes were noted for other genes and histological parameters, suggesting that these factors were not influenced by the conditions examined in this study. For comprehensive reference and potential interest in future research, graphical representations of all the analyzed parameters can be found in **Resource 4** (**Annex VI**), including those genes and histological parameters for which no differences were observed between the different conditions analyzed. It is important to note that of the 126 implants placed in the horses, a total of 8 implants could not be recovered. This occurred due to the behavior of the horses, leading to suture dehiscence because of scratching or grooming interactions between the horses.

3.1 Changes in gene expression of markers related to equine MSCs immunomodulation and immunogenicity

Allogeneic MSC-naïve, MSC-primed or MSC-chondro were administered to autologous, MHC-matched and MHC-mismatched horses. Each type of MSCS was encapsulated in alginate and 3 scaffolds/recipient were placed subcutaneously and recovered after 1, 3 and 6 weeks. The procedure was repeated to assess the effect of a second administration.

The expression changes of most important genes involved in the immunomodulatory properties of equine MSCs was analyzed (*IL6*, *COX2*, *IDO1*, *iNOS2*, and *VCAM1*). Additionally, the expression of genes related to antigen presentation, including *MHC-I*, *MHC-II*, *CD80*, and *CD40*, was analyzed to investigate the impact of different conditions on MSC immunogenicity.

3.1.2 Gene expression changes between MSC types and over time

In vivo exposure of the three types of MSCs (MSC-naïve, MSC-primed and MSC-chondro) resulted in different changes in the expression of the genes analyzed. Statically significant differences were observed for all the genes analyzed except for *MHC-I*. In the MHC-matched group, at T1, T3, and T4, MSC-chondro displayed higher *COX2* expression compared to MSC-naïve ($p < 0.05$). Similarly, in the MHC-mismatched group, MSC-chondro exhibited higher *COX2* expression than MSC-naïve at T1, T4, and T6 ($p < 0.05$ in all cases). Also, in the MHC-mismatched group, *COX2* expression in MSC-primed was significantly downregulated compared to MSC-naïve ($p < 0.05$) and MSC-chondro ($p < 0.01$) at T1 and T6. Additionally, at T4 in the MHC-mismatched group, *COX2* expression was upregulated in MSC-chondro compared to MSC-primed ($p < 0.05$). Assessing the changes in gene expression over the different time-points, MSC-chondro in the MHC-matched group showed a significant overexpression of *COX2* at T1 compared to T2 and T3 ($p < 0.05$ in both cases) (**Fig. 3**).

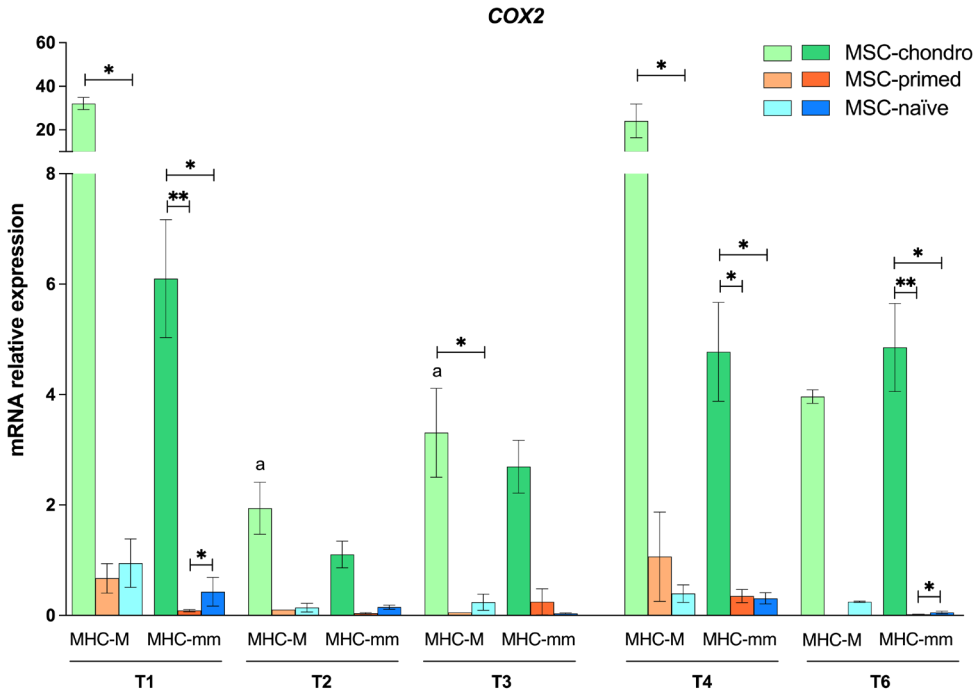


Fig. 3. Changes in cyclooxygenase 2 (*COX2*) expression in equine mesenchymal stem cells (MSCs) following administration to MHC-matched (MHC-M) and MHC-mismatched (MHC-mm) horses. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using each baseline MSCs type as reference sample (value 1). Significant differences between cell-types at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). Significant differences between time-points compared to T1 are represented by lower case letter [a] ([a], $p < 0.05$).

IDO1 expression was significantly higher in MSC-chondro compared to MSC-naïve in the MHC-matched group at T1 and T4 ($p < 0.05$ in both cases). Furthermore, at T4, MSC-primed exhibited higher *IDO1* expression compared to MSC-naïve in both the MHC-matched and MHC-mismatched groups ($p < 0.01$ in both cases). In addition, in the MHC-mismatched group, MSC-primed displayed higher *IDO1* expression compared to MSC-chondro at T4 ($p < 0.05$) and MSC-naïve at T6 ($p < 0.05$). However, at T6, the levels of *IDO1* expression decreased compared to T1 and T4, suggesting a reduction in the immunomodulatory effect over time (**Fig. 4**).

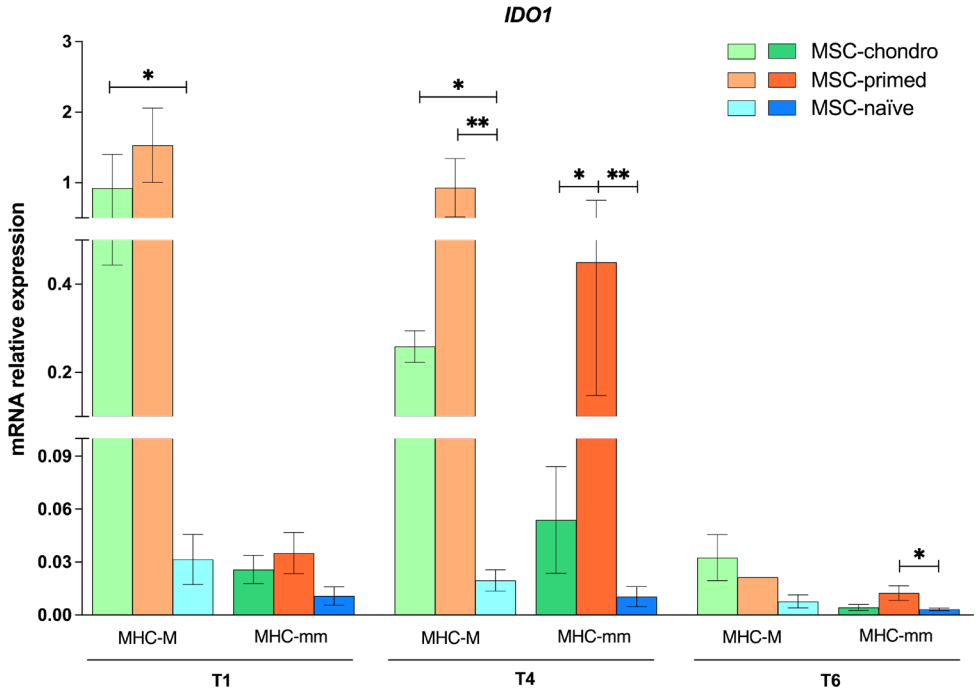


Fig. 4. Changes in indoleamine 2,3-dioxygenase 1 (*IDO1*) expression by equine mesenchymal stem cells (MSCs) following administration to MHC-matched (MHC-M) and MHC-mismatched (MHC-mm) horses. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using each baseline MSCs type as reference sample (value 1). Significant differences between cell-types at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$).

For both *IL6* and *iNOS2* significant differences were only observed in the MHC-mismatched group. At T1 and T4, *IL6* expression in MSC-chondro was significantly higher compared to MSC-primed ($p < 0.01$) and MSC-naïve ($p < 0.05$) (**Fig. 5A**). Likewise, for *iNOS2*, MSC-chondro exhibited higher expression than MSC-primed ($p < 0.05$) at T4, where MSC-primed displayed generally low values (**Fig. 5B**). Notably, at T1, T4, and T6, *iNOS2* and *IL6* expression in MSC-primed was significantly lower than in MSC-naïve ($p < 0.05$). Furthermore, at T5, both *IL6* and *iNOS2* expression were higher in MSC-chondro compared to MSC-primed ($p < 0.05$), and at T6, the difference remained significant ($p < 0.01$) (**Fig. 5**).

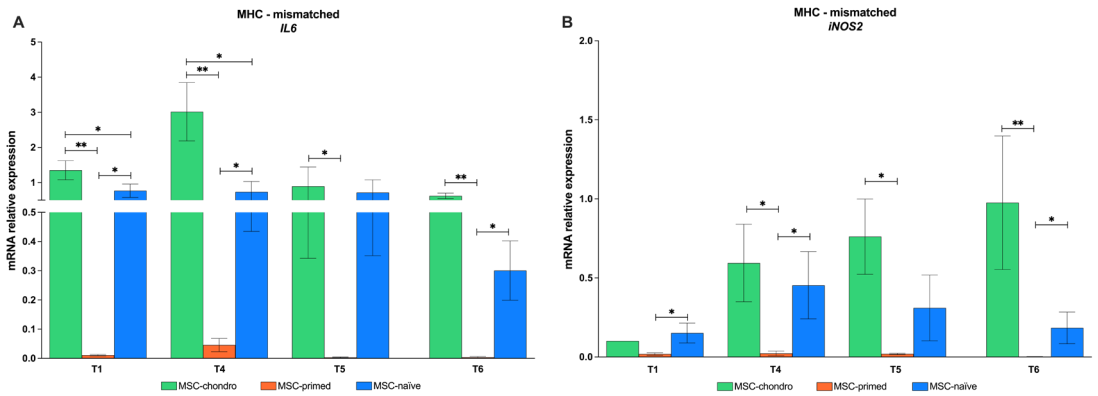


Fig. 5. Changes in interleukin 6 (*IL6*) (A) and inducible nitric oxide synthase 2 (*iNOS2*) (B) expression by equine mesenchymal stem cells (MSCs) following administration to MHC-mismatched horses. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using each baseline MSCs type as reference sample (value 1). Significant differences between cell-types at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$).

In terms of *VCAM1*, at T1 and T4, MSC-naïve exhibited an increase in *VCAM1* expression compared to MSC-chondro ($p < 0.05$) and MSC-primed ($p < 0.01$) in the MHC-matched group. Following the initial administration, MHC-matched animals receiving MSC-naïve exhibited an overexpression of *VCAM1* at T1 compared to T2 and T3 ($p < 0.05$ in both cases). Upon re-exposure, an upregulation of *VCAM1* was observed at T4 and T5, with a significant increase observed specifically at T5 compared to T2 ($p < 0.05$) (**Fig. 6A**).

In the MHC-mismatched group, a significant upregulation of *VCAM1* in MSC-naïve compared to MSC-chondro was observed only after re-exposure (T4) ($p < 0.05$). However, in the MHC-mismatched group, significant downregulation of *VCAM1* expression was observed in MSC-primed compared to MSC-chondro and MSC-naïve at T1 ($p < 0.05$ in both cases), T4 (MSC-chondro, $p < 0.05$; MSC-naïve, $p < 0.01$), T5 (MSC-chondro, $p < 0.05$; MSC-naïve, $p < 0.01$), and T6 ($p < 0.05$ in both cases) (**Fig. 6B**).

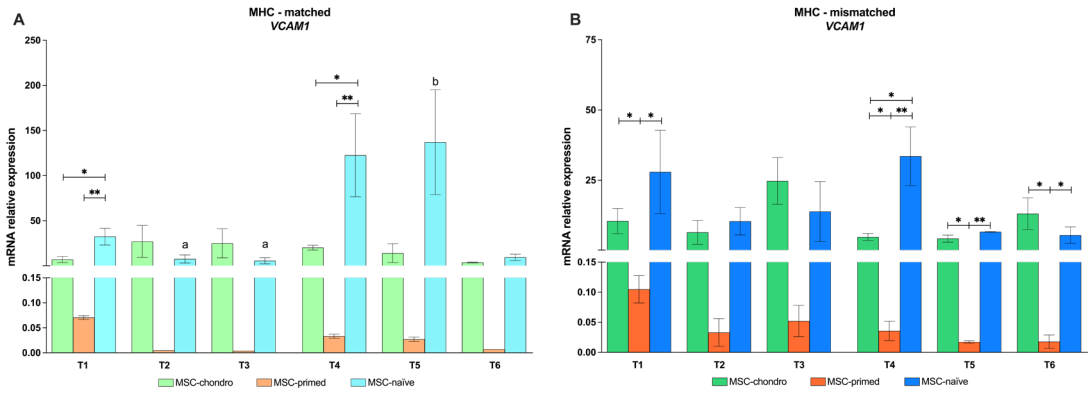


Fig. 6. Changes in vascular cell adhesion molecule 1 (*VCAM1*) expression by equine mesenchymal stem cells (MSCs) following administration to MHC-matched (A) and MHC-mismatched (B) horses. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using each baseline MSCs type as reference sample (value 1). Significant differences between cell-types at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). Significant differences between time-points compared to T1 and T2 are represented by lower case letter [a] and [b], respectively ([a], [b], $p < 0.05$).

Regarding the immunogenic genes, at T1 in the MHC-matched group, MSC-chondro exhibited significantly higher *CD40* expression compared to MSC-primed and MSC-naïve, but at T4, *CD40* expression was significantly lower than MSC-naïve ($p < 0.05$ in all cases). In the MHC-mismatched group, at both the initial (T1) and final (T6) implants, *CD40* expression was consistently lower in MSC-primed compared to MSC-chondro and MSC-naïve ($p < 0.05$ in all cases) (**Fig. 7A**).

Regarding *CD80*, its expression showed consistent increases in MSC-chondro of the MHC-mismatched group throughout all time points. Additionally, in the MHC-mismatched group, *CD80* expression in MSC-naïve was significantly lower than in MSC-chondro and MSC-primed at the first and fourth scaffolds ($p < 0.05$ in all cases). Furthermore, in the MHC-mismatched group, an increase in *CD80* expression was observed in MSC-chondro compared to MSC-primed at T1 and compared to MSC-naïve at T6 ($p < 0.05$ in both cases). In the MHC-matched group, a significant upregulation of *CD80* expression was observed in MSC-chondro compared to MSC-naïve at T1, T3, and T4 ($p < 0.05$ in all cases) (**Fig. 7B**).

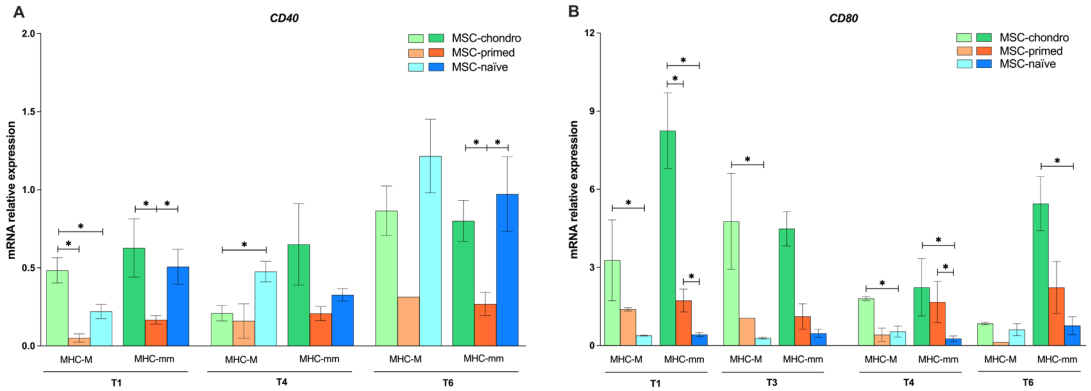


Fig. 7. Changes in costimulatory molecules *CD40* (A) and *CD80* (B) expression by equine mesenchymal stem cells (MSCs) following administration to MHC-matched (MHC-M) and MHC-mismatched (MHC-mm) horses. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using each baseline MSCs type as reference sample (value 1). Significant differences between cell-types at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$).

The administration of MSC-naïve to the MHC-matched group showed an overexpression of *MHC-I* on the first scaffolds (T1), which decreased at subsequent time points and was statistically significant compared to T3 ($p < 0.05$). Furthermore, after the second administration, *MHC-I* expression was upregulated at T4 compared to T6 in both allogenic groups that received MSC-naïve scaffolds ($p < 0.05$ in both cases) (**Fig. 8**). Therefore, the highest *MHC-I* expression was observed after the first administration (T1), and although the expression was higher than at T4, the difference was not statistically significant. Moreover, except for T1, the *MHC-I* expression was downregulated compared to the control implant at all other time points in the MHC-matched group. Graphical representations of gene expression for the other conditions analyzed, for which no differences were observed, can be found in **Resource 4** (**Annex VI**).

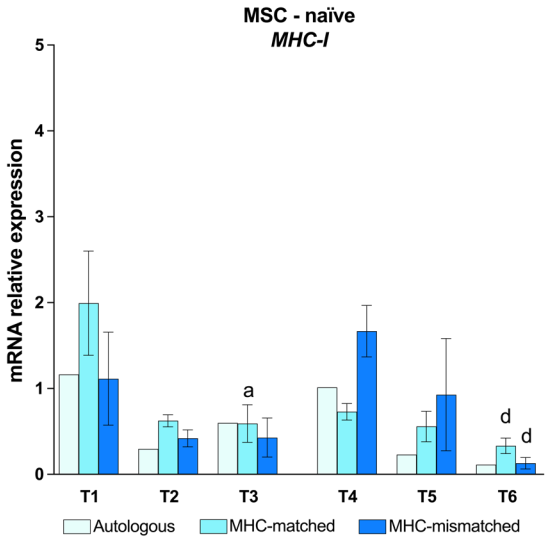


Fig. 8. Changes in MHC-I expression by equine mesenchymal stem cells unstimulated (MSCs-naïve) following administration to autologous, MHC-matched, and MHC-mismatched horses over different time points. Significant differences between time-points compared to T1 and T4 are represented by lower case letter [a] and [d], respectively ([a], [d], $p < 0.05$).

Regarding *MHC-II* expression, in the MHC-matched group, an increase in *MHC-II* expression was observed in MSC-primed and MSC-chondro compared to MSC-naïve on the first scaffold (T1) ($p < 0.05$ in both cases) and on the second scaffold ($p < 0.05$ in MSC-chondro) (**Fig. 9A**).

In the MHC-mismatched groups, a trend towards higher *MHC-II* expression could be observed in MSC-primed along all the time-points analyzed. Specifically, MSC-primed exhibited the highest expression of *MHC-II* expression compared to MSC-chondro and MSC-naïve at T1 ($p < 0.05$ in both cases) and compared to MSC-chondro at T5 ($p < 0.05$). On the last scaffold (T6), MSC-naïve downregulated *MHC-II* expression compared to MSC-chondro and MSC-primed ($p < 0.05$) (**Fig. 9B**).

Regarding the changes over different time-points, *MHC-II* expression in MSC-naïve MHC-matched group showed an increase at the last time-point analyzed after each exposure (T3 and T6). At T3, there was a significant overexpression compared to T1 and T2 ($p < 0.05$ in both cases), while at T6, there was a significant overexpression compared to

T3 ($p < 0.05$), T4 ($p < 0.01$), and T5 ($p < 0.05$) (**Fig. 9A**). Similarly, in the MHC-mismatched groups, a significant overexpression of *MHC-II* was observed at T6 compared to T4 for both MSC-naïve and MSC-primed scaffolds ($p < 0.05$ in both cases). Furthermore, in MSC-primed scaffolds administered to the MHC-mismatched group, although there was a significant downregulation of *MHC-II* after re-exposure at T4 compared to T1 ($p < 0.05$), a significant increase was observed at T5 compared to T4 ($p < 0.05$) (**Fig. 9B**).

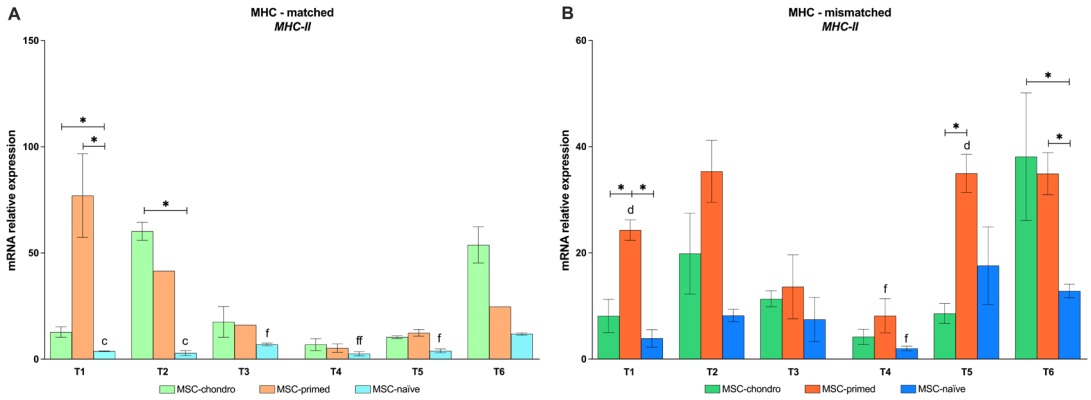


Fig. 9. Changes in *MHC-II* expression by equine mesenchymal stem cells (MSCs) following administration to MHC-matched (A) and MHC-mismatched (B) horses. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using each baseline MSCs type as reference sample (value 1). Significant differences between cell-types at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$). Significant differences between time-points compared to T3, T4 and T6 are represented by lower case letter ^{c}, ^{d} and ^{f}, respectively (^{c}, ^{d}, ^{f}, $p < 0.05$).

3.1.3 Influence of MHC compatibility on MSC gene expression

The influence of MHC matching on gene expression was analyzed, showing significant differences in MSC-chondro and MSC-naïve when administered to MHC-matched or MHC-mismatched recipients. For MSC-chondro, significant differences were observed primarily in immunomodulatory genes, with increased expression in the MHC-matched group. Specifically, *COX2*, *IDO1*, and *IL6* exhibited higher expression in the MHC-matched group compared to the MHC-mismatched group on the first scaffolds ($p < 0.05$) (**Fig. 10A**). Similarly, at the first time after re-exposure (T4), an upregulation in *COX2*, *IDO1*, and *VCAM1* expression was observed in MSC-chondro administered to the MHC-matched group ($p < 0.05$) (**Fig. 10A**).

In the case of MSC-naïve, a significant increase in gene expression of the immunomodulatory molecules *iNOS2* at T1 and *COX2* at T6 was observed in the MHC-matched group over the MHC-mismatched group ($p < 0.05$) (**Fig. 10B**). Interestingly, none of the three cell types (MSC-naïve, MSC-primed, and MSC-chondro) showed a significant upregulation of immunomodulatory molecules in the MHC-mismatched group compared to the MHC-matched group. However, when analyzing the expression of immunogenic molecules in the MSC-naïve groups, a significant increase in the expression of *CD40*, *CD80*, and *MHC-II* at T2, *MHC-I* at T4, and *MHC-II* at T5 was observed in the MHC-mismatched group compared to the MHC-matched group ($p < 0.05$ at all time points) (**Fig. 10B**). While MHC-matched groups showed upregulation of immunomodulatory

molecule expression, MHC-mismatched groups exhibited increased expression of immunogenic molecules.

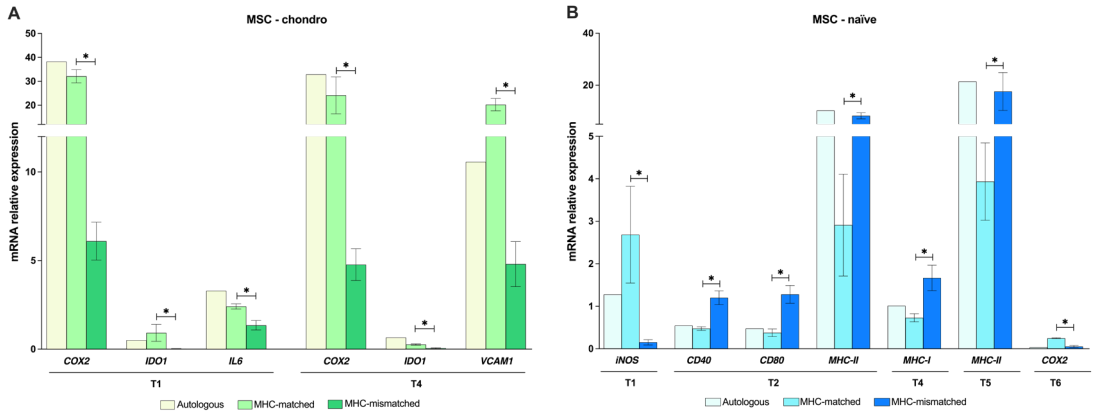


Fig. 10. Changes in immunomodulatory and immunogenic expression by MSC-chondro (A) and MSC-naïve (B) groups following administration to autologous, MHC-matched and MHC-mismatched horses. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using each baseline MSCs type as reference sample (value 1). Significant differences between groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$).

3.2 Changes induced by MSCs on recipient immune response

The local immune response induced by MSC-MSC-naïve, MSC-primed or MSC-chondro conditions was evaluated after each MSCs type was encapsulated in alginate and placed 3 scaffolds/recipient subcutaneously in autologous, and MHC-matched or mismatched horses and recovered at different time-points. The analysis of all retrieved implants revealed the presence of MSCs embedded within the scaffolds. While there were instances where complete removal of the implants was not possible in certain horses, fragments of alginate were consistently identified during the examination of the capsules. These findings confirm the persistent integration of MSCs within the scaffolds throughout the study period.

3.2.1 Influence of MSCs type on local immune response over time

The effect of the type of MSCs received (MSC-naïve, MSC-primed and MSC-chondro) showed relevant differences when lymphocytes and eosinophils were analyzed. In contrast, the different types of MSCs administered induced the same immune response in terms of vascular proliferation, fibroblast proliferation, hemorrhage, presence of PMN and histiocytes.

Our findings demonstrate a significant impact of *in vivo* administration of MSCs on lymphocyte levels in both MHC-matched and mismatched horses. Specifically, in MHC-matched animals, MSC-primed and MSC-chondro attracted a greater number of lymphocytes compared to MSC-naïve. Conversely, in the MHC-mismatched groups, MSC-primed exhibited the lowest lymphocyte scores after the first administration. However, a notable early increase in lymphocytes induced by both allogenic MSC-primed compared to MSC-naïve and MSC-chondro was observed after re-exposure at T4. Importantly, this

increase was statistically significant only in the MHC-mismatched group ($p < 0.05$ in both cases) (**Fig. 11B**).

Following the initial exposure, MSC-naïve induced the highest scores of eosinophils in both allogeneic groups. This increase was statistically significant at T2 compared to MSC-chondro in the MHC-mismatched group ($p < 0.05$), at T3 compared to MSC-chondro in the MHC-matched group ($p < 0.05$), and also at T3 compared to MSC-primed in the MHC-mismatched group ($p < 0.05$) (**Fig. 11C**, **11D**). However, after the second administration, a significant decrease in eosinophil scores was observed in MSC-naïve scaffolds compared to MSC-primed ($p < 0.05$) and MSC-chondro ($p < 0.01$) in the MHC-matched groups (**Fig. 11C**). In contrast, in MHC-mismatched groups, there was a decrease in eosinophil scores in MSC-primed scaffolds at T4 compared to both MSC-chondro and MSC-naïve ($p < 0.01$ in both cases) (**Fig. 11D**).

Both allogenic MSC-chondro scaffolds induced the highest lymphocyte scores at T2 and T5 compared to all other time points, but the increase was statistically significant only for MHC-matched group at T4 ($p < 0.05$ over both time points) (**Fig. 11A**). The same significant trend was observed in MSC-naïve scaffolds at T2 and T5 compared to T4 ($p < 0.05$ in both cases) in MHC-mismatched horses (**Fig. 11B**). Regarding MSC-primed scaffolds, lymphocytes were significantly increased in MHC-mismatched horses at T4 and T5 over T1 ($p < 0.05$ in both cases) and at T6 over T3 ($p < 0.05$) (**Fig. 11B**).

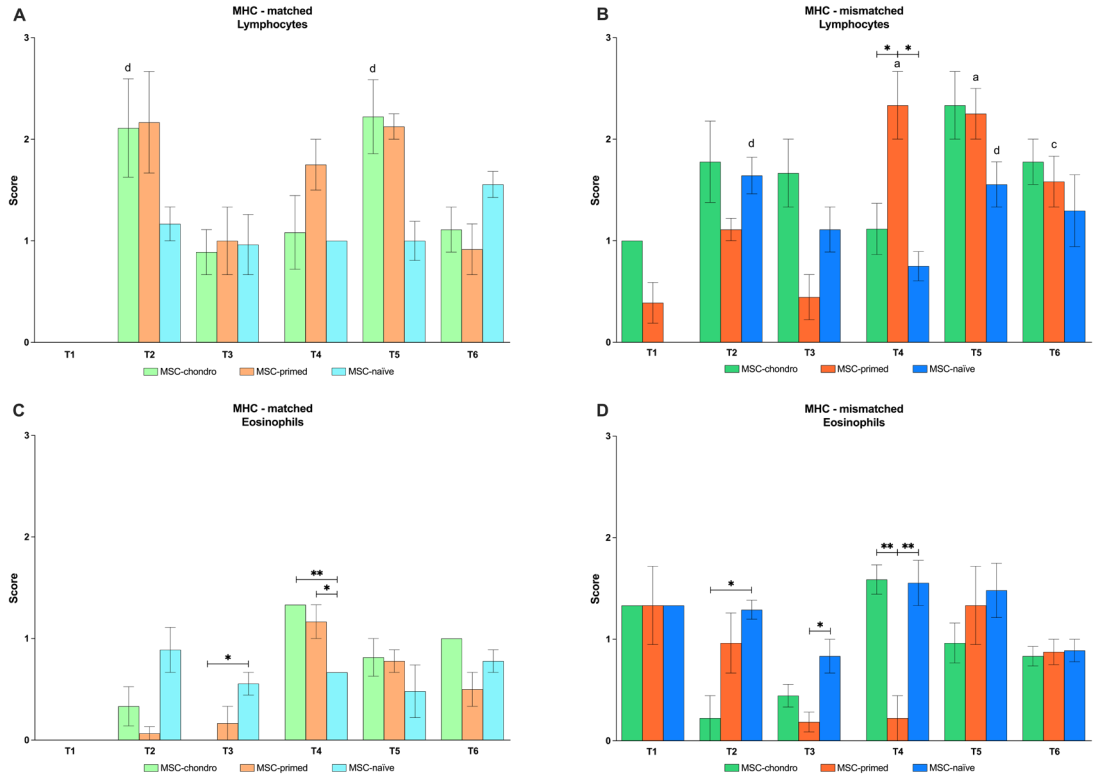


Fig. 11. Evolution of lymphocytes and eosinophils scores along the time in MHC-matched (A, C) and MHC-mismatched (B, D) recipients following the administration of different MSCs types. Scores are normalized over acellular control (value 1) and represented as mean ± S.E.M. Significant differences between groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). Significant differences between time-points compared to T1, T3 and T4 are represented by lower case letter [a], [c] and [d], respectively ([a], [c], [d], $p < 0.05$).

Fibrosis induced by MSCs scaffolds reached higher values than acellular controls in the second implants after both administrations (T2 and T5) and combinations. After the first administration, the observed increase in fibrosis scores at T2 in all MSCs implants was only significant compared to T3 in MHC-matched groups that received MSC-chondro and MSC-naïve scaffolds ($p < 0.05$ in both cases) (**Fig. 12A**).

After re-exposure, the MSCs scaffolds exhibited an overall increase in fibrosis scores at T5, although this increase was less pronounced than at T2, and the difference was statistically significant in the MHC-matched group that received MSC-naïve implants ($p < 0.05$) (**Fig. 12A**). Additionally, the fibrosis increase observed at T5 compared to T4 in all MSC types and allogenic settings was also statistically significant in both MSC-chondro groups ($p < 0.05$ in both cases) (**Fig. 12A**, **12B**). Similar to the observations after the first administration, a decrease in fibrosis scores was observed at the third scaffold (T6) compared to the second scaffold (T5), and this decrease was statistically significant for all MHC-mismatched horses ($p < 0.05$ in all cases) (**Fig. 12B**) and for MSC-chondro in the MHC-matched group ($p < 0.05$) (**Fig. 12A**).

The presence of PMN increased after the second administration (T4) for all cell types and combinations but decreased over time below acellular controls. Notably, a significant decrease in PMN cells induced by MSC-naïve was observed at T5 ($p < 0.01$) and T6 ($p < 0.05$) compared to T4 in MHC-mismatched group (**Fig. 12D**). This decreasing trend compared to T4 was also observed in MSC-chondro scaffolds at T5 in MHC-matched horses ($p < 0.05$) and at T6 in MHC-mismatched horses ($p < 0.05$). Moreover, this decrease was also significant in MSC-primed scaffolds administered to both allogeneic groups at T4 compared to T5 ($p < 0.05$ in both cases).

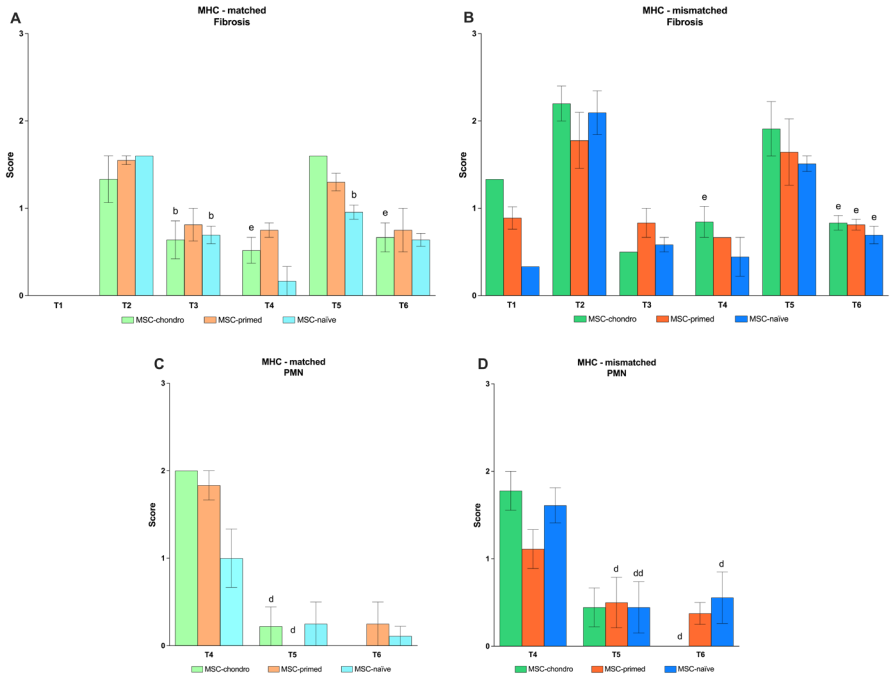


Fig. 12. Evolution of fibrosis and polymorphonuclear (PMN) cells scores along the time in MHC-matched (A, C) and MHC-mismatched (B, D) recipients following the administration of different MSCs types. Scores are normalized over acellular control (value 1) and represented as mean ± S.E.M. Significant differences between time-points compared to T2, T4 and T5 are represented by lower case letter ^{b}, ^{d} and ^{e}, respectively (^{b}, ^{d}, ^{e}, $p < 0.05$).

3.2.2 Influence of MHC compatibility in the local immune response

When comparing the allogeneic groups, MSC-chondro demonstrated a significant increase in PMN scores at the second (T2) and third (T3) scaffolds during the first administration in the MHC-mismatched group compared to MHC-matched scaffolds ($p < 0.05$ in both cases). Additionally, MSC-chondro significant increase histiocyte scores in the MHC-mismatched horses at T2 and T6 compared to the MHC-matched group ($p < 0.05$ in both cases). Furthermore, the observed low levels of hemorrhage and fibrosis at T2 caused by MSC-chondro in the MHC-matched group were significantly different from the MHC-mismatched group ($p < 0.05$ in both cases) (**Fig. 13A**).

Similarly, consistent trends were observed in MSC-naïve scaffolds, where the observed decrease in hemorrhage at T2 and T4 in the MHC-matched group was statistically significant compared to the corresponding scaffolds in the MHC-mismatched group ($p < 0.05$ in both cases). In terms of multinucleated cell presence, a significant increase was observed in MSC-naïve scaffolds of the MHC-mismatched group compared to the MHC-matched group at T2 and T3 ($p < 0.05$ in both cases) (**Fig. 13C**).

Additionally, at T3, MSC-naïve scaffolds administered to the MHC-matched group induced significant vascular proliferation compared to the MHC-mismatched group ($p < 0.05$). After re-exposure to MSC-naïve scaffolds, the MHC-mismatched groups exhibited higher eosinophil scores at T4 and T5 compared to the MHC-matched groups ($p < 0.05$ in both cases). Notably, at T5, the MHC-mismatched group also displayed more fibrosis than the MHC-matched group when MSC-naïve scaffolds were administered ($p < 0.05$ in both cases) (**Fig. 13C**).

Regarding MSC-primed scaffolds, a significant increase in histiocytes scores was observed after both administrations in MHC-mismatched horses, and this increase was significantly higher than the MHC-matched scores at T2 and T5 ($p < 0.05$). However, different patterns were observed in the other parameters among horses that received MSC-primed scaffolds. When administered in MHC-mismatched horses, MSC-primed scaffolds had minimal impact on the presence of multinucleated cells, whereas in MHC-matched horses, a significant increase was observed at T3 ($p < 0.01$), T5 ($p < 0.05$), and T6 ($p < 0.05$) (**Fig. 13B**). Furthermore, a significant increase in eosinophils was observed after the second exposure at T4 in MHC-matched horses compared to MHC-mismatched groups ($p < 0.05$). Additionally, MSC-primed scaffolds induced less hemorrhage at T6 in the MHC-mismatched group compared to MHC-mismatched scaffolds ($p < 0.05$) (**Fig. 13B**).

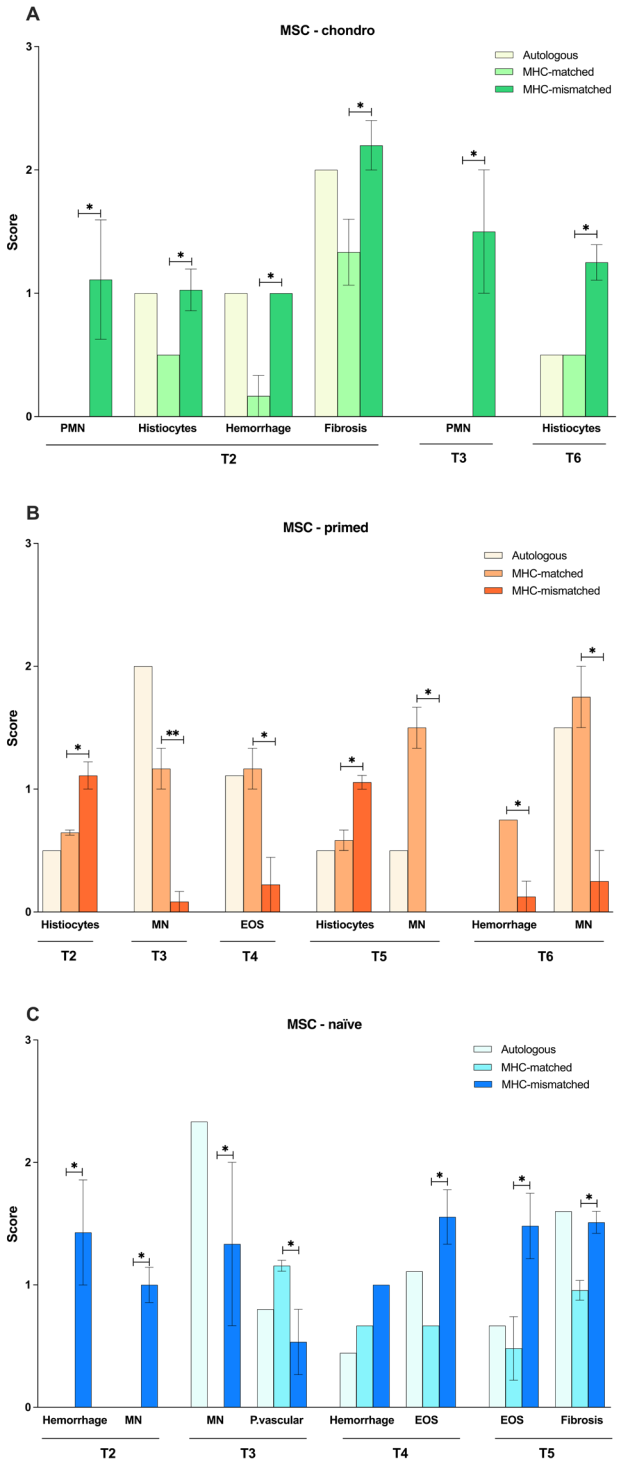


Fig. 13. Differences in polymorphonuclear (PMN) cells, histiocytes, hemorrhage, fibrosis, multinucleated (MN) cells, eosinophils (EOS) and vascular proliferation scores along in autologous, MHC-matched and MHC-mismatched recipients following the MSC-chondro (A), MSC-primed (B) and MSC-naïve (C) administrations. Scores are normalized over acellular control (value 1) and represented as mean ± S.E.M. Significant differences between groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$).

Histological analysis of lymphocytes, fibroblastic proliferation and synovial metaplasia showed no significant differences in the three types of MSCs in any of the implants analyzed.

4 Discussion

The immunomodulation and immunogenicity of equine MSCs play a crucial role in their therapeutic effectiveness and ability to avoid detection by the immune system during allogeneic administration (Voga et al. 2020). The immune properties of MSCs are heavily influenced by their surroundings, and the response to these changes can either improve their potency (i.e., increased regulatory capacity) or compromise their safety and effectiveness (i.e., immune targeting and elimination) (Berglund et al. 2017).

The use of encapsulated MSCs has been recently proposed as a treatment strategy for focal joint defects in equine and other species *in vivo* (Maihöfer et al. 2021; Ribitsch et al. 2021). This approach not only provides a potential therapeutic benefit, but it also enables the analysis of changes that occur to MSCs post-administration. Understanding how MSCs respond to various stimuli is critical for optimizing cell therapies for veterinary and human patients. Although many studies have explored the effects of licensing MSCs with different cytokines and ligands and their impact on various immune cell populations, little is known about how immune cells cause changes in equine MSCs *in vivo* that may have therapeutic implications.

To the best of authors' knowledge, this is the first study conducted in equine species that evaluates both the local immune response of the recipient and the reaction of MSCs after repeated administration to autologous and allogeneic MHC-matched and mismatched recipients.

Sodium alginate has been utilized in numerous studies as a synthetic extracellular matrix to encapsulate cells (Ho et al. 2022), and it is deemed a viable option for encapsulating MSCs for further analysis. It is worth noting that mammalian cells are unable of interacting with the polysaccharides present in alginate, which is favorable for maintaining the hydrogel's integrity and confining the MSCs within it.

However, this property also enables the transfer of nutrients and metabolites between the organism and the encapsulated cells, facilitating investigation into the interplay between the animal's immune response and the responses of the MSCs (Watts et al. 2013). In line with previous research, our study found that the porosity of the alginate implants facilitated fluid absorption, nutrient diffusion, and did not impact MSCs viability (de Vries-van Melle et al. 2014; Santos et al. 2018; de Souza et al. 2021). Additionally, several authors concur that encapsulating MSCs within an alginate scaffold is critical to maintaining cell retention at the site of injury and shielding them from an allogeneic immune response *in vivo* (Ryan et al. 2014).

Prior to engaging into further discussion of our results, it is important to mention the limitations of this study. Importantly, the aim of our work was to evaluate the local immune response of the recipients to MSCs and the changed in the MSCs gene expression following *in vivo* administration. To retrieve the MSCs, a minor subcutaneous lesion was created to place the MSCs scaffolds in each horse. The response of the recipients to the subcutaneous administration of the implants was similar to the response following an injury and subsequent healing of a cutaneous wound. Consequently, several of the patterns

initially observed were due to the natural tissue repair process, including hemostasis, inflammation, proliferation, and remodeling. While the study did not intend to evaluate the response to therapeutic treatment with MSCs, the potential effect should not be overlooked. To avoid the "scaffold effect" and "natural wound healing effect", acellular implants were used to isolate the "MSCs effect" in the recipient horses. Despite this, the differences observed in tissue repair parameters, regulated by MSCs, could be used in future studies to evaluate the therapeutic effect.

The limited sample size was a potential factor that could impact the statistical analysis results, as it was difficult to purchase MHC-homozygote MSCs donors due to the high variability of MHC-haplotypes (Sadeghi et al. 2018). In addition, should be considered the challenges of working with a large species like horses, considering their unique requirements and size. To this consideration must be added the hierarchical and social behaviour of the horses, which meant that 8 of the implants could not be recovered. Although efforts were made to keep the disruptive horses separate, it was too late for some of the implants.

Despite the similarity in age, origin, breed, and weight of all the horses involved in this study, there was significant inter-individual variability in gene expression. To mitigate this issue, the findings were normalized based on the gene expression of each donor, which was assessed after analyzing the control implants.

It should be noted that while scaffolds can prevent immune cell infiltration into the alginate structure, they can still infiltrate around the alginate capsules, leading to the formation of a fibrotic capsule (Doloff et al. 2017). This capsule could impede nutrient and oxygen transport to the alginate microstructure, resulting in reduced viability of encapsulated cells. However, some studies have shown that encapsulated MSCs can survive for weeks *in vivo* in both equine and murine species (de Vries-van Melle et al. 2014; Santos et al. 2019). Previous studies have also found that larger and rounder implants can attenuate foreign reactions and fibrosis (Veiseh et al. 2015). Therefore, strategies to reduce the fibrotic capsule and improve nutrient and oxygen transport may enhance the therapeutic potential of encapsulated MSCs. Under the conditions of our study, autologous and MHC-matched horses did not develop fibrous capsule after the first administration (T1). However, in the acellular and MHC-mismatched groups, regardless of the type of MSCs administered, a fibrous capsule was observed at T1. The development of a fibrous capsule, although it may seem to influence MSCs gene expression, was not found to be correlated with the observed gene overexpression at different time points.

The evaluation of gene expression showed that MSC-chondro and MSC-naïve tended to increase the expression of immunomodulatory genes, indicating a potential for a better therapeutic effect with these MSC types. Furthermore, the results suggested that MSC-chondro have immunomodulatory properties, contrary to previous studies in horses (Van Hecke et al. 2021) and rodents (Ryan et al. 2014). However, the observed decrease in *COX2* and *IL6* expression after T1 in MSC-chondro suggested that the immunomodulatory properties of differentiated MSCs may not be sustained over time.

It is notable that many of the MSC types and combinations showed an overexpression of immunomodulatory genes following each administration (T1 and T4), but this tended to decrease over time when compared to the basal scaffolds. These observations suggest that the downregulation of immunomodulatory genes may be due to inconsistent exposure to an inflammatory environment, as many of these genes are typically induced by inflammation, as we observed at T1 and T4.

Although previous *in vitro* studies have shown an increase in *COX2*, *iNOS2*, and *IL6* expression in MSCs after cytokine priming (Cassano et al. 2018a; Caffi et al. 2020; Connard et al. 2021; Lee et al. 2021), our *in vivo* study found that MSC-primed downregulated the expression of most immunomodulatory genes over time in both allogenic groups. These results suggest that the effect of pro-inflammatory cytokine treatment may decrease over time after MSC-primed administration *in vivo*. Another possible explanation could be that MSC-primed did not encounter a sufficiently inflammatory environment, as seen in injury situations described by other authors (Barrachina et al. 2018a).

In agreement, the histological analysis results indicate that the local immune response induced by MSCs was not relevant. However, the *in vivo* administration of MSCs had an impact on lymphocyte scores, and different types of MSCs may have varying effects on lymphocytes over time. Interestingly, lymphocyte arrival was earlier in horses that received allogeneic MSC-primed scaffolds after the second administration. In the typical order of arrival of immune cells after an injury, neutrophils and macrophages arrive first, and lymphocytes arrive later (Short et al. 2022). The lymphocytes increase observed in horses that received MSC-primed may be attributed to a combination of factors, including the low expression of immunomodulatory genes such as *IL6* and *COX2*, which are responsible for inhibiting T cell proliferation by equine MSCs (Carrade Holt et al. 2014), as well as increased immunogenic properties.

When analyzing immunogenic markers, it is important to consider the type of MSCs and their compatibility with the donor, as these factors could be related to the recognition of MSCs and the generation of immune response after repeated administration (Schnabel et al. 2014; Berglund y Schnabel 2017; Kamm et al. 2021). While MSC-chondro and MSC-naïve showed the highest expression, the lower change expression of *CD80* and *MHC-II* in MSC-naïve suggests they may be more immune evasive. On the other hand, MSC-chondro and MSC-primed showed an upregulation of *MHC-II* expression throughout all scaffolds, with the highest expression occurring at later times.

This suggests that the immunomodulatory effects of MSCs occur earlier, followed by immunogenic effects later, supporting the concept that MSCs are immune evasive. Additionally, these findings reject the idea that fibrous capsules prevent MSCs from interacting with the recipient as the highest expression of immunogenic genes was not at T1 and T4.

Based on the findings of our study, it appears that MSCs priming can enhance their ability to evade the immune system (Ankrum et al. 2014; Berglund et al. 2017). Even immune evasion would be more efficient by MSC-naïve and MSC-primed applied in MHC-

matched horses. However, it is important to note that the downregulation of immunomodulatory genes in MSC-primed may not be entirely adequate to achieve optimal immune evasion.

In terms of compatibility, the MHC-matched group showed an upregulation of immunomodulatory genes in both MSC-naïve and MSC-chondro, while the MHC-mismatched group exhibited an upregulation of immunogenicity genes in MSC-naïve and MSC-primed. However, there were no significant differences in MSC-primed gene expression based on the type of receptor (**Resource 4**, **Annex VI**). Therefore, under the conditions of this study, it seems that MHC compatibility did not have a substantial impact on the immunogenicity of MSC-chondro.

The overexpression of immunogenic markers and the induced local immune response in MSC-naïve MHC-mismatched group compared to MHC-matched group could be observed after both administrations, which would make them less immune evasive. Hence, upon re-exposure, the administration of MSC-naïve scaffolds resulted in the most pronounced increase in eosinophilic score within the MHC-mismatched group. While eosinophils play a beneficial role in tissue engineering by promoting wound healing (Weller y Spencer 2018), it is worth noting that they can also contribute to the expansion of B cells. Additionally, in both mice and humans, eosinophils also support increased numbers of peripheral B cells; and in mice, eosinophils promote B cell survival, proliferation and immunoglobulin secretion by a contact-independent mechanism(Weller y Spencer 2018). Moreover, in a previous investigation, the intra-articular administration of MSC-naïve was conducted in both MHC-matched and MHC-mismatched horses, resulting in an increased humoral and local immune response (Rowland et al. 2021). This finding further emphasizes the significance of MHC compatibility when implementing therapy using MSCs of this type.

Nevertheless, it is noteworthy that there was a significant increase in MHC-I expression after the first administration of MSC-naïve, even in cases where the recipient was MHC-matched. This finding was unexpected, as it suggests that the recipient's immune system recognized the MSCs as foreign. This increase in *MHC-I* expression could be attributed to the local inflammatory response following the surgical procedure, which activates the expression of this marker. However, upon re-exposure at T4, the MHC-matched group exhibited downregulated *MHC-I* expression, indicating a potential development of immune tolerance over time (Schnabel et al. 2014).

Regarding *MHC-II* expression, our study revealed an upregulation over control scaffolds after the first administration for all types of MSCs and conditions. Interestingly, the highest expression was observed in the final implants (T5 and T6) in the MHC-mismatched combinations of MSC-primed and MSC-naïve. This progressive increase in *MHC-II* expression over time suggests a potential role in triggering immune responses, as *MHC-II* has greater immunogenic potency compared to *MHC-I* and is associated with lower immune tolerance (Berglund et al. 2017).

Previous *in vitro* studies have emphasized the importance of selecting MSCs donors with low *MHC-II* expression to minimize immune response (Schnabel et al. 2014). However,

our *in vivo* findings demonstrate that MHC expression in MSCs may undergo changes after administration. Our findings also suggest that MHC expression in MSCs can be influenced by the local inflammatory environment and may not solely reflect the baseline characteristics of the donor cells. It should be noted, however, that not all gene expression detected through RT-qPCR necessarily corresponds to the secretion of the respective molecule by MSCs (Cassano et al. 2018a).

While interesting variations in the expression of immunomodulatory and immunogenic genes were observed, these changes did not have a significant impact on the local immune response of the recipient horses when comparing the three types of MSCs. However, when evaluating histological parameters in the allogeneic groups over time, it was noticeable that the local response elicited by MSCs administered to MHC-matched horses was comparatively lower in MSC-naïve and MSC-chondro scaffolds. These findings suggest that MHC compatibility may play a role in attenuating the local immune response induced by MSCs, particularly with the use of MSC-naïve and MSC-chondro in MHC-matched settings.

It is noteworthy that during the time-points characterized by increased fibrosis (T2 and T5), MHC-mismatched horses displayed higher levels of fibrosis compared to MHC-matched horses. This observation, particularly in MSC-naïve MHC-mismatched scaffolds at T5, suggests that the immunogenicity of MSCs may be heightened upon re-exposure, leading to a more pronounced local response in the recipient.

Neutrophils, as the first line of defense in the innate immune system, are known to play a crucial role in the immune response (Short et al. 2022). In our study, it was observed that most MHC-mismatched scaffolds attracted a higher number of neutrophils compared to MHC-matched implants across various time points. However, it is important to note that all autologous and allogenic groups induced higher neutrophil scores at T4 compared to the acellular control, indicating the potential chemoattractive properties of MSCs at the site of injury. Previous studies also reported the presence of neutrophils surrounding alginate scaffolds (Rezaa Mohammadi et al. 2018). However, when considering the other time points analyzed in our study, the neutrophil scores were generally lower compared to the acellular scaffolds. In the study conducted by Mohammadi et al. (2018), it was suggested that macrophages and neutrophils played a significant role in the formation of a thick fibrotic layer around alginate scaffolds in immunocompetent mice. In our study, a reduced histiocyte reaction was observed in MHC-matched horses that received MSC-chondro and MSC-primed compared to MHC-mismatched and acellular horses. This finding indicates that MHC-mismatched MSCs exhibited less control over the histiocyte reaction, suggesting a potential limitation in their ability to regulate this immune response.

Among the three MSCs types evaluated in this study, MSC-chondro demonstrated the most favorable immunomodulatory profile. Interestingly, administering MSC-chondro to MHC-mismatched horses did not lead to an increase in their immunogenicity, indicating that MHC-mismatched administration is a viable option for these cells. However, it is

worth noting that MHC matching did reduce the local immune response induced by MSC-chondro after administration.

In contrast, MSC-naïve administered to MHC-mismatched horses displayed a higher immunogenic profile and a lower immunomodulatory profile. Moreover, the MHC-mismatched group exhibited a stronger local immune response after both administrations. Based on these findings, it is recommended to prioritize MHC-matched donors when considering MSC-naïve administration to optimize the immune response.

Interestingly, MSC-primed administration triggered an early immune response accompanied by a decrease in the immunomodulatory profile and an increase in the immunogenic profile. These results suggest that MSC-primed cells may have a more complex immune response compared to the other types evaluated.

5 Conclusions

Overall, the study highlights the importance of considering MHC matching in MSC administration. MSC-chondro may be a suitable option for MHC-mismatched scenarios, while MSC-naïve administration should prioritize MHC-matched donors to minimize immune reactions. Further research is needed to fully understand the immunological implications of MSC-primed administration and to optimize the selection of MSCs for allogeneic cell therapies.

These findings underscore the necessity for in vivo investigations to elucidate the intricate interactions between MSCs and the recipient's immune system. Such studies are crucial for the development of safer and more effective cell therapies. Further research is needed to explore additional factors and mechanisms that could contribute to the immune evasiveness of MSCs and optimize their therapeutic potential.

6 Statements and Declarations

6.1 Acknowledgments

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6.3 Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

6.4 Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection, analysis and interpretation of data were performed by Alina Cequier, Mª Belén Serrano, Mª Teresa Soler-Monsó and Laura Barrachina. The first draft of the manuscript was written by Alina Cequier and Mª Belén Serrano and all authors commented on previous versions of the manuscript. Clinical support and sample collection were performed by Antonio Romero, Arantza Vitoria, Sara Fuente and Francisco José Vázquez. Elvira Bernad and Mirta García-Martínez provided technical support. Antonio Romero, Francisco José Vázquez, Jorge Albareda and Clementina Rodellar provided important conceptual guidance. Clementina Rodellar and Pilar Zaragoza were responsible for obtaining funding.

All authors approved the final version of the article to be published and agree to be accountable for all aspects of the work in ensuring that accuracy or integrity of any part of the work are appropriately investigated and resolved.

6.5 Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

6.6 Ethics approval

The animal study protocol was approved by the Ethic Committee for Animal Experiments of the University of Zaragoza (Project License PI 15/16, 27th May 2016). The care and use of animals were performed in accordance with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for scientific purposes.

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MANUSCRITO V

Effect of inflammation, differentiation, and MHC compatibility on the immunogenicity of allogeneic equine mesenchymal stem cells (MSCs)

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Abstract

Background: The effectiveness and safety of mesenchymal stem cells (MSCs) in therapy can be affected by their immunomodulation and immunogenicity, making them crucial factors to consider. The development of an immune response after the allogeneic administration of MSCs has been demonstrated in different species, including horses, which is highly relevant both as patient and as translational model. Further understanding of allogenic MSCs interactions with the immune system *in vivo* is required as these interactions can be influenced by factors like matching or mismatching of donor-receptor major histocompatibility complex (MHC) and the levels of MHC expression, which can vary under different circumstances.

Methods: The study investigated the immunogenicity of equine MSCs in basal conditions (MSC-naïve), pro-inflammatory primed (MSC-primed) or chondrogenically differentiated (MSC-chondro) on relevant lymphocyte subpopulation after co-cultures, in both MHC-matched and MHC-mismatched allogeneic settings, before and after two administrations of each MSCs condition *in vivo*. Assessing the overall cellular immune response systemically and the development of memory cells by re-exposing the lymphocytes of each recipient to the specific type of MSCs they received from their donors.

Results: Helper and cytotoxic T lymphocytes proliferation was observed in response to all types of MHC-mismatched MSCs, with a stronger and continuous effect in MSC-primed co-cultures, as well as in MSC-naïve after the second administration. In MHC-mismatched co-cultures, both MSC-chondro and MSC-naïve promoted B lymphocyte activation, while both allogenic MSC-primed had less effect on B cells over time.

Conclusions: While MSC-primed showed enhanced immunogenicity by promoting sustained proliferation of helper and cytotoxic T cells, their immunomodulatory properties would allow them to prevent the induction of a humoral immune response. Importantly, equine MSCs do not lose their regulatory ability neither increase their immunogenicity after chondrogenic differentiation but have reduced capacity to stimulate Treg cells and can stimulate the proliferation of B cells. The immune response elicited against equine allogeneic MSCs and the factors influencing it must be further studied to develop more effective and safer cell therapies.

Keywords: horse, allogeneic, immunogenicity, flow cytometry, immune response, co-culture, major histocompatibility complex, repeated administration, haplotype, mixed lymphocyte reaction.

Background

Mesenchymal stem cells (MSCs) are adult multipotent stem cells that have attracted significant interest in the field of regenerative medicine due to their distinct biological properties (Voga et al. 2020; Alvites et al. 2022). Since the underlying causes of certain diseases are similar in both humans and animals, the findings obtained from MSCs research can be valuable for both human and veterinary patients, promoting the concept of "One Medicine, One Health" (Lo Monaco et al. 2018; Fortier et al. 2020). Horses are particularly significant in translational medicine for the treatment of various pathologies, particularly those impacting the musculoskeletal system (Ribitsch et al. 2020; Jiang et al. 2021; Knott et al. 2022). Furthermore, the paracrine activity of MSCs, encompassing their ability to modulate the immune system, has broadened the scope of their possible application to include pathologies with inflammatory and immune involvement (Mishra et al. 2020). Various research studies focusing on the treatment of diverse diseases in horses, including asthma, metabolic syndrome, abnormal wound healing, and endotoxemia, have yielded an interesting results with potential applications in human medicine (Lange-Consiglio et al. 2019; Cequier et al. 2021).

In order to promote One Medicine strategies in the regenerative medicine field, further investigation into the immune properties of equine MSCs is necessary. The immune regulatory mechanisms of MSCs primarily rely on their paracrine activity (Voga et al. 2020; Foo et al. 2021) which is not only associated with their therapeutic effects but also with their ability to evade immune recognition when administered allogenically. It is now widely agreed that MSCs are not truly immune-privileged but that they are immune-evasive. This emphasizes the need of considering their recognition and elimination by the immune system in the allogeneic setting (Berglund et al. 2017). Allogeneic application presents several advantages over autologous therapy, including the potential for faster and wider availability of well-characterized MSCs, particularly when autologous cells are not suitable (Kot et al. 2019; Aldrich et al. 2021; Berglund et al. 2021).

Thus, the development of safe and effective allogeneic treatments requires further investigation into the immunogenicity of MSCs. In both human and horses, it has been reported that allogeneic MSCs may trigger cellular and humoral immune responses, which can limit their therapeutic effects and potentially lead to adverse reactions (García-Sancho et al. 2017; Rowland et al. 2021). Additionally, the development of immune memory mechanisms could influence the outcome of repeated administration of allogeneic MSCs (García-Sancho et al. 2017; Barrachina et al. 2020). The matching or mismatching for the major histocompatibility complex (MHC) influences the recognition of the donor's stem cells by the recipient's immune system. However, the MHC matching/mismatching is not always taken into account when designing studies or clinical trials, but it has been reported as a potential key factor in several species, including

human, horses and other species (Isakova et al. 2014; Schnabel et al. 2014; Rowland et al. 2018; Kot et al. 2019).

In recent years, there has been a growing interest in the establishment of haplobanks using human-induced pluripotent stem cells (iPSCs)(Sullivan et al. 2020), and similar strategies have also been employed in equine MSC studies (Rowland et al. 2021; Cequier et al. 2022a). This approach involves selecting homozygous individuals as donors, enabling compatibility with a wider range of heterozygous individuals. This strategy avoids the potential production of cellular and humoral response specifically targeting the human (HLA) or equine leukocyte antigen (ELA) carried by the MSCs that could compromise their efficacy *in vivo* (García-Sancho et al. 2017; Rowland et al. 2021; Sanabria-de la Torre et al. 2021). Furthermore, such recognition can be more or less easily triggered by the expression level of the MHC, which can be intrinsic to the donor and/or modified by different factors, like inflammation and differentiation (Ryan et al. 2014; Barrachina et al. 2020).

However, the clinical relevance of the immune recognition of allogeneic MSCs remains to be fully elucidated and research conducted in horses not only facilitates the optimization of treatments in equine species but also offers valuable insights into the immune response to allogeneic MSCs. Such studies hold significant translational value for advancing medical knowledge and cellular therapies. Actually, the administration of allogeneic MSCs has demonstrated safety and effectiveness in various conditions (Ardanaz et al. 2016; Kot et al. 2019; Rowland et al. 2021; Van Hecke et al. 2021). This suggests that there may be a balance between the immunogenicity and the immunomodulatory properties of allogeneic MSCs, which helps them evading the immune system and exerting their therapeutic effects (Ankrum et al. 2014). Therefore, it is crucial to understand how different factors like MHC matching/mismatching, inflammation, or MSC differentiation, can modify this balance and their contribution to the immune recognition of MSCs. Furthermore, the implications in the *in vivo* scenario need to be clarified in order to advance the development of allogeneic cell therapies, to which it can greatly contribute investigating in veterinary species.

To better understand the fate of MSCs when they interact with the immune system *in vivo*, we aimed at assessing the effect of pro-inflammatory priming, chondrogenic differentiation and MHC compatibility on the systemic immune response to MSCs after single and repeated administration. Specifically, this study evaluated the effect of MSCs under different conditions (MSC-naïve, MSC-primed, and MSC-chondro) on relevant lymphocyte subpopulation after co-cultures, in both MHC-matched and MHC-mismatched allogeneic settings, before and after two administrations of each MSCs condition *in vivo*.

Priming equine MSCs with pro-inflammatory cytokines like interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) can enhance their immunomodulatory properties, leading to improved regulatory effects (Cassano et al. 2018; Caffi et al. 2020). However, this process can also increase their immunogenicity by inducing MHC expression, limiting their allogeneic use (Ankrum et al. 2014; Barrachina et al. 2018a). In equine

MSCs, it has been reported that low doses of IFNγ and TNFα during short priming periods allows maintaining a balance between the immunomodulatory and immunogenic profiles *in vitro* (Barrachina et al. 2017).

Differentiation of MSCs has been suggested to decrease their regulatory capabilities and increase their immunogenicity, as they would lose their 'stemness' when they become a specialized cell type (Lohan et al. 2014; Ryan et al. 2014). An increase in the expression of MHC-I and/or MHC-II could pose a greater risk of allo-recognition if MSCs are administered in a differentiated state (Barrachina et al. 2018b), especially if their regulatory abilities are also depleted during differentiation.

Considering the possible effect of different factors, our hypothesis was that the immunomodulatory capacity of MSCs induced by proinflammatory cytokines would allow them to evade the recipient's immune system regardless of MHC compatibility. Conversely, the differentiation of equine MSCs into chondrocytes may affect their immunomodulatory properties. This process could potentially trigger an immune response against the differentiated cells, and the intensity of this response could also be influenced by MHC incompatibility. To the best of the authors' knowledge, this is the first *in vivo* study that simultaneously investigate the effect of all these three factors for MSC administration. Additionally, this study uses a large animal species with higher translational potential, and which can also benefit as patient.

Methods:

Study design:

In this study, MSCs were repeatedly administered *in vivo* in MHC-matched and MHC-mismatched horses and the systemic cellular immune response was analyzed serially in a modified one-way mixed lymphocytes reaction (MLR) system *in vitro*. Bone marrow MSC donors consisted of three horses homozygotes for their MHC haplotypes, specifically HapPRE10/HapPRE10, HapPRE11/HapPRE11, and HapMAI04/HapMAI04 (Cequier et al. 2022a). The recipient horses were categorized into two groups: MHC-matched and MHC-mismatched with the donors. In the MHC-matched group, there were eight heterozygous horses that shared one haplotype with one of the donors: three HapPRE10 heterozygotes, three HapPRE11 heterozygotes and two HapMAI04 heterozygotes. For the MHC-mismatched group, nine horses were selected, all of which carried haplotypes distinct from HapMAI04, HapPRE10, and HapPRE11. Within each group (MHC-matched and MHC-mismatched), animals were arranged in three subgroups (2-3 animals/subgroup) to investigate MSCs in three conditions: basal (MSC-naïve), proinflammatory-primed (MSC-primed) and chondrogenically differentiated (MSC-chondro) MSCs. Furthermore, each homozygous donor (n = 3) also acted as an autologous control (**Fig. 1**).

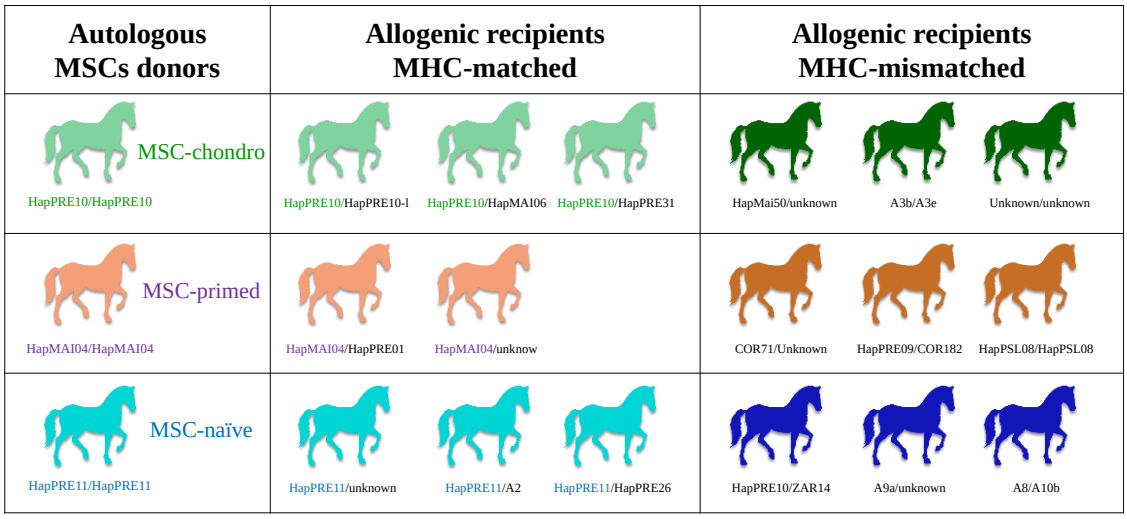


Fig. 1. Study design. Groups of horses (autologous, allogenic MHC-matched and allogenic MHC-mismatched) and types of MSCs received (MSC-chondro, MSC-primed and MSC-naïve). Below each horse its MHC haplotype is indicated.

Each animal received 3 alginate scaffolds placed subcutaneously on the neck and each scaffold contained 5×10^6 MSCs of the corresponding type (MSC-naïve, MSC-primed or MSC-chondro). The scaffolds were extracted at different time-points: 1 (T1), 3 (T2) and 6 (T3) weeks following the implantation. One month after the removal of the last scaffold, each horse was re-exposed to the same type and dose of BM-MSCs (MSC-naïve, MSC-primed and MSC-chondro; MHC-matched or MHC-mismatched) using the contralateral side of the neck and the same assessments were performed at the same time-points (T4: just prior to re-exposure to the same MSCs; T5: one week after the re-exposure; T6: three weeks after the re-exposure and T7: six weeks after the re-exposure). This was done to mimic a second administration in order to explore the effects of immune memory mechanisms that may have developed after the first administration. Schematic timeline and methods are shown in **Fig. 2A**. It should be noted that this route of administration was used to be able to retrieve the implants at the above-mentioned times to study the local immune response (unpublished data).

In this study, a modified one-way MLR assay was used to evaluate the cellular immune response at the systemic level and the possible development of cellular-based immune memory. Briefly, peripheral blood lymphocytes (PBLs) were obtained from all the recipients and re-exposed *in vitro* to the same MSCs administered *in vivo* (MSC-naïve, MSC-primed, MSC-chondro; MHC-matched or MHC-mismatched). In this co-culture system, PBLs were not mitogen-activated to assess the immunogenic potential of MSCs in terms of inducing a proliferative response and stimulating the production of IFNγ by T cells.

To do this, PBLs were stained with carboxyfluorescein succinimidyl ester (CFSE) to evaluate their proliferation after 5 days of co-culture. At this point, PBLs were collected and labelled with a selection of antibodies to analyze the changes in the frequency and proliferation of the main lymphocyte subsets (T cells, cytotoxic and helper cells, regulatory T lymphocytes and B cells) by flow cytometry. At the same time, co-culture

supernatants were collected to determine the secretion of IFNγ by ELISA, serving as an indicator of T cell activation **Fig. 2B**.

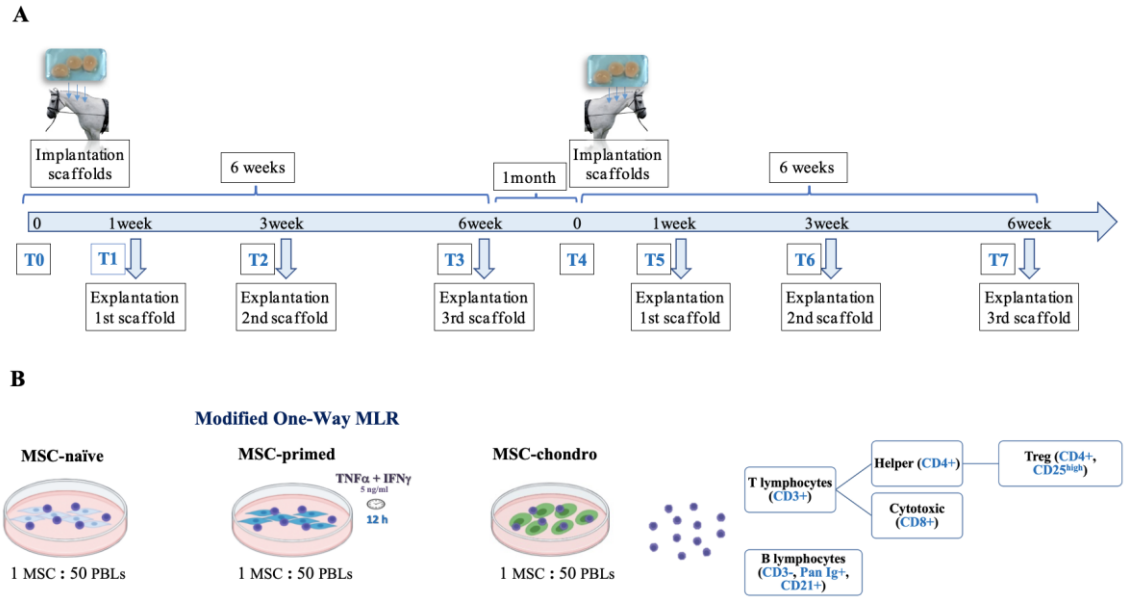


Fig. 2. Schematic representation of the study design (A) showing the three MSCs scaffolds with the same type of MSCs subcutaneously placed in recipient horses. The scaffolds were removed at different time-points (1, 3 and 6 weeks) and one month following the removal of the last scaffold, each animal was re-exposed to the same type of MSCs. Peripheral blood was collected from all recipients at different time-points. (B) Modified one-way mixed lymphocyte reaction (MLR) assays were performed by co-culturing PBLs of recipients with MSC-naïve, MSC-primed and MSC-chondro previously received. After co-culture, PBLs and supernatant were collected to evaluate, the frequency and proliferation of each lymphocyte subset by flow cytometry and the secretion of IFNγ.

Animal selection by MHC-haplotyping

Twenty healthy horses aged between 2 to 12 years (12 geldings, 7 mares and 1 stallion), with no previous history of receiving MSCs or transfusions, were selected based on their MHC haplotypes as determined by microsatellite typing using a validated panel of 10 highly polymorphic intra-MHC regions, as previously described (Sadeghi et al. 2018; Barrachina et al. 2020). The methodology for DNA isolation, multiplex PCRs, and fragment analysis was performed exactly as previously reported by our group (Cequier et al. 2022a).

Definitive haplotypes were established for animals homozygous or with direct relatives (parent-offspring pairs or half-sibling groups) and the remaining animals were assigned with provisional haplotypes based on previously known ones reported in bibliography (Rowland et al. 2018; Sadeghi et al. 2018; Holmes et al. 2019; Barrachina et al. 2020). The premise of using homozygous individuals for matching with different heterozygous individuals is the same proposed to create haplo-banks of human induced pluripotent stem cells (iPSCs) (Sullivan et al. 2020), and also used in equine MSC studies (Rowland et al. 2021; Pezzanite et al. 2022).

All the procedures involving animals were carried out under the Project License PI 15/16 approved by the in-house Advisory Ethics Committee for Animal Research from the

University of Zaragoza. The care and use of animals were performed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63. All animals were kept on paddocks of the facilities of the Animal Research Service of the University of Zaragoza, with free access to water and fed with ad libitum grass hay.

Preparation of experimental MSCs for *in vivo* conditions: MSC-naïve, MSC-primed and MSC-chondro

Equine bone marrow MSCs were obtained and characterized, as previously described (Barrachina et al. 2017) as part of a previous study of our group (Cequier et al. 2022a). In brief, equine MSCs (n = 3) were isolated and expanded at 5,000 cells/cm_2 in conventional MSCs medium consisting in low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/mL streptomycin and 100 U/mL penicillin (all form Sigma–Aldrich) at 37 °C and 5% CO_2.

The MSCs were then suspended at a concentration of 5×10^6 MSCs/ml in phosphate buffered saline (PBS, Gibco, Thermo Fisher) and combined in a 1:1 ratio with a 3% alginate solution. This resulted in a final concentration of 1.5% alginate in a total volume of 2 mL per scaffold as previously described (Watts et al. 2013). Scaffold polymerization was achieved as described earlier (de Vries-van Melle et al. 2014; Leijs et al. 2017; Santos et al. 2018) for 30 minutes into sterile 102 mM $CaCl_2$ (Sigma–Aldrich) at 37 °C in 5% CO_2 conditions. For MSC-naïve and MSC-primed, scaffolds were cultured in conventional MSCs culture medium for 48 hours. For MSC-primed scaffolds, 12 hours before placement, 5 ng/mL of each pro-inflammatory cytokines TNFα and IFNγ (R&D Systems) were added to the conventional MSCs media as described earlier (Barrachina et al. 2018a). For MSC-chondro, chondrogenesis was induced according to the methodology previously described encapsulation (de Vries-van Melle et al. 2014; Ryan et al. 2014) for 21 days.

To ensure the removal of any xenogeneic antigens that might affect the immune response, all scaffolds (MSC-naïve, MSC-primed, and MSC-chondro) were washed twice with PBS and, 24 hours prior to placement, the FBS was substituted with autologous serum obtained from each recipient. This substitution aimed to render the MSCs devoid of xenogeneic antigens (Pezzanite et al. 2015). Subsequently, just before the intervention, scaffolds were again washed three times with PBS.

Autologous, MHC-matched and MHC-mismatched recipients received subcutaneous implantation of three alginate scaffolds containing the same type of MSCs (MSC-naïve, MSC-primed, or MSC-chondro) on the neck (T0). The implants were subsequently removed at specific time intervals, namely 1, 3, and 6 weeks post-implantation (T1, T2, T3). After one month, each animal received a second administration of the same MSCs type on the contralateral neck (T4). Subsequently, the implants were removed at the same time intervals as before (T5, T6, T7). The purpose of this experimental design was to examine the impact of a secondary administration on the studied parameters.

Co-cultures for lymphocyte proliferation assays: Modified one-way MLR

Preparation of MSC-naïve, MSC-primed and MSC-chondro for modified one-way MLR co-cultures

One-way MLRs were conducted before each administration and serially after each one for a total of 8 time-points (T0 to T7). Prior to each time-point, cryopreserved MSCs (*n* = 3) were thawed and seeded at 5000 cells/cm_2 in the basal MSCs medium at 37 °C and 5% CO_2 for 72 h to recover from freezing. Subsequently, MSCs were detached with 0.25% trypsin-EDTA (Sigma–Aldrich) and seeded into a 24-well plate at 20,000 MSCs per well.

The co-cultures were carried out following the methodology previously validated by our group (Cequier et al. 2022a). For MSC-naïve and MSC-primed, plating was done 24 h prior to co-cultures to allow MSCs to attach to the well. For the MSCs-primed condition, MSCs were exposed for 12 h to 5 ng/mL of equine recombinant TNFα (R&D Systems) plus 5 ng/mL of equine recombinant IFNγ (R&D Systems), added to the basal culture medium, right before adding the PBLs.

For MSC-chondro, plating was performed with the same number of cells, but 14 days before the co-cultures to induce differentiation. Chondrogenic differentiation was induced with the StemPro™ Chondrogenesis Differentiation Kit (Thermo Fisher) using the micro-mass system during 14 days (Bussche y Van De Walle 2014), before adding PBLs.

For each donor, two replicates of each MSC type were prepared to run each co-culture with the PBLs of the corresponding recipients in duplicate. Prior to adding the PBLs, wells were washed with PBS.

Isolation of PBLs and CFSE labelling

At the designated time-points and prior to any other intervention in the animals, blood was collected aseptically via jugular venipuncture and PBLs were isolated using the carbonyl iron granulocyte depletion method, followed by density gradient centrifugation with Lymphoprep™ (Fisher Scientific) as previously used by our group (Cequier et al. 2022a). This isolation technique has been reported to provide an enriched lymphocyte population (95–99%) (Pezzanite et al. 2015).

Subsequently, PBLs were labelled with 2.5 μM CFSE (Sigma–Aldrich) in order to evaluate lymphocyte proliferation by assessing CFSE dilution using flow cytometry (Schnabel et al. 2014; Cassano et al. 2018). After the staining procedure, PBLs were counted in a hemocytometer chamber using Trypan Blue 0.4% and adjusted to 10×10^6 live cells/mL in PBL medium (consisting of RPMI 1640 medium supplemented with 10% FBS, 0.1 mM 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin, all from Sigma–Aldrich).

Modified one-way MLR

To assess the ability of MSCs to induce the proliferation of resting lymphocytes, modified one-way MLRs were performed. Stimulator MSCs were previously seeded onto 24-well plates at a density of 20,000 cells per well in duplicate for each condition (MSC-naïve, MSC-primed, MSC-chondro). Autologous, MHC-matched and mismatched responder CFSE-stained PBLs were then added at 1×10^6 PBL per well following the experimental design aforementioned, thus resulting in a MSC:PBL ratio of 1:50 (Schnabel et al. 2014; Colbath et al. 2017; Berglund 2018; Cequier et al. 2022a).

For each experimental condition, appropriate controls were established in duplicate. In a 96-well U-bottom plate, 500,000 PBLs per well were seeded as controls for each recipient: unlabeled PBLs to account for the background, CFSE-labelled PBLs alone to set the non-proliferating gate, and CFSE-labelled PBLs stimulated with 10 µg/mL of the mitogen phytohemagglutinin isoform P (PHA, Sigma–Aldrich) as internal controls to corroborate that PBLs were able to proliferate (Ranera et al. 2016; Cequier et al. 2022a).

In addition, classic MLRs were established for each recipient. Briefly, MHC-matched and mismatched PBLs were used as stimulators by treating them with 50 µg/mL mitomycin C (Sigma–Aldrich) (37 °C 30 min incubation followed by 2 washes with PBS) to inhibit proliferation (Paterson et al. 2014; Rowland et al. 2018). Stimulator PBLs were plated at 500,000 PBLs/well in 96-well plates immediately before the addition of 500,000 CFSE-stained responder PBLs (ratio 1:1) to create MHC-matched and mismatched MLRs for all the PBLs tested with MSCs (Cequier et al. 2022a).

All the co-cultures performed along the whole experiment were always carried out with freshly isolated PBLs. All the co-cultures were maintained for 5 days without media exchange, after which analyses were performed, as will be detailed below.

Analysis of lymphocyte proliferation and subpopulations

After 5 days in modified one-way MLR co-cultures, the PBLs were collected from the 24-well plates, centrifuged at 310× g for 5 min, resuspended in PBS, and split for the two flow multi-color panels. The supernatants were collected and centrifuged at 500× g for 15 min to remove any contaminating cell and were immediately frozen at –20 °C for further ELISA analysis as detailed below.

Similarly, control PBLs (alone) in 96-well plates were centrifuged at 310× g for 5 min. The supernatants were collected, processed, and stored as described above, and the cells were suspended in PBS. The experimental PBLs were then transferred to the same 96-well plates alongside the controls for antibody staining for flow cytometry.

Two multi-color panels of markers were previously designed by our group to allow assessment of different lymphocyte subpopulations, along with the proliferation (CFSE dilution) (Cequier et al. 2022a). In panel 1, anti-CD3 (primary rat anti-human, 1:100, MCA1477, Bio-Rad; secondary mouse anti-rat, 1:100, 12-4812-82, Invitrogen), anti-Pan Ig (primary mouse anti-horse, 1:100, MCA1899, Bio-Rad; secondary goat anti-mouse, 1:200, A-21036, Invitrogen) and anti-CD21 (1:40, 561357, BD Pharmigen) were used to

assess the global T and B cell populations. In panel 2, anti-CD8 (1:5, MCA2385, Bio-Rad), anti-CD4 (primary mouse anti-horse, 1:200, MCA1078, Bio-Rad; secondary rat anti-mouse, 1:200, 406619, BioLegend) and anti-CD25 (primary goat anti-human, 1:50, AF-223, R&D Systems, secondary donkey anti-goat, 1:400, 705-605-003, Jackson Immuno Research) antibodies were used to assess cytotoxic, helper and regulatory T cells, respectively.

All the primary antibodies were selected based on previous reports and tested to correctly label equine cells. Primary antibodies were used directly conjugated with fluorochromes or in combination with appropriate secondary antibodies (Kol et al. 2015; Hillmann et al. 2019; Kamm et al. 2021b). The methodology for staining the equine PBLs with these antibodies has been described in detail in a previous publication of our group that can be openly accessed, and was exactly the same than in this study (Cequier et al. 2022a).

All samples were analyzed in a Gallios flow cytometer (Beckman Coulter, Madrid, Spain), acquiring a minimum of 10,000 events per sample. Flow cytometry data was analyzed with FCS Express 7 Flow software (De Novo Software, Pasadena, CA, USA). Unstained PBLs and secondary controls (cells stained with secondary antibodies alone) were used to assess fluorescence background and to establish gates for each marker. Compensation controls were performed using single color stains, while fluorescence minus one (FMO) controls were used to determine the fluorescence spread from other channels. Viability staining was performed with Ghost dye Violet 450 (Tonbo Biosciences, Bio-Rad) for all PBLs.

The gating strategy was also the same the same than in a previous report from our group (Cequier et al. 2022a). Briefly, the lymphocyte population was initially gated in the forward and side scatter (FSC × SSC) plot, with doublets being excluded from analysis, followed by exclusion of dead cells by incorporating a viability stain. In the first panel, live cells were further gated as T cells (CD3+) or B cells (CD3−/pan Ig+/CD21+) (Tomlinson et al. 2018). In panel 2, live cells were gated to differentiate between cytotoxic T cells (CD8+/CD4−) and helper T cells (CD4+/CD8−). Furthermore, the subpopulation of regulatory T cells (Treg) was gated from the CD4+ cell population based on their high expression of CD25.

To evaluate the proliferation of PBLs in each specific lymphocyte subset, CFSE dilution was analyzed in cells gated as aforementioned. Autofluorescence (background) was determined using unstimulated and unstained PBLs. Unstimulated and CFSE-labelled PBLs were used to establish the non-proliferating population, considering cells to the left (lower fluorescence intensity) as the proliferating population.

To account for inter-individual variability, the percentage and proliferation of each lymphocyte subpopulation after *in vitro* exposure to MSCs was normalized to the MLR-matched controls.

Interferon gamma secretion assay

Supernatants collected were used to evaluate IFNγ production by using a commercially available ELISA kit (Equine IFN-gamma DuoSet ELISA, R&D Systems, REF: DY1586), as previously reported (Paterson et al. 2014; Clark et al. 2016; Cequier et al. 2022a). The supernatants from the classical MLRs with MHC-matched or mismatched PBLs as stimulators were used as the negative and positive control, respectively. All supernatants were diluted 1:1 in reagent diluent. All the procedures were performed as per manufacturer's instructions and concentrations determined using a standard curve, including a blank.

The standard curve was set from 62.5 pg/mL to 8000 pg/mL of IFNγ. All the samples and points of the standard curve were run in triplicate. All the colorimetric assays were analyzed on a microplate reader (Biotek Synergy HT, Winooski, VA, USA) and read immediately at 450 nm with wavelength correction set to 540 nm. The duplicate readings for each standard, control, and sample were averaged, and the average zero standard optical density was extracted. The standard curve was created generating a four-parameter logistic curve-fit and the concentrations extrapolated were multiplied by the dilution factor.

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics version 26 statistical package. Analytical statistical tests were used to test for differences in each lymphocyte subpopulation and in IFNγ secretion as the dependent variables of the study.

The independent variables were "MHC group" (two categories: MHC-matched and MHC-mismatched), "MSC type" (three categories: MSC-naïve, MSC-primed and MSC-chondro) and "time" (eight categories: T0, T1, T2, T3, T4, T5, T6, T7). Each dependent variable was analysed individually and each of the independent variables as a factor to study the differences as follows: differences between MSC types at each time within each MHC group of receptors, differences between MHC groups of receptors for each MSC type at each time, differences over time within each MHC group of receptors for each MSCs type.

The existence of outlier samples was evaluated with the Grubbs test (alpha = 0.05) and the results were analyzed by Shapiro–Wilk test to assess normality of data. Levene's test was used to test the equality of variances. When data followed a normal distribution and had homogeneous variances, the parametric test ANOVA was used, followed by Bonferroni comparisons test as a post hoc. In normally distributed data with unequal variances, Welch's t-test was used. In non-normal data and variables with more than three groups, Kruskal-Wallis or Friedman tests followed by Dunn's test were used as post hoc, for independent (differences between MSC types) or related (differences over time) samples, respectively. The Mann-Whitney U test was used for comparisons between two groups (differences between MHC groups). The significance level was set at $p < 0.05$ for all analyses. GraphPad Prism 9.2 was used for graphical representation (San Diego, CA, USA).

Results

Changes in the frequency and proliferation of lymphocytes subpopulations after *in vitro* exposure to MSCs (modified one-way MLR assay)

CD3+ T Lymphocytes: Proliferation patterns shift in the CD3+ T cell population, while frequencies remain unaltered

The study evaluated the effect of different MSCs types and combinations on the activation of CD3+ T cells after two allogenic administrations. To this end, the percentage of CD3+ T cells was measured at different time-points using *in vitro* co-culture with the same MSCs administered *in vivo* (**Fig. 3**). After first administration, the percentage of CD3+ T lymphocytes remained similar under the different conditions, so neither the type of combination nor the type of MSCs seemed to produce relevant changes in the global T cell population along the time.

Throughout all the times analyzed, the presence of MSC-chondro resulted in a decrease in CD3+ T cells compared to MLR-matched, MSC-naïve and MSC-primed in both allogeneic combinations (**Fig. 3A**, **3B**). Overall, MSC-primed tended to increase the percentage of CD3+ regardless of the type of combination along all time-points. Specifically, in both MHC-matched and mismatched settings, MSC-primed increased the CD3+ population compared to MSC-chondro at T4 ($p < 0.05$; MHC-mismatched) (**Fig. 3B)** and T5 ($p < 0.05$; MHC-matched and mismatched) (**Fig. 3A**, **3B**).

After the second administration at T5, MSC-primed MHC-matched also showed a significant increase compared to MSC-naïve ($p < 0.05$) (**Fig. 3A**). From T4 and after the re-exposure (T5, T6 and T7), the percentage of T cells was significantly lower in MSC-chondro compared to MSC-naïve in the MHC-mismatched scenario ($p < 0.05$ in all cases) (**Fig. 3B**). However, in the MHC-matched combination, MSC-chondro only promoted a significant reduction compared to MSC-naïve at T6 and T7 ($p < 0.05$ in both cases) (**Fig. 3A**). Overall, mismatched combinations tended to further increase the percentage of T cells, but only MSC-chondro showed a significant increase ($p < 0.05$) compared to the matched setting at T7 (**Fig. 3C**). Although, before second administration at T4, the mismatched MSC-chondro co-cultures reduced the percentage of CD3+ T cells compared to MHC-matched group ($p < 0.05$), but not exceeding the MLR-matched values (**Fig. 3C**).

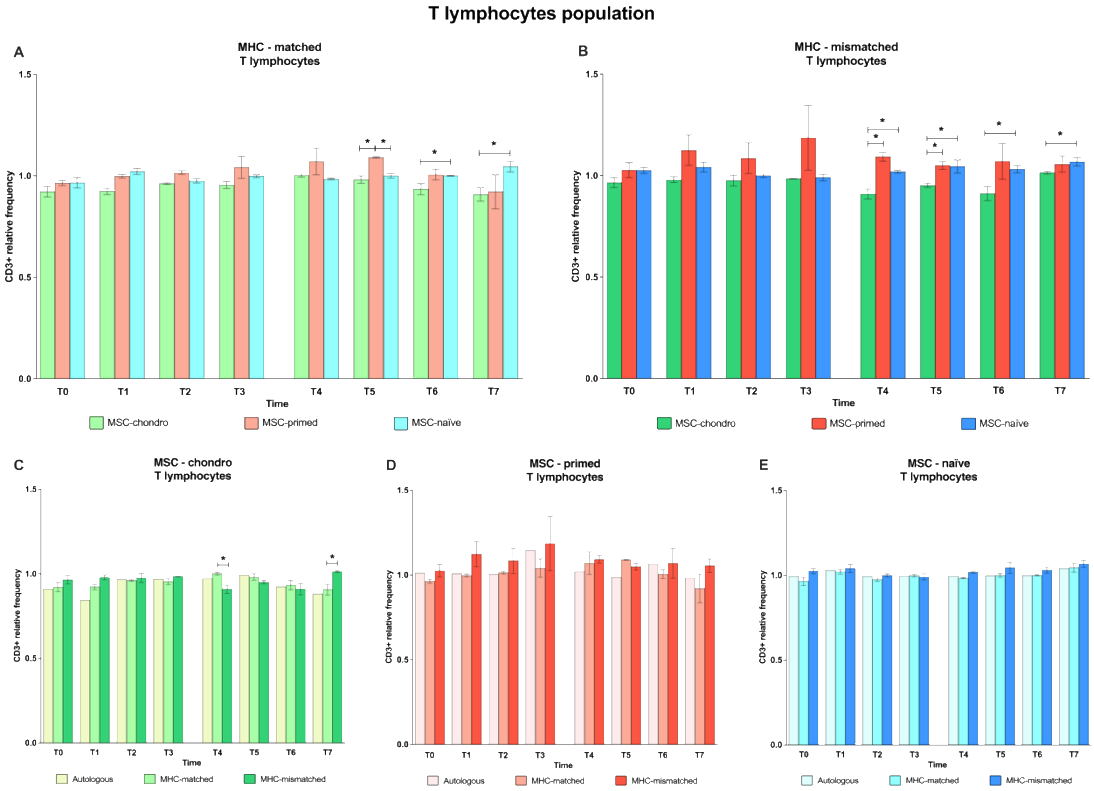


Fig. 3. Mean ± SEM of the relative frequency of CD3+ T lymphocytes in the immunogenicity assays (modified one-way mixed lymphocyte reaction) in MHC-matched (A) and MHC-mismatched (B) recipients following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Changes along time of non-activated PBLs from autologous, MHC-matched and MHC-mismatched recipients exposed *in vitro* to MSC-chondro (C), MSC-primed (D) and MSC-naïve (E). Data from each PBL recipient is normalized over the negative control (MLR M−, matched MLR) consisting of responder PBLs from the same donor exposed to MHC-matched stimulator PBLs (value 1), to account for inter-individual variability. Significant differences between cell-type and groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$).

In spite of the limited changes in the frequency of CD3+ T cells, their proliferation was stimulated upon *in vitro* exposure to MSCs in several conditions. Overall, results showed that CD3+ proliferation increased after the second administration in all MSCs types and combinations, even in all autologous control groups (**Fig. 4**). Regarding the effect of different MSC types (naïve, primed and chondro), CD3+ proliferation was not significantly different among the three cell types when the co-cultures were MHC-matched. Nonetheless, MSC-primed induced the highest proliferative response of CD3+ after the first administration and MSC-chondro after the second one (**Fig. 4A**).

In contrast, MHC-mismatched co-cultures promoted a higher T cell response, showing a significantly increased proliferation at both T0 and T1 when T cells were co-cultured with MSC-chondro compared to MSC-naïve ($p < 0.05$) (**Fig. 4B**). Interestingly, MSC-primed induced a T cell increase over MSC-chondro and MSC-naïve at T2 and T3, but the increase was statistically significant only at T3 ($p < 0.01$ in both cases) (**Fig. 4B**). Before second administration at T4, MSC-naïve MHC-mismatched co-cultures showed the highest T cell increase compared to MSC-chondro ($p < 0.05$) and MSC-primed. Moreover, after re-

exposure (T5), also a CD3+ proliferation increase was observed for MSC-naïve over MSC-primed and MSC-chondro ($p < 0.05$ in both cases). However, at the last time-point analyzed (T7), a T cell proliferation increase over MSC-chondro was observed for MSC-primed ($p < 0.01$) and MSC-naïve ($p < 0.05$) (**Fig. 4B**).

For MSC-chondro, MHC-matched co-cultures showed a significant increase in CD3+ proliferation at T5 compared to T1 ($p < 0.05$), whereas MHC-mismatched groups showed a significant T cell increase at T1 compared to T0 ($p < 0.05$), T2 ($p < 0.01$) and T3 ($p < 0.05$) (**Fig. 4C**). Furthermore, at the last time-point T7, a significant increase in CD3+ proliferation was observed compared to T4 in MSC-chondro MHC-mismatched co-cultures ($p < 0.05$) (**Fig. 4C**). Interestingly, MHC-mismatched co-cultures induced a significantly higher T cell proliferation than MHC-matched ones at T1 ($p < 0.05$) (**Fig. 4C**).

Allogenic MSC-primed co-cultures followed a similar trend before after each administration (T1 and T5). Interestingly, MSC-primed co-cultured with MHC-mismatched PBLs induced a significant T cell proliferation increase at T3 over T0 and T1 ($p < 0.01$ in both cases) and also at T7 over T4 and T5 ($p < 0.05$ in both cases) (**Fig. 4D**). Regarding differences between allogenic MSC-primed co-cultures, although a significant T cell increase was observed in MHC-mismatched at T0 ($p < 0.05$), CD3+ proliferation was similar between both allogenic co-cultures along time but T3 ($p < 0.01$) and T7 ($p < 0.05$) where a significant increase was observed in MHC-mismatched group (**Fig. 4D**).

For MSC-naïve, CD3+ proliferation was similar for both allogenic groups along the first administration time-points, although MHC-matched co-cultures showed a significant increase at T1, T4, and T5 compared to T0 ($p < 0.05$ in all cases). However, after re-exposure, a significant proliferation increases in CD3+ T cells was observed for the MHC-mismatched group at T5, T6, and T7 over T0 and T1 ($p < 0.05$ for all times) (**Fig. 4E**). Additionally, after the second administration, MHC-mismatched co-cultures showed a significant increase in CD3+ proliferation at T5 ($p < 0.05$), T6 ($p < 0.05$) and T7 ($p < 0.01$) over the MHC-matched co-cultures (**Fig. 4E**).

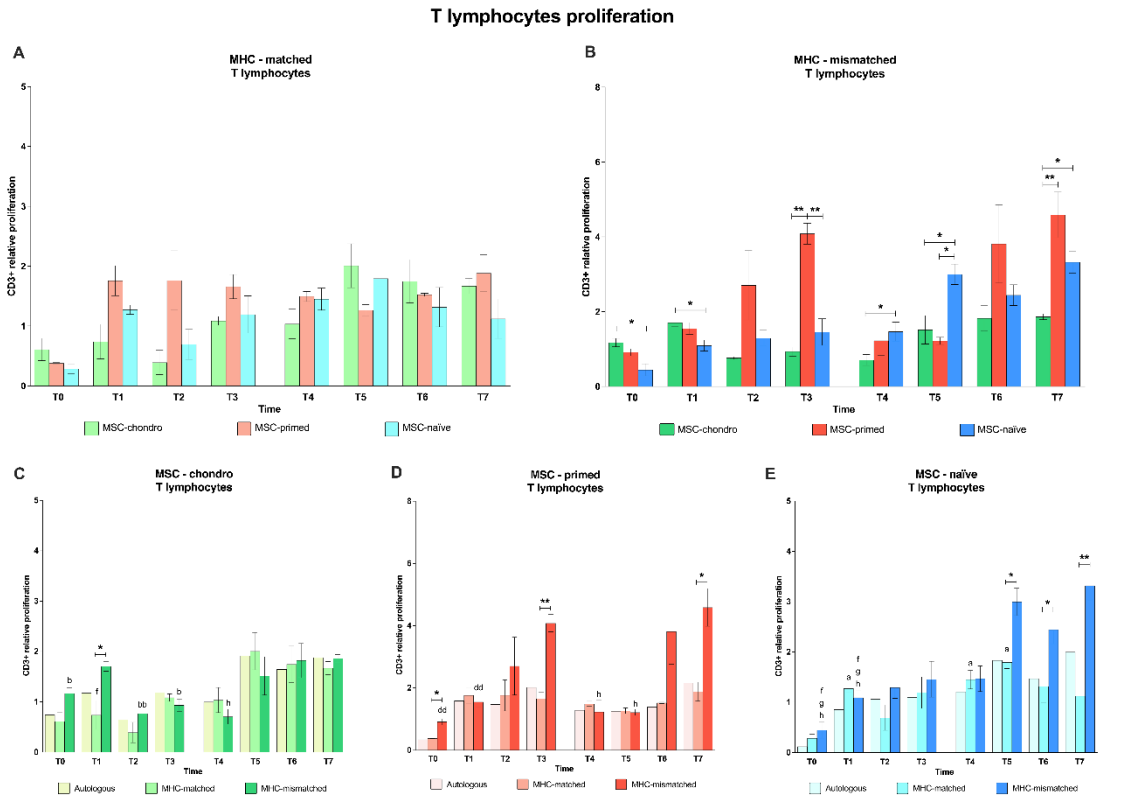


Fig. 4. Mean ± SEM of the relative proliferation of CD3+ T lymphocytes in the immunogenicity assays (modified one-way mixed lymphocyte reaction) in MHC-matched (A) and MHC-mismatched (B) recipients following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Changes along time of non-activated PBLs from autologous, MHC-matched and MHC-mismatched recipients exposed *in vitro* to MSC-chondro (C), MSC-primed (D) and MSC-naïve (E). Proliferation of each PBL donor is normalized over the proliferation observed in the negative control (MLR M−, matched MLR) consisting of responder PBLs from the same donor exposed to MHC-matched stimulator PBLs (value 1), to account for inter-individual variability. Significant differences between cell-type and groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). Significant differences between time-points are represented by lower case letter: T0, [a]; T1, [b]; T3, [d]; T5, [f]; T6, [g] and T7, [h] ([a], [b], [f], [g], [h], $p < 0.05$; [bb], [dd], $p < 0.01$).

CD8+ Cytotoxic and CD4+ Helper T Lymphocytes: MSC-primed induce helper and cytotoxic lymphocytes, while MSC-chondro and MSC-naïve manage to evade them after first administration

The present study also investigated the CD4+ T and CD8+ T cell responses along the time when administering allogeneic MSC-primed, MSC-chondro and MSC-naïve in MHC-matched or mismatched recipients. When recipients' lymphocytes were exposed to the same MSCs *in vitro* (modified one-way MLRs), the percentage of CD8+ T cells tended to increase after the first administration of MSC-primed compared to MSC-chondro and MSC-naïve, for both MHC-matched and MHC-mismatched settings (**Fig. 5**). These differences were statistically significant in MSC-primed compared to MSC-naïve from T1 to T3 timepoints for the MHC-mismatched setting ($p < 0.05$ in all cases) (**Fig. 5B**). Interestingly, after re-exposure, MSC-chondro showed the highest increase in the percentage of CD8+ T cells for both allogenic co-cultures at T5 and T6, although the

increase was statistically significant only for the MHC-matched co-culture at T5 over MSC-primed ($p < 0.05$) and MSC-naïve ($p < 0.01$) (**Fig. 5A**). Moreover, this increase at T5 was statistically significant compared to T0 and T1 ($p < 0.05$ in both cases) in both MSC-chondro MHC-matched and MHC-mismatched co-cultures (**Fig. 5C**).

Overall, MHC-mismatched combinations tended to increase the percentage of cytotoxic T cells compared to MHC-matched ones, but only MSC-primed showed a significant increase at T2 ($p < 0.05$) compared to the MHC-matched group (**Fig. 5D**). Interestingly, while the CD8+ T cell population hardly changed over time in the MSC-primed MHC-matched co-culture, a significant increase was observed in the MHC-mismatched co-cultures one week after the first administration (T1 over T0 and T4; $p < 0.05$ in both cases). This increase was also observed after the second administration, but it was not statistically significant until three weeks of re-exposure (T6 over T5; $p < 0.05$) (**Fig. 5D**).

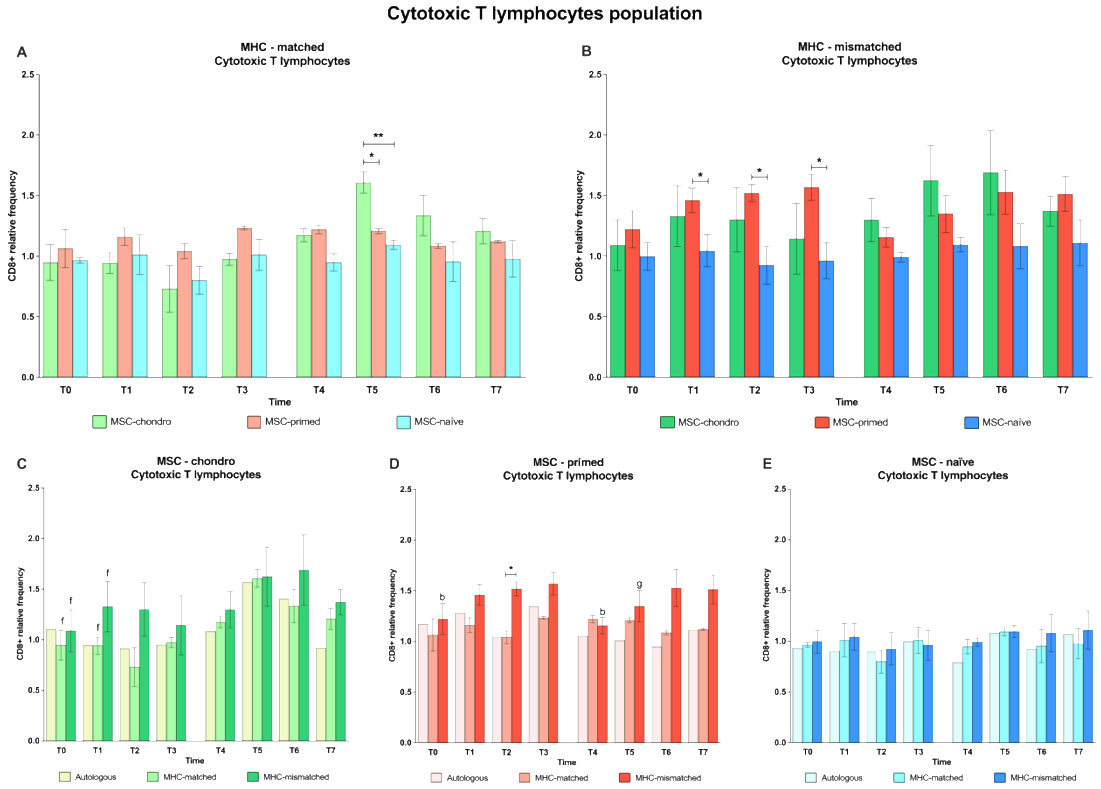


Fig. 5. Mean ± SEM of the relative frequency of CD8+ cytotoxic T cells in the immunogenicity assays (modified one-way mixed lymphocyte reaction) in MHC-matched (A) and MHC-mismatched (B) recipients following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Changes along time of non-activated PBLs from autologous, MHC-matched and MHC-mismatched recipients exposed *in vitro* to MSC-chondro (C), MSC-primed (D) and MSC-naïve (E). Data from each PBL recipient is normalized over the negative control (MLR M−, matched MLR) consisting of responder PBLs from the same donor exposed to MHC-matched stimulator PBLs (value 1), to account for inter-individual variability. Significant differences between cell-type and groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$; ** $p < 0.01$). Significant differences between time-points are represented by lower case letter: T2, ^{b}; T5, ^{f} and T6, ^{g} (^{b}, ^{f}, ^{g}, $p < 0.05$).

In terms of CD4+ T cells, for the MHC-matched co-culture, both MSC-chondro and MSC-primed reduced the percentage of helper T cells over the MLR matched used as control along all the time-points, while MSC-naïve consistently showed a higher percentage of helper T cells over the other MSCs types (T3 and T6: $p < 0.05$ in all cases) (**Fig. 6A**). In the MHC-mismatched groups, a similar trend of increasing the percentage of CD4+ T cells by MSC-naïve was observed over MSC-primed at T3 ($p < 0.05$) and over MSC-chondro at T0, T5, T6 and T7 ($p < 0.05$ in all cases) (**Fig. 6B**).

In MSC-chondro, the CD4+ percentage increased in the MHC-mismatched co-cultures one week after each administration (T1 and T5). This percentage decreased at three weeks but increased again at 6 weeks after each administration (T3 and T7) compared to the other timepoints and to the MHC-matched co-cultures and the MLR-matched control (**Fig. 6C**). Specifically, a significant increase in CD4+ percentage was observed at T3 over T1 and T4 ($p < 0.05$), but a significant decrease in helper T cells was observed at T6 compared to T5 ($p < 0.05$) (**Fig. 6C**).

Similarly, MSC-primed MHC-mismatched co-cultures also promoted a significant increase in CD4+ T cells after the second administration at T6 and T7 compared to T2 and T3, respectively ($p < 0.05$ in both cases) (**Fig. 6D**).

In MSC-naïve, a significant CD4+ T cells increase was observed after the first administration in both allogeneic groups (T3 compared to T1; $p < 0.05$ in both cases). In addition, MSC-naïve MHC-matched co-cultures induced a significant CD4+ increase at T6 compared to T2 ($p < 0.05$) (**Fig. 6E**).

Regarding the effect of MHC-matched and MHC-mismatched combination, CD4+ and CD8+ T cells exhibited a similar trend, with higher levels induced by MHC-mismatched MSCs of any type. After the first administration, both MSC-primed and MSC-naïve MHC-mismatched co-cultures resulted in a significant increase in the percentage of helper T cells compared to the MHC-matched setting at T1 ($p < 0.05$ in both cases) (**Fig. 6D**, **6E**), and at T3 for MSC-chondro ($p < 0.05$) (**Fig. 6C**). After the second administration, a similar increase in CD4+ cells was observed in MHC-mismatched co-cultures at T5 for MSC-chondro and MSC-naïve ($p < 0.05$ in both cases) (**Fig. 6C**, **6E**), and at T7 for MSC-primed ($p < 0.05$) (**Fig. 6D**).

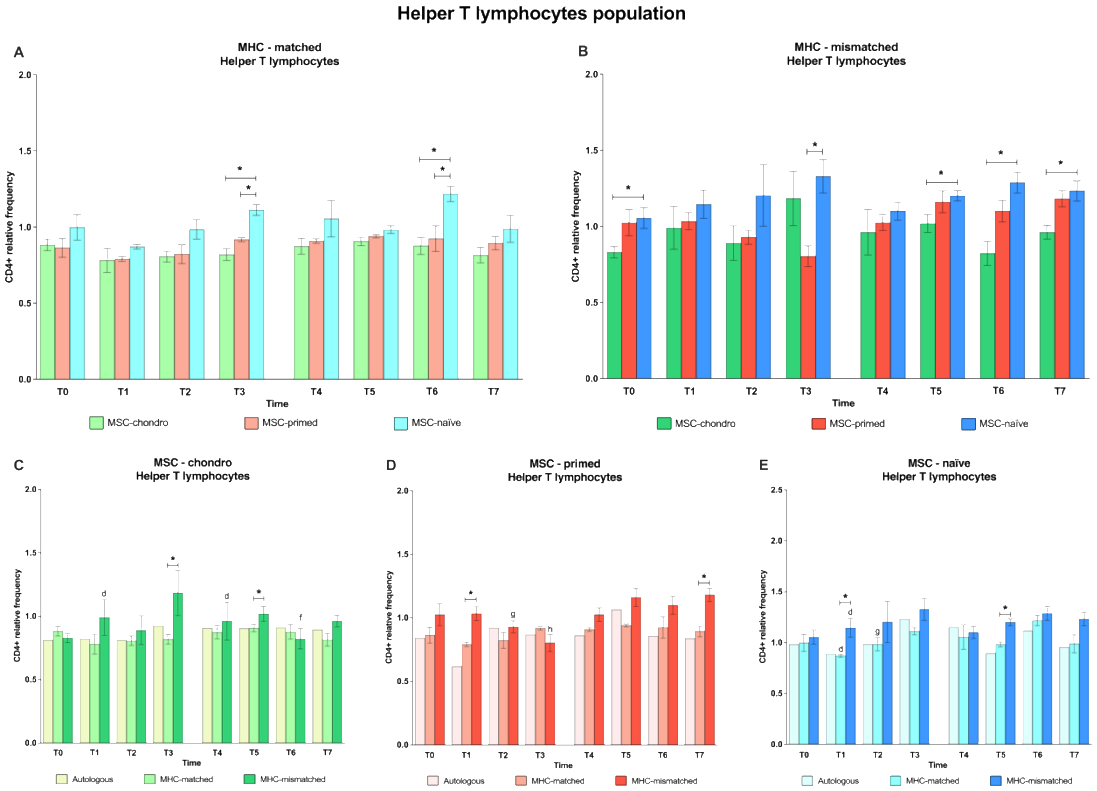


Fig. 6. Mean ± SEM of the relative frequency of CD4+ helper T cells in the immunogenicity assays (modified one-way mixed lymphocyte reaction) in MHC-matched (A) and MHC-mismatched (B) recipients following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Changes along time of non-activated PBLs from autologous, MHC-matched and MHC-mismatched recipients exposed *in vitro* to MSC-chondro (C), MSC-primed (D) and MSC-naïve (E). Data from each PBL recipient is normalized over the negative control (MLR M−, matched MLR) consisting of responder PBLs from the same donor exposed to MHC-matched stimulator PBLs (value 1), to account for inter-individual variability. Significant differences between cell-type and groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$). Significant differences between time-points are represented by lower case letter: T3, ^{d}; T5, ^{f}; T6, ^{g} and T7, ^{h} (^{d}, ^{f}, ^{g}, ^{h}, $p < 0.05$).

The findings regarding the proliferation of helper and cytotoxic cells reinforced the results observed in terms of their frequencies as MSC-primed clearly promoted the highest CD4+ T and CD8+ T cell response in both allogeneic settings along the time, followed by MSC-chondro and MSC-naïve (**Fig. 7, 8**). Importantly, MHC-matched combinations induced lower proliferation of helper and cytotoxic T cells for the three types of MSCs. Furthermore, the proliferative response after re-exposition to MHC-mismatched MSCs was more marked for MSC-chondro when compared to the first administration.

Overall, in the MHC-matched combination, CD4+ T and CD8+ T cell proliferation was higher in the presence of MSC-primed compared to MSC-naïve and MSC-chondro at almost all timepoints (T0, T4, T5 and T7, $p < 0.05$ in both cell types; T1 and T3, $p < 0.01$ in both cell types) (**Fig. 7A, 8A**). Moreover, higher CD4+ proliferation was observed in MSC-primed over MSC-chondro and MSC-naïve at T2 ($p < 0.01$) and T6 ($p < 0.05$) (**Fig. 8A**). It is worth noting that in the MHC-matched co-cultures a significant increase in helper T cells was induced by MSC-naïve over MSC-chondro at T2 ($p < 0.01$) and T7 ($p < 0.05$) (**Fig. 8A**).

Regarding MHC-mismatched groups, CD8+ T and CD4+ T cell proliferation followed a similar pattern as the MHC-matched groups; MSC-primed also induce a statistically significant T cell proliferation over MSC-chondro (T0, T1, T4 and T6, $p < 0.05$ in both T cell types; CD8+: T2, T3, and T7, $p < 0.01$; CD4+: T2, T3, and T7, $p < 0.05$) and MSC-naïve (T0, T1 and T3, $p < 0.05$ in both T cell types; T2 and T7, $p < 0.01$ in both T cell types; CD8+: T4 and T6, $p < 0.05$; CD4+: T6, $p < 0.01$) (**Fig. 7B, 8B**). In addition, in the MHC-mismatched groups, proliferation of CD4+ T cells was higher after re-exposure to MSC-naïve compared to MSC-primed at T5 ($p < 0,05$) and compared to MSC-chondro at T5, T6 and T7 ($p < 0.05$ in all cases) (**Fig. 8B**). However, no statistically significant proliferation differences in CD8+ T cells were observed between MSC-chondro and MSC-naïve in either allogenic co-culture at any timepoint (**Fig. 7B**).

In MSC-chondro MHC-matched co-cultures, a significant increase in helper T cell proliferation was observed one week after the first administration (T1) compared to T0 ($p < 0.05$), but not exceeding the MLR-matched values (**Fig. 8C**). In contrast, MSC-chondro MHC-mismatched co-cultures induced greater changes in CD4+ and CD8+ proliferation rates over time (**Fig. 7C, 8C**). Specifically, a significant increase in CD4+ proliferation was observed at T3 compared to T2 ($p < 0.01$), exceeding MLR-matched values. Such proliferation progressively increased from T3, peaked at T5, and decreased at T6 and T7 compared to T4 and T5, respectively ($p < 0.05$ in both cases) (**Fig. 8C**). In contrast, the increase in CD8+ proliferation in MSC-chondro was also progressive but peaked at T6 (statistically significant over T4 ($p < 0.05$), T5 ($p < 0.01$), and T7 ($p < 0.05$) (**Fig. 7C**). Higher cytotoxic and helper T cell proliferation was observed in MHC-mismatched MSC-chondro co-cultures compared to MHC-matched ones at T0, T3, and T4 ($p < 0.05$ in all cases) (**Fig. 7C, 8C**). Furthermore, CD8+ proliferation induced by MSC-chondro was also significantly higher in the MHC-mismatched setting compared to the MHC-matched at T2 ($p < 0.05$) and T6 ($p < 0.01$) (**Fig. 7C**).

Cytotoxic T cells proliferation was significantly increased in MSC-primed MHC-matched co-cultures at T1 and T3 over T0, T4, and T5 ($p < 0.05$ in all cases). In MSC-primed MHC-mismatched co-cultures, a significant CD8+ proliferation increase was observed at T2, T3 and T7 over T0 (T2 and T7, $p < 0.01$; T3, $p < 0.05$), T1, T4 and T5 ($p < 0.05$ in all time-points) (**Fig. 7D**). In contrast, helper T cell proliferation in MSC-primed MHC-matched co-cultures only increased at T2 over T0, T4 and T6 ($p < 0.05$), while MHC-mismatched co-cultures showed highly variable differences along the time (**Fig. 8D**). Specifically, MHC-mismatched co-cultures induced a significant CD4+ proliferation increase at T2 and T3 over before and after each administration (T0, $p < 0.01$; T1, T4 and T5, $p < 0.05$ in all cases). However, the highest increase in T helper proliferation was observed at the last time points after the MHC-mismatched administration of MSC-primed (T6: T0, T1, T3, T4, T5, $p < 0.01$; T2, $p < 0.05$; T7: T0, T1, T2, T3, T4, $p < 0.05$; T5, $p < 0.01$) (**Fig. 8D**). The proliferation of CD4+ T and CD8+ T cells exposed to MSC-primed in the MHC-mismatched scenario was also higher compared to the MHC-matched co-cultures along the time (T2, $p < 0.05$; T6 (CD8+, $p < 0.05$; CD4+, $p < 0.01$) and T7, $p < 0.01$) (**Fig. 7D, 8D**). Additionally, significant differences were observed in CD8+ proliferation at T0 and T3 ($p < 0.05$ in both cases) (**Fig. 7D**).

On the other hand, MSC-naïve co-cultures overall induced a similar CD4+ proliferation after the first and the second exposure. However, after the second administration in the MHC-mismatched group, an increase in CD4+ proliferation was observed at all times compared to the first exposure (over T3: T5 and T6, $p < 0.05$; T7 $p < 0.01$) (**Fig. 8E**), especially at the last time-point T7 (over T0, T1 and T4; $p < 0.05$) (**Fig. 8E**). The response of cytotoxic T cells was less marked against MSC-naïve MHC-mismatched, with a non-significant increase at T2 and T6 (**Fig. 7E**).

When considering the effect of combination, it was observed that MSC-naïve MHC-mismatched co-cultures increased the proliferation of CD4+ and CD8+ cells compared to MHC-matched co-cultures at the baseline (T0, before any administration) ($p < 0.05$ for CD4+ cells) (**Fig. 8E**). However, after the first exposure (T1 to T3), there were no significant differences in T cell proliferation between MHC-matched and MHC-mismatched allogenic co-cultures for MSC-naïve. Nevertheless, similarly to that observed at T0, prior to the second administration (T4), the proliferation rates induced by MSC-naïve were higher in the MHC-mismatched setting (**Fig. 7E, 8E**). Furthermore, the proliferative response against MHC-mismatched MSC-naïve increased in the subsequent time-points over the MHC-matched co-cultures. Specifically, the proliferation of helper and cytotoxic T cells was significantly higher at T4, T5, and T7 for both T cells ($p < 0.05$ in all times) (**Fig. 7E, 8E**), and also at T6 for CD4+ cells ($p < 0.01$) (**Fig. 8E**).

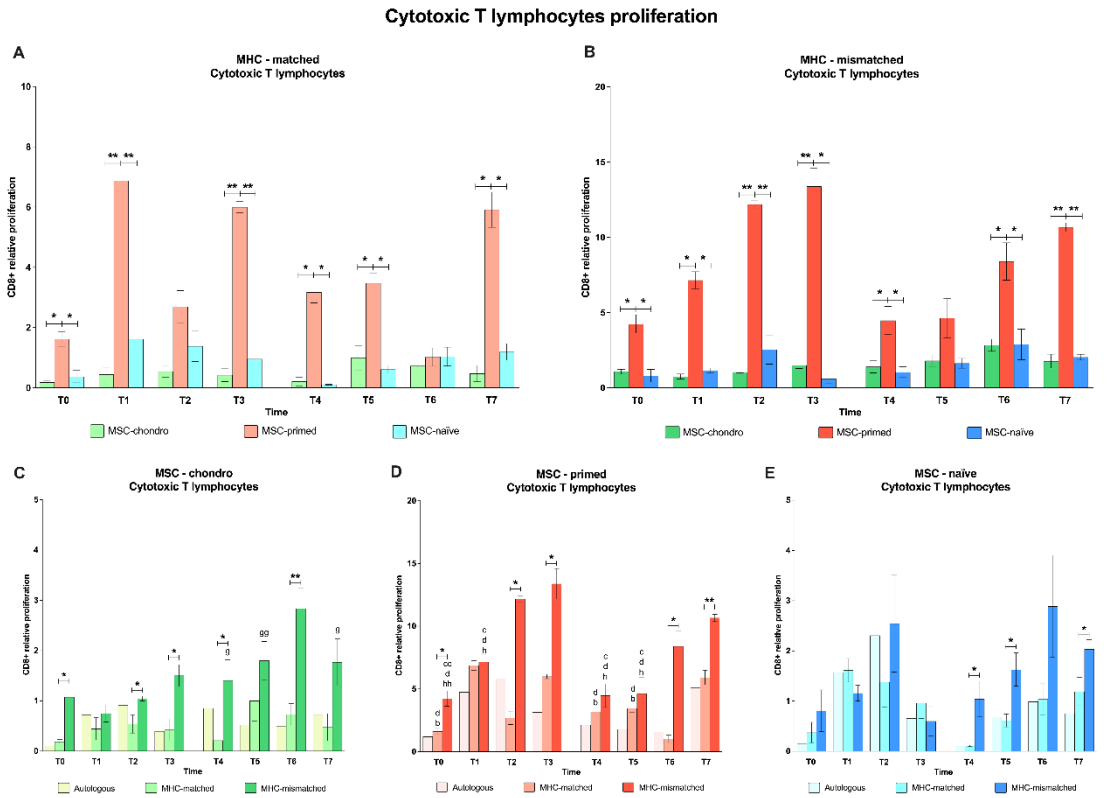


Fig. 7. Mean ± SEM of the relative proliferation of CD8+ cytotoxic T cells in the immunogenicity assays (modified one-way mixed lymphocyte reaction) in MHC-matched (A) and MHC-mismatched (B) recipients following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Changes along time of non-activated PBLs from autologous, MHC-matched and MHC-mismatched recipients exposed *in vitro* to MSC-chondro (C), MSC-primed (D) and MSC-naïve (E). Proliferation of each PBL donor is normalized over the proliferation observed in the negative control (MLR M−, matched MLR) consisting of responder PBLs from the same donor exposed to MHC-matched stimulator PBLs (value 1), to account for inter-individual variability. Significant differences between cell-type and groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). Significant differences between time-points are represented by lower case letter: T1, ^{b}; T2, ^{c}; T3, ^{d}; T6, ^{g} and T7, ^{h} (^{b}, ^{c}, ^{d}, ^{g}, ^{h}, $p < 0.05$; ^{cc}, ^{gg}, ^{hh}, $p < 0.01$).

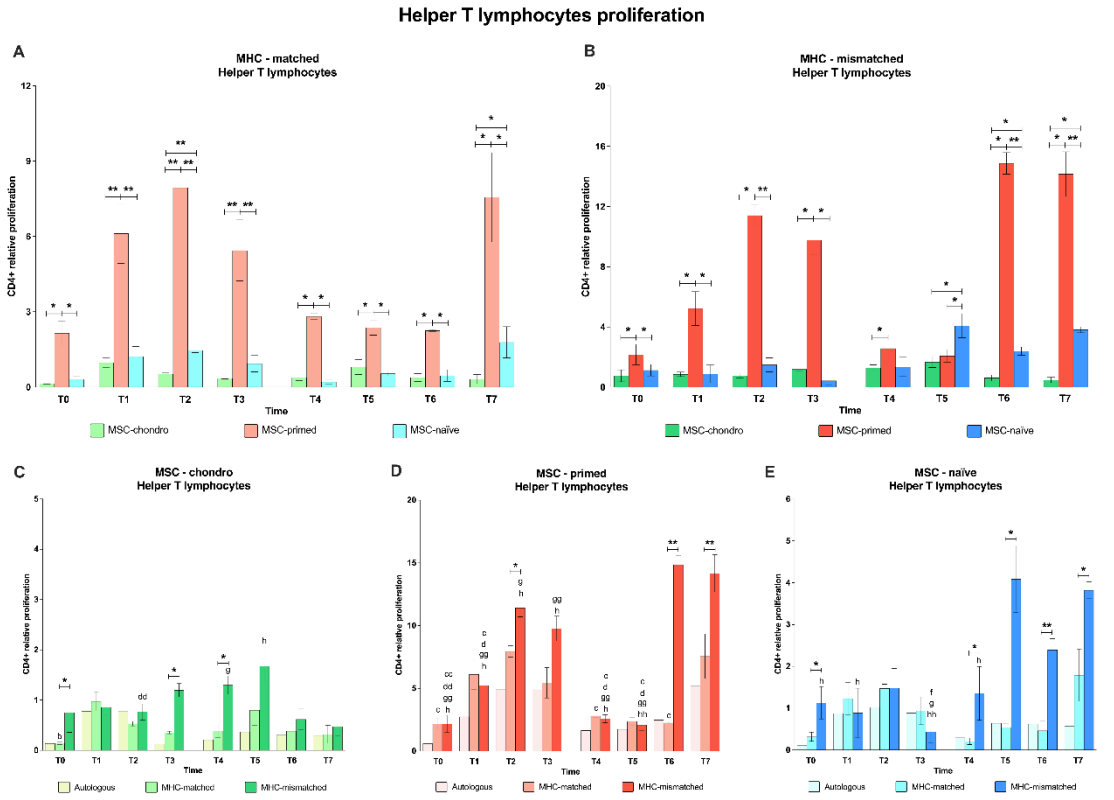


Fig. 8. Mean ± SEM of the relative proliferation of CD4+ helper T cells in the immunogenicity assays (modified one-way mixed lymphocyte reaction) in MHC-matched (A) and MHC-mismatched (B) recipients following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Changes along time of non-activated PBLs from autologous, MHC-matched and MHC-mismatched recipients exposed *in vitro* to MSC-chondro (C), MSC-primed (D) and MSC-naïve (E). Proliferation of each PBL donor is normalized over the proliferation observed in the negative control (MLR M−, matched MLR) consisting of responder PBLs from the same donor exposed to MHC-matched stimulator PBLs (value 1), to account for inter-individual variability. Significant differences between cell-type and groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). Significant differences between time-points are represented by lower case letter: T1, ^{b}; T2, ^{c}; T3, ^{d}; T5, ^{f}; T6, ^{g} and T7, ^{h} (^{b}, ^{c}, ^{d}, ^{f}, ^{g}, ^{h}, $p < 0.05$; ^{cc}, ^{dd}, ^{gg}, ^{hh}, $p < 0.01$).

CD4+ CD25^{high} Regulatory T Cells: MSC-primed cells have a greater capacity to induce Treg, particularly in the MHC-mismatched condition, whereas MSC-chondro barely induce Treg

The effect of MSCs co-culture combinations on the percentage of Treg was also analyzed in this study. MSC-primed was found to increase the percentage of Treg over MSC-naïve and MSC-chondro in all co-culture combinations and timepoints (**Fig. 9A**, **9B**).

Comparison between cell types showed that before any administration (T0), allogenic MSC-primed co-cultures significantly increase Treg over MSC-chondro ($p < 0.05$ in both cases). In addition, MSC-naïve MHC-mismatched co-cultures showed a Treg increase over MSC-chondro at T0 ($p < 0.05$) (**Fig. 9A**, **9B**).

It is noteworthy that the Treg increase observed for MSC-primed and MSC-naïve MHC-matched groups remained significant for all time-points that could be analyzed compared to MSC-chondro (MSC-primed: T3, T4, T5, T6, T7, $p < 0.01$; MSC-naïve: T3, T5 and T7, $p < 0.01$; T4 and T6, $p < 0.05$) (**Fig. 9A**). For MHC-mismatched groups, the same Treg patterns were observed in MSC-primed and MSC-naïve compared to MSC-chondro (MSC-primed: T1, T2, T3, T4, T5, T6 and T7; $p < 0.01$; MSC-naïve: T1, T5 and T7, $p < 0.01$; T6, $p < 0.05$) (**Fig. 9B**). Interestingly, from T1 to T7, the percentage of Treg was always statistically higher in MSC-primed than in MSC-naïve for both allogenic MHC-matched (T3, T5 and T7, $p < 0.05$, T4 and T6, $p < 0.01$) and MHC-mismatched (T0 and T5, $p < 0.05$; T1, T2, T3, T4, T5, T6, and T7; $p < 0.01$) settings (**Fig. 9A**, **9B**).

The results showed that MSC-primed MHC-mismatched significantly increased the population of Treg over T0 at all timepoints analyzed (T1, T2, T3, T4, T5, T6, T7, $p < 0.05$) (**Fig. 9D**). Furthermore, the Treg increase induced by MSC-primed MHC-mismatched co-cultures was found to be significant at the latest time-points (T6 and T7) compared to T1, T4 and T5 ($p < 0.05$). Regarding the effect of combination, MHC-mismatched MSC-primed increased the Treg population over MHC-matched co-cultures at T0, T2, T6, and T7 ($p < 0.05$) (**Fig. 9D**).

Regarding the effect induced by MSC-naïve on the Treg population, MHC-mismatched co-cultures tended to increase this population over MHC-matched, (significant differences at T0 ($p < 0.01$) and T3 ($p < 0.05$) (**Fig. 9E**). In the MHC-mismatched co-cultures the Treg increased steadily over time, particularly after the second exposure, and a significant increase was observed at T5 and T7 compared to T0 ($p < 0.05$ in both cases) (**Fig. 9E**). However, the opposite happened in MSC-naïve MHC-matched group, where highest values were observed before the first administration at T0 over T4 ($p < 0.05$), T6 ($p < 0.01$) and T7 ($p < 0.05$). In addition, significantly higher percentage of Tregs were observed at T3 compared to T7 in MSC-naïve MHC-matched co-cultures ($p < 0.05$) (**Fig. 9E**).

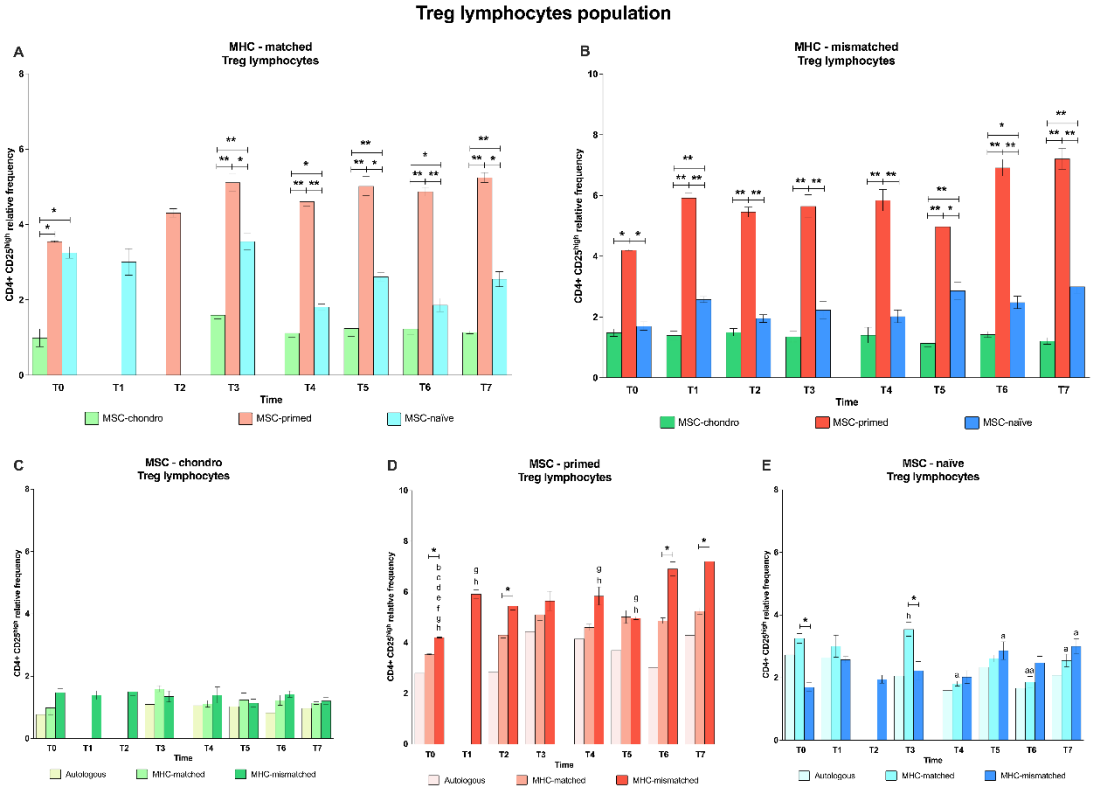


Fig. 9. Mean ± SEM of the relative frequency of CD4+ CD25^{high} regulatory T cells in the immunogenicity assays (modified one-way mixed lymphocyte reaction) in MHC-matched (A) and MHC-mismatched (B) recipients following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Changes along time of non-activated PBLs from autologous, MHC-matched and MHC-mismatched recipients exposed *in vitro* to MSC-chondro (C), MSC-primed (D) and MSC-naïve (E). Data from each PBL recipient is normalized over the negative control (MLR M−, matched MLR) consisting of responder PBLs from the same donor exposed to MHC-matched stimulator PBLs (value 1), to account for inter-individual variability. Significant differences between cell-type and groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). Significant differences between time-points are represented by lower case letter: T0, ^{a}; T1, ^{b}; T2, ^{c}; T3, ^{d}; T4, ^{e}; T5, ^{f}; T6, ^{g} and T7, ^{h} (^{a}, ^{b}, ^{c}, ^{d}, ^{e}, ^{f}, ^{g}, ^{h}, $p < 0.05$; ^{aa}, $p < 0.01$).

Consistent with the findings regarding Treg frequencies, MSC-primed tended to increase Treg proliferation, whereas MSC-chondro showed minimal induction of Treg proliferation, regardless of the combination (**Fig. 10A, 10B**). In terms of the effect of cell type in the first administration, Treg frequencies and proliferation was only compared in the MHC-matched condition at T0 and T3 due to the lack of anti-CD25 antibody when T1 and T2 time-points were analyzed. A significantly higher Treg proliferation induced by MSC-primed over MSC-naïve at T3 ($p < 0.05$) and MSC-chondro at T0 and T3 ($p < 0.05$ in both cases) was observed in MHC-matched co-cultures (**Fig. 10A**). In the MHC-mismatched group, Treg proliferation was significantly higher in MSC-primed compared to MSC-chondro and MSC-naïve at T0 ($p < 0.05$), T1, T2, and T3 ($p < 0.01$ in all times) (**Fig. 10B**). In addition, in both the allogenic MHC-matched and mismatched co-cultures, MSC-primed significantly induced proliferation of Treg compared to MSC-chondro and MSC-naïve at T4 ($p < 0.05$), T6 (MHC-matched, $p < 0.01$; MHC-mismatched, $p < 0.05$), and T7 ($p < 0.01$ in all cases) (**Fig. 10A, 10B**). However, while a significant Treg

proliferation increase was observed in MSC-primed over MSC-chondro and MSC-naïve in MHC-matched co-cultures at T5 ($p < 0.05$ in both cases) (**Fig. 10A**), MSC-naïve induced the most Treg proliferation at T5 in MHC-mismatched co-cultures (**Fig. 10B**).

The proliferation Treg results showed that MSC-chondro tended to reduce Treg proliferation rates over time, but an increase was observed over the MLR-matched control at T6 for both allogenic co-cultures (**Fig. 10C**). This increase at T6 was also statistically significant compared to T0, T3, and T7 ($p < 0.05$ in all cases) for the MHC-matched co-cultures and compared to T1 and T5 ($p < 0.05$ in all cases) for MHC-mismatched co-cultures (**Fig. 10C**). In addition, a higher Treg proliferation was observed in the MHC-mismatched combination compared to the MHC-matched one along time-points, and the increase was statistically significant at T3 ($p < 0.01$) (**Fig. 10C**).

Regarding MSC-primed co-cultures, in the MHC-mismatched group, Treg proliferation increased at three weeks after each administration (T2 over T0 and T1; $p < 0.05$; T6 over T4 and T5; $p < 0.05$) (**Fig. 10D**). The highest Treg proliferation was observed at T3, followed by a significant decrease in Treg at T4 ($p < 0.01$) and T5 ($p < 0.05$). At T5, the Treg proliferation was also significantly lower than at T1 ($p < 0.05$) and T7 ($p < 0.01$) (**Fig. 10D**). On the other hand, in the MHC-matched group, Treg proliferation rates remained similar along the time in MSC-primed co-cultures but increased at T3 and T6, however there were no significant differences over any other time. Additionally, significant differences between MHC-matched and mismatched groups were observed for MSC-primed, with higher proliferation in the MHC-mismatched combination compared to the MHC-matched one at T2, T3, and T7 ($p < 0.05$ in all cases) (**Fig. 10D**).

For allogenic MSC-naïve co-cultures, similar Treg proliferation rates were observed after the first administration. However, a Treg increase was observed in the MHC-mismatched co-cultures in the re-exposure (from T4 to T6), and it was significant at T5 over T0, T1, T2, T3, and T7 ($p < 0.05$ in all cases) (**Fig. 10E**). Furthermore, the proliferation induced by MSC-naïve in the MHC-mismatched setting at T4, T5, and T6 was significantly higher compared to the MHC-matched group ($p < 0.05$ in all cases) (**Fig. 10E**).

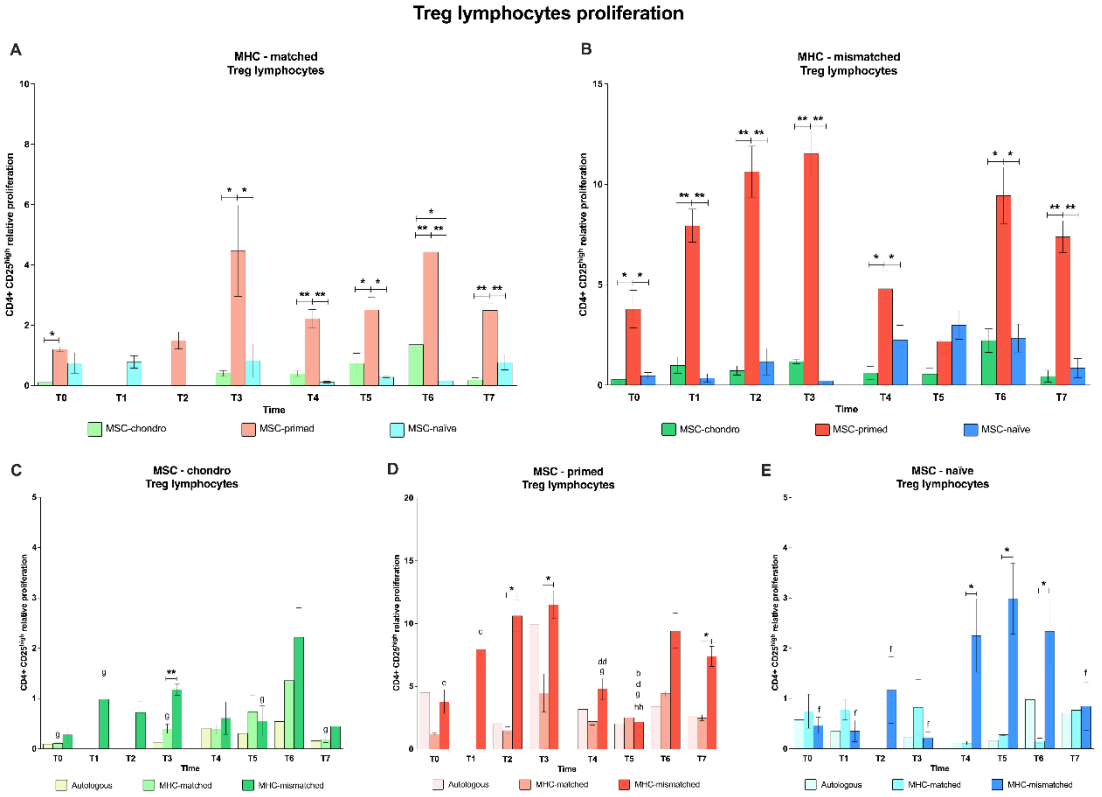


Fig. 10. Mean ± SEM of the relative proliferation of CD4+ CD25^{high} regulatory T cells in the immunogenicity assays (modified one-way mixed lymphocyte reaction) in MHC-matched (A) and MHC-mismatched (B) recipients following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Changes along time of non-activated PBLs from autologous, MHC-matched and MHC-mismatched recipients exposed *in vitro* to MSC-chondro (C), MSC-primed (D) and MSC-naïve (E). Proliferation of each PBL donor is normalized over the proliferation observed in the negative control (MLR M−, matched MLR) consisting of responder PBLs from the same donor exposed to MHC-matched stimulator PBLs (value 1), to account for inter-individual variability. Significant differences between cell-type and groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). Significant differences between time-points are represented by lower case letter: T1, ^{b}; T2, ^{c}; T3, ^{d}; T5, ^{f}; T6, ^{g} and T7, ^{h} (^{b}, ^{c}, ^{d}, ^{f}, ^{g}, $p < 0.05$; ^{hh}, $p < 0.01$).

CD3- Pan-Ig+ CD21+ B Cells: MSC-chondro and MSC-naïve promote the activation of B cells, whereas MSC-primed appears to have no stimulatory effect on them

The study found that in both allogenic combinations, MSC-chondro and MSC-naïve tended to increase the percentage of B cells over MSC-primed, especially after the second exposure (**Fig. 11**). B cells increase over MSC-primed was statistically significant for MSC-chondro in MHC-matched combinations at T6 over MSC-primed ($p < 0.05$) (**Fig. 11A**). In addition, in the MHC-mismatched co-cultures, both MSC-chondro and MSC-naïve co-cultures resulted in an increase in B cells compared to MSC-primed from T4 to T7 ($p < 0.05$ in all cases) (**Fig. 11B**).

Moreover, after re-exposure, an increase in B cells percentage was observed for MSC-chondro and MSC-naïve in both allogenic groups compared to the first exposure, but these differences were only statistically significant for MSC-chondro (**Fig. 11C**, **11E**). Specifically, a statistically significant increase in B cells was observed at T5 and T7

compared to T0 for MSC-chondro MHC-mismatched co-cultures ($p < 0.05$ in both times) and at T6 over T0 and T2 ($p < 0.05$ in both cases) for MHC-matched co-cultures (**Fig. 11C**). On the other hand, for MSC-naïve, although there were no significant differences after re-exposure, a B cells increase was observed at T3 over T0 in both MHC-matched ($p < 0.05$) and MHC-mismatched ($p < 0.01$) co-cultures (**Fig. 11E**). Overall, the study found that mismatched combinations tended to further increase the percentage of B cells over time, although the type of combination did not significantly increase the percentage of B cells.

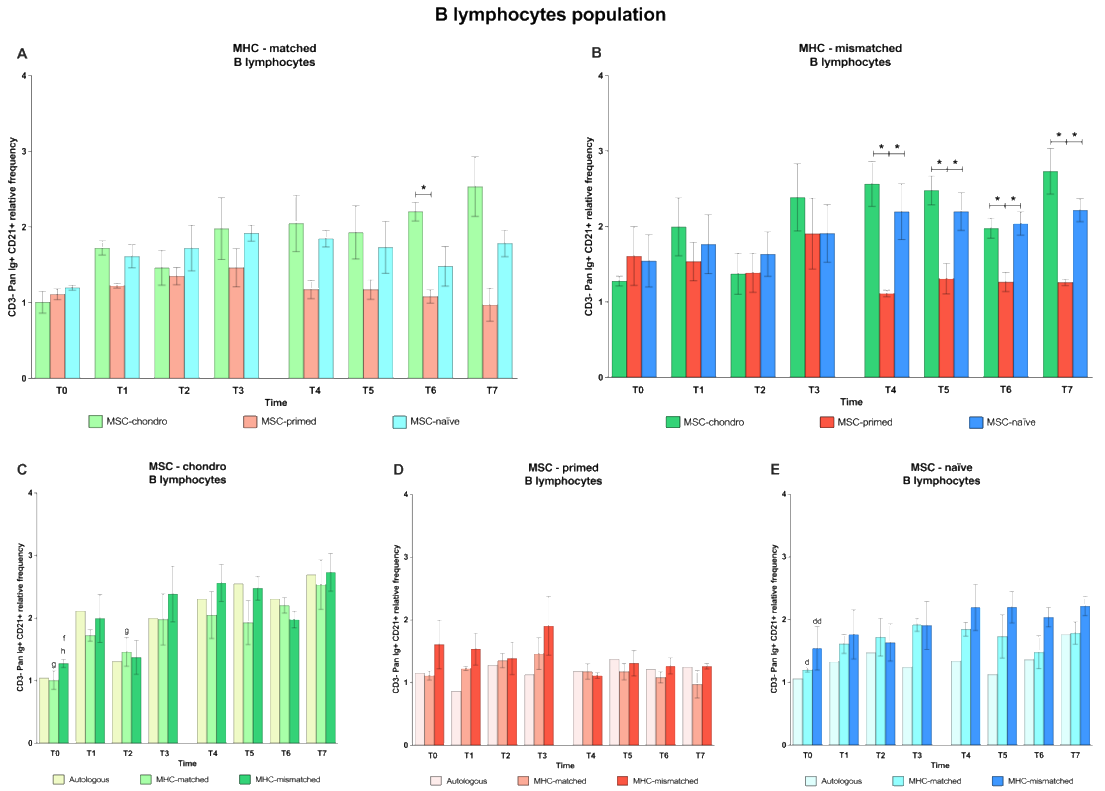


Fig. 11. Mean ± SEM of the relative frequency of CD3−-Pan-Ig+ CD21+ B cells in the immunogenicity assays (modified one-way mixed lymphocyte reaction) in MHC-matched (A) and MHC-mismatched (B) recipients following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Changes along time of non-activated PBLs from autologous, MHC-matched and MHC-mismatched recipients exposed *in vitro* to MSC-chondro (C), MSC-primed (D) and MSC-naïve (E). Data from each PBL recipient is normalized over the negative control (MLR M−, matched MLR) consisting of responder PBLs from the same donor exposed to MHC-matched stimulator PBLs (value 1), to account for inter-individual variability. Significant differences between cell-type at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$). Significant differences between time-points are represented by lower case letter: T3, ^{d}; T5, ^{f}; T6, ^{g} and T7, ^{h} (^{d}, ^{f}, ^{g}, ^{h}, $p < 0.05$; ^{dd}, $p < 0.01$).

In line with the findings from the analysis of B cells frequencies, the highest proliferation of B cells was observed in the co-cultures with MSC-chondro. In addition, the proliferation of B cells induced by MSC-naïve and MSC-primed was similar along time-points and combinations in the first exposure. However, following the second administration, both MSC-chondro and MSC-naïve promoted the highest proliferation of

B cells, whereas MSC-primed displayed the lowest proliferation rate in both allogenic groups (**Fig. 12**).

Regarding the differences between cell types in their ability to induce B cell proliferation, results showed that at T0 and T3, MSC-chondro induced a significantly higher B cell proliferation compared to MSC-primed and MSC-naïve in both allogenic groups ($p < 0.05$ in all cases) (**Fig. 12A**, **12B**). Additionally, in the MHC-mismatched group, MSC-chondro also induced a significant B cells proliferation increase at T1 compared to both MSC-naïve and MSC-primed ($p < 0.01$ in both cases) and at T2 compared to MSC-naïve ($p < 0.05$) (**Fig. 12B**). Also, in MHC-mismatched co-cultures, MSC-naïve showed an increase in B cell proliferation from T4 that became equal to MSC-chondro. The observed increase in B cell proliferation in the MSC-chondro and MSC-naïve co-cultures was statistically significant compared to MSC-primed at T4 and T5 ($p < 0.05$ in all cases) (**Fig. 12B**).

The pattern of B cells proliferation for MSC-chondro was similar between autologous, MHC-mismatched, and MHC-matched groups along almost all time-points analyzed. However, for MHC-mismatched MSC-chondro, an increase in B cells proliferation was observed at T1 compared to MHC-matched setting ($p < 0.05$) and compared to T2 ($p < 0.05$), but this increase was not repeated after re-exposure (T5) (**Fig. 12C**). In addition, at the last timepoint of the first exposure (T3), an increase in B cells proliferation induced by both allogenic MSC-chondro co-cultures was observed, but the increase was statistically significant only at T3 for MHC-matched over T2 ($p < 0.05$ in all cases) (**Fig. 12C**).

A similar pattern was observed for MSC-primed between autologous and allogenic groups over time-points. However, in the MHC-matched group, an increase in B cells proliferation induced by MSC-primed was observed at T1 over T0 and T5 ($p < 0.05$ in both cases) (**Fig. 12D**).

For MSC-naïve, both allogenic groups showed similar proliferation rates for B cells after the first administration. However, an increase was observed in MHC-mismatched groups from T4 and upon re-exposure to the same type of cells, and this increase was statistically significant at T4 over T1 ($p < 0.05$), also at T5 over T0 ($p < 0.05$) and at T6 over T0 and T2 ($p < 0.05$ in both cases) (**Fig. 12E**). It is worth noting that at the last timepoint (T7) in both allogenic MSC-naïve groups, an increase in B cells proliferation was observed that was statistically higher than at T0 ($p < 0.05$ in both groups), T1 (MSC-naïve MHC-mismatched, $p < 0.05$), T2 ($p < 0.05$ in both groups), T3 (MHC-mismatched, $p < 0.01$; MHC-matched, $p < 0.05$) (**Fig. 12E**). Despite the changes observed after re-exposure, these differences were only significant between MHC-mismatched and MHC-matched MSC-naïve conditions at T4 and T6 ($p < 0.05$ in both cases) (**Fig. 12E**).

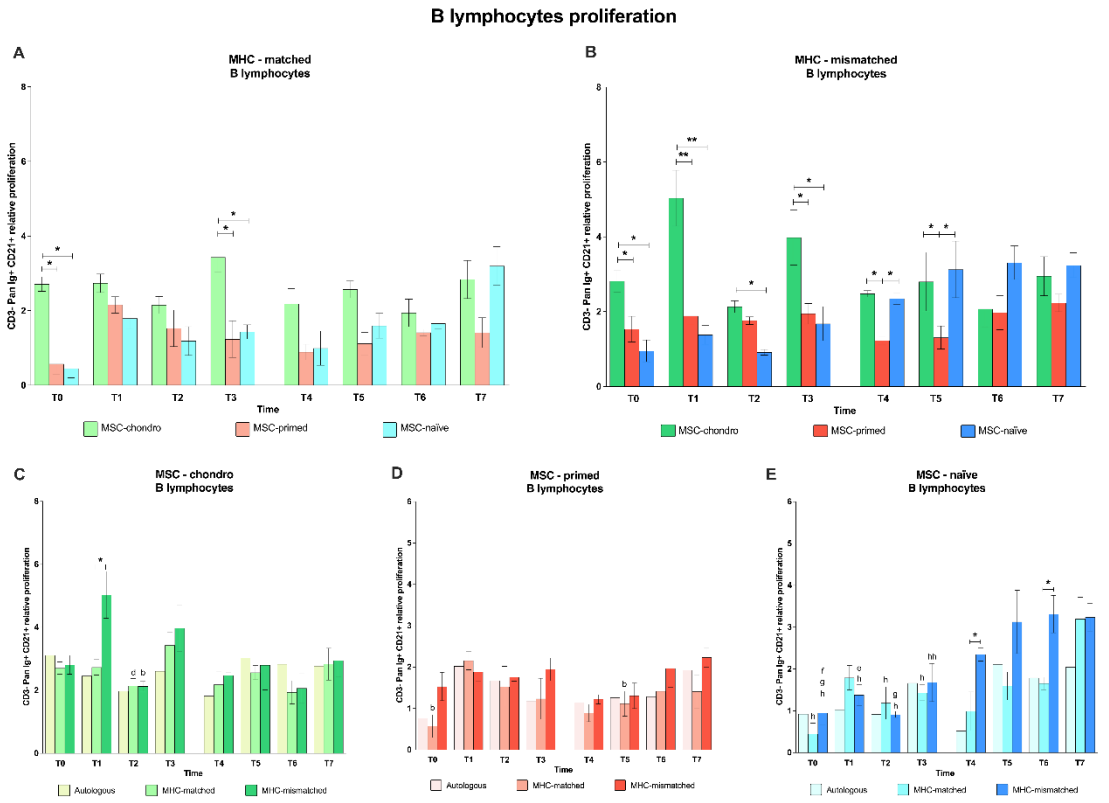


Fig. 12. Mean ± SEM of the relative proliferation of CD3−-Pan-Ig+ CD21+ B cells in the immunogenicity assays (modified one-way mixed lymphocyte reaction) in MHC-matched (A) and MHC-mismatched (B) recipients following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Changes along time of non-activated PBLs from autologous, MHC-matched and MHC-mismatched recipients exposed *in vitro* to MSC-chondro (C), MSC-primed (D) and MSC-naïve (E). Proliferation of each PBL donor is normalized over the proliferation observed in the negative control (MLR M−, matched MLR) consisting of responder PBLs from the same donor exposed to MHC-matched stimulator PBLs (value 1), to account for inter-individual variability. Significant differences between cell-type and groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). Significant differences between time-points are represented by lower case letter: T1, ^{b}; T3, ^{d}; T4, ^{e}; T5, ^{f}; T6, ^{g} and T7, ^{h} (^{b}, ^{d}, ^{e}, ^{f}, ^{g}, ^{h}, $p < 0.05$; ^{hh}, $p < 0.01$).

Interferon Gamma (IFNγ) production: MSC-primed induce IFNγ production, whereas MSC-chondro induce minimal IFNγ secretion

The concentration of IFNγ was measured in co-culture supernatants from all modified one-way MLR assays as reflection of lymphocyte activation. The values of IFNγ secretion produced by both allogenic MSC-primed co-cultures were higher compared to the other MSCs types and to the positive control (MLR-mismatched) along all the time-points analyzed.

Overall higher IFNγ secretion was observed in the co-cultures with MSC-primed, followed by MSC-naïve and MSC-chondro in both allogenic settings and along all the time-points (**Fig. 13**). Specifically, the IFNγ secretion induced by the MSC-primed was significantly higher compared to MSC-chondro in both MHC-matched and MHC-mismatched allogenic co-cultures at all the times analyzed on the first exposure: T0 ($p < 0.05$ in both cases), T1 ($p < 0.01$ in both cases), T2 (MHC-matched, $p < 0.01$; MHC-

mismatched, $p < 0.05$), T3 ($p < 0.05$ in both cases) and even before re-exposition (T4, $p < 0.05$ in both cases) (**Fig. 13A, 13B**). Moreover, MSC-primed MHC-mismatched also induced higher secretion of IFNγ over MSC-chondro at T6 ($p < 0.05$) and T7 ($p < 0.01$) (**Fig. 13B**). Although the IFNγ concentration detected in the MSC-naïve co-cultures was consistently higher than that observed in the MSC-chondro assays, the difference between them was only significant one week after each administration (T1, $p < 0.01$; T5, $p < 0.05$) in the MHC-matched co-cultures (**Fig. 13A**). Nevertheless, the presence of IFNγ in the supernatants from MSC-naïve co-cultures was lower compared to MSC-primed in both MHC-matched and MHC-mismatched settings before the first administration at T0 ($p < 0.05$ in both cases) and at T2, T3 and T4 in MHC-matched co-cultures ($p < 0.05$ in all cases) (**Fig. 13A**). Interestingly, IFNγ decreased in MHC-mismatched co-cultures with MSC-naïve compared to MSC-primed at T1 ($p < 0.05$) and T7 ($p < 0.01$) (**Fig. 13B**).

While IFNγ values in the MSC-chondro MHC-matched co-cultures were generally similar to the negative control (unstimulated PBLs alone) throughout the different times, those of the MHC-mismatched group were higher until T5 (**Fig. 13C**). However, a decrease in IFNγ concentration was observed at T6 and T7 compared to T1 ($p < 0.05$ in both cases) and compared to the equal times after the first administration (T2, $p < 0.01$; T3, $p < 0.05$) (**Fig. 13C**). Even the IFNγ decrease observed in the MHC-mismatched co-cultures was significantly lower at T6 compared to T5 ($p < 0.05$). In addition, a significantly higher IFNγ concentration was observed in the MHC-mismatched combination compared to the MHC-matched one after each administration (T1 and T5, $p < 0.05$) (**Fig. 13C**).

Regarding MSC-primed co-cultures, after the first administration, the values were very similar between autologous and allogenic co-cultures. However, after the second administration, IFNγ values in the MHC-mismatched co-cultures were significantly increased at T7 compared to T0, T1, T4 and T5 ($p < 0.05$ in all cases) (**Fig. 13D**). In contrast, after re-exposure, IFNγ values in MHC-matched co-cultures decreased compared to the first administration, with a significant decrease observed at T5 compared to T1 ($p < 0.05$) and at T6 compared to T2 ($p < 0.05$) (**Fig. 13D**). Although IFNγ values in the MHC-matched co-cultures were consistently lower compared to the MHC-mismatched co-cultures after re-exposure to MSC-primed, this decreased was statistically significant only at T7 ($p < 0.05$) (**Fig. 13D**).

MSC-naïve induced significantly higher IFNγ secretion at T1 compared to baseline and to later times after both the first and second administration in MHC-matched co-cultures (T0, T3, T4 and T5, $p < 0.05$ in all cases) (**Fig. 13E**). Interestingly, and even though IFNγ tended to be higher in MHC-mismatched co-cultures, MHC-matched MSC-naïve promoted higher levels at T1 ($p < 0.01$). Afterwards, in the MHC-mismatched co-cultures, IFNγ secretion increased progressively but only at T3 it was significantly higher than the baseline ($p < 0.05$) and also than in the MHC-matched group ($p < 0.05$) (**Fig. 13E**). However, after the second administration, a greater increase of IFNγ in MHC-mismatched over MHC-matched was observed (T4, T5 and T7, $p < 0.05$ in all cases). Moreover, this increase after the re-exposure to MHC-mismatched MSC-naïve was significantly higher at T7 than at T0, T1 and T3 ($p < 0.05$ in all cases) (**Fig. 13E**).

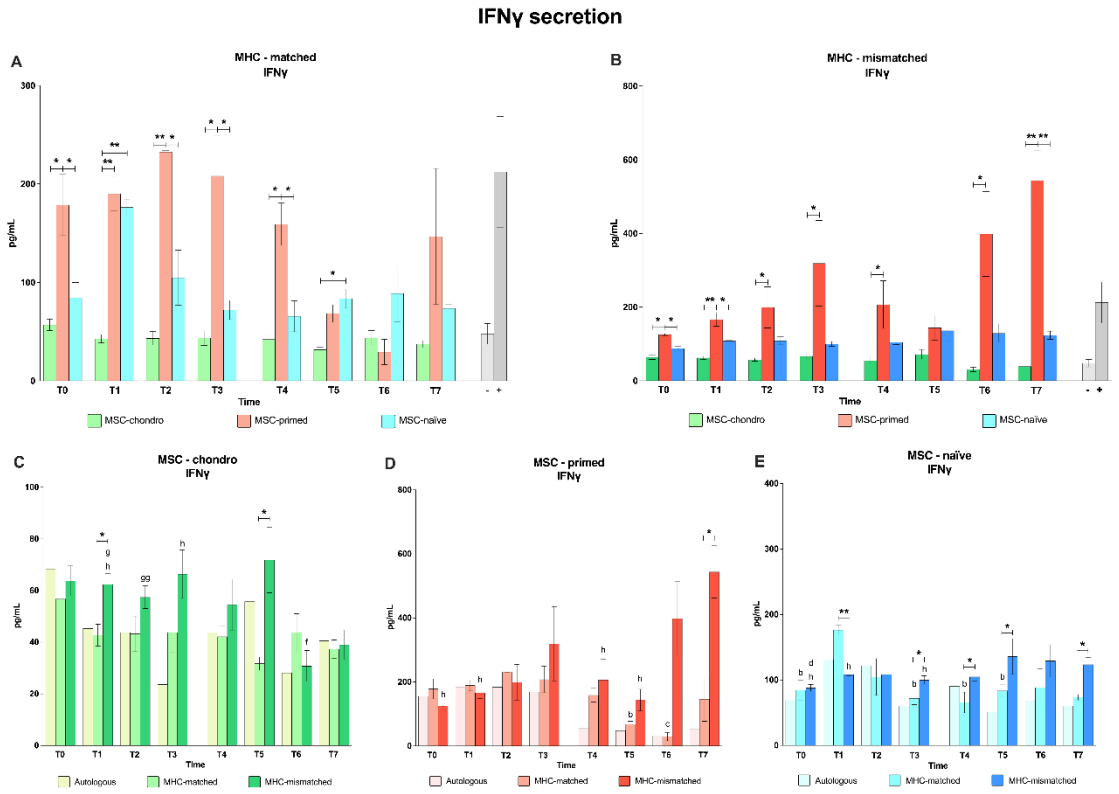


Fig. 13. Mean ± SEM concentration (pg/mL) of interferon gamma (IFNɣ) in co-culture supernatants from immunogenicity assays (modified one-way mixed lymphocyte reaction) in MHC-matched (A) and MHC-mismatched (B) recipients following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Concentration of IFNɣ in the supernatant from autologous, MHC-matched and MHC-mismatched co-cultures exposed *in vitro* to MSC-chondro (C), MSC-primed (D) and MSC-naïve (E). Significant differences between cell-type and groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). Significant differences between time-points are represented by lower case letter: T1, ^{b}; T2, ^{c}; T3, ^{d}; T5, ^{f}; T6, ^{g} and T7, ^{h} (^{b}, ^{c}, ^{d}, ^{f}, ^{g}, ^{h}, $p < 0.05$; ^{gg}, $p < 0.01$).

Discussion

To the best of authors' knowledge, this is the first study in the equine species assessing *in vivo* the systemic immune cellular response to allogenic MHC-matched and MHC-mismatched MSCs under different conditions: chondrogenic differentiated, pro-inflammatory primed and basal MSCs. The study aimed to investigate the effect of repeated allogeneic administration of different types of MSCs (MSC-primed, MSC-chondro, and MSC-naïve) on the percentages and proliferation of T and B cells subsets after *in vitro* re-exposure to the same cells (modified one-way MLR), in both MHC-matched and MHC-mismatched recipients.

Despite the limited number of studies on the ability of equine MSCs to stimulate an immune response in resting lymphocytes *in vitro* (Schnabel et al. 2014; Colbath et al. 2017; Kamm et al. 2021b), there is also a lack of research on the effects of MSCs on specific lymphocyte subpopulations in the horse. This is the first *in vivo* study in which the systemic cellular response and the development of cellular immune memory of different lymphocyte populations after co-culture with MSCs *in vitro* have been analyzed sequentially following two administrations of MSCs.

Overall, and in agreement with previous reports, co-culture assays conducted in this study showed that allogeneic MSCs do not stimulate marked changes on the proliferation and frequency of different lymphocyte subpopulations before any administration. Consequently, we hypothesized that the immunomodulatory mechanisms of the different MSC types would enable them to evade the immune system response to a varying degree upon administration, and we expected to observe this reflected in the co-cultures. Nevertheless, evaluating the immune response at various time points after repeated administration of equine MSCs *in vivo* revealed that MSCs primed with pro-inflammatory cytokines exhibited higher immunogenicity compared to MSC-naïve and MSC-chondro in both allogenic co-cultures.

We also hypothesized that MHC-matched MSCs would generate similar results than autologous ones, while MHC-mismatched MSCs would present increased immunogenicity. This effect was barely observed when analyzing the relative frequencies of the lymphocyte subsets, but it was clear when studying the proliferation rates: MHC-mismatched MSCs of any type tended to induce more T lymphocyte proliferation compared to MHC-matched MSCs. This effect was particularly obvious with MSC-primed at almost all time-points and for most of the T cell populations, as well as with MSC-naïve after the second administration. Additionally, although MSC-chondro cells induced the lowest effect on immune T cell proliferation, MHC-mismatched MSC-chondro co-cultures induce greater changes in cytotoxic proliferation rates over time compared to MHC-matched co-cultures.

This findings are consistent with previous results, where an increase in proliferation of T cells was observed when co-cultured with MHC-mismatched MSCs expressing high levels of MHC-II compared to MHC-matched MSCs (Schnabel et al. 2014) and MHC-mismatched MSCs with low MHC class II expression (Kamm et al. 2021b). Our findings with MSC-primed and MSC-naïve support this, suggesting that MHC-mismatched MSCs with elevated MHC-II expression induce greater lymphocyte activation *in vitro*. However, when Colbath *et al*. (2016) investigated the immunogenicity of autologous and allogeneic equine MSCs *in vitro*, their findings indicated that allogeneic MSCs were not inherently more immunogenic than autologous MSCs, as both types induced similar mild lymphocyte proliferation. In contrast, our *in vivo* study demonstrated a higher immunogenic response in cytotoxic and helper T lymphocytes over time in MHC-mismatched co-cultures, particularly when MSCs were primed. This finding suggests that the results observed *in vitro* do not always accurately reflect the immune response that occurs *in vivo*.

Before delving into further discussion regarding our findings, it is crucial to acknowledge the limitations of this study. The sample size utilized in the presented experiments is limited due to the challenges inherent in working with a large animal species like horses (Ribitsch et al. 2020), and particularly small in the MSC-primed administered to MHC-matched group (n = 2). Additionally, it is important to note that autologous group consisted of only three horses, as they were involved solely for control purposes as previously described (Pezzanite et al. 2015; Berglund y Schnabel 2017).

Allogenic administrations activated CD3+ T cells

Our results suggests that the activation of T cells by equine MSCs in different scenarios may depend on the MHC compatibility and the time after administration. The type of MSCs can have an impact on CD3+ T cell population, with MSC-chondro barely modifying CD3+ T cells and MSC-primed tending to increase their percentage regardless of the MHC compatibility throughout all times analyzed using *in vitro* co-cultures. However, the effect of the type of MSCs on T cell response is more marked in MHC-mismatched co-cultures. Furthermore, a second *in vivo* administration of the same allogeneic MSCs increased the CD3+ T cell proliferation rate in all the subsequent *in vitro* co-cultures, especially in MSC-primed and MSC-naïve MHC-mismatched co-cultures. However, we did not find significant differences in CD3+ proliferation among the three cell types in MHC-matched co-cultures, suggesting that MHC matching plays a crucial role in preventing immune cell recognition. In addition, although MSC-primed MHC-mismatched hardly induced CD3+ proliferation early in both exposures, in the long term it induces the highest CD3+ proliferation over MSC-chondro and MSC-naïve, suggesting that the effect of different MSC types on T cell activation may change over time.

Moreover, after the first administration (T1), MSC-naïve induced a proliferative response of T cells in both allogenic and autologous co-cultures compared to the baseline (T0), in line with what has been described by other authors following intradermal administration of equine MSCs (Carrade et al. 2011). In addition, MSC-naïve also showed the highest increase in MHC-mismatched co-cultures after (T5) re-exposure over MSC-primed and MSC-chondro, indicating that MSC-naïve can induce a stronger T cell response in a mismatched setting and may be more effective in re-stimulating T cells.

MSC-primed induce helper and cytotoxic lymphocytes, while MSC-chondro and MSC-naïve MSCs manage to evade them after first administration

Both MSC-naïve and MSC-chondro co-cultures induced the activation of T cells but prevent a higher proliferation of both cytotoxic and helper T cells in a similar way, suggesting a further activation of their immunomodulatory properties over MSCs-primed. Although the possibility of lower immunogenicity exerted by MSC-chondro and MSC-naïve after their administration should not be ruled out.

Our results suggest that equine MSCs administered *in vivo* seem to induce cytotoxic and helper T cell populations able to respond against them when the MSCs are encountered again, and different MSCs types and MHC compatibility can have varying effects on T cell populations over time. By this way, both allogenic MSC-primed co-cultures tend to induce a CD8+ and CD4+ T cells response compared to MSC-chondro and MSC-naïve co-cultures after the first administration. Previous research (Pigott et al. 2013) assessed the immune response after 120 days of autologous or allogeneic intra-articular injection of MSC-naïve in horses, and in agreement with our results, CD4+ population neither increased in MSC-naïve co-cultures.

However, the analysis of lymphocyte proliferation would provide more information than the population study (Cequier et al. 2022a). Interestingly, after the second administration (T5), MSC-chondro and MSC-naïve induces a higher response of both T cells after the second administration in the MHC-mismatched group. In contrast, also after the second administration, the cytotoxic and helper T cells did not increase their response against the both MSC-primed co-cultures compared to the first administration. However, it can be concluded that MSC-primed are the most immunogenic cell types in our study as induced the highest CD4+ T and CD8+ T cell response compared to MSC-chondro and MSC-naïve co-cultures in both allogeneic settings over time points. The increased immunogenicity of MSC-primed cells can be attributed to the overexpression of MHC-I and MHC-II following MSC priming, as reported in previous studies in the equine species (Schnabel et al. 2014; Cequier et al. 2022b).

Overall, the use of MHC-matched combinations prevents the increase of the percentage and proliferation of helper and cytotoxic T cells, especially after the second administration for the three-cell type administered. The lower immunogenicity displayed by MSC-chondro when administered in MHC-mismatched animals, compared to the other cell types, is consistent with findings reported in the equine species (Van Hecke et al., 2021) regarding the efficacy and safety of chondrogenically pre-differentiated MSCs after repeated administrations. Nonetheless, it remains to be determined whether the therapeutic effects of MSC-chondro after *in vivo* administration would be facilitated by MHC matching. Under the condition of our study, both allogenic MSC-chondro always induced the least proliferation of CD4+ and CD8+, but MHC-mismatched co-cultures were significantly higher than MHC-matched ones.

In addition, the finding in our study highlight the importance of considering MHC-matching when selecting MSCs-primed for therapy in allogeneic settings, as they would presumably be more immunoevasive. Furthermore, the administration of MSCs in inflammatory environments naturally enhances their immunomodulatory properties, which can be further amplified through the use of pro-inflammatory cytokines (Sivanathan et al. 2017; Voga et al. 2020). However, the immunogenic behavior observed with pre-stimulation may also lead to an increased immune response from the allogeneic MHC-mismatched recipient. However, further research is needed to determine the clinical relevance of these findings in the context of MSC primed-based therapies because the observed increase response in helper and cytotoxic at specific time points (three weeks after each administration). These observations may indicate that the administration of repeated doses of MSC-primed should be separated in time, as continuous re-exposure may impair its effectiveness.

Another possible explanation of the increase in T helper and cytotoxic T-lymphocyte proliferation during the later stages (3 to 6 weeks) after each administration could be attributed to the removal of each implant, leading to a decline in total *in vivo* MSCs. Each animal received a total of 15 million MSCs per administration, with 5 million being removed at every time point. Consequently, the upsurge in *in vitro* immunogenicity noticed during the later stages might be clarified by the dwindling immunomodulatory properties *in vivo*, which occurred as a result of the decreasing amount of MSCs administered. This hypothesis could

have been corroborated by co-culturing MSCs and PBLs 24/48 hours after removal of each MSC implant.

The results also indicate that after the second administration, the MSC-naïve MHC-mismatched group showed a sustained increase in helper and cytotoxic proliferation over time compared to the first exposure and compared to the matched group for both T cells. This finding has potential implications for the clinical use of MSCs, as the heightened cellular immune response observed in MHC-mismatched settings could potentially lead to the elimination of MSCs following a second administration. Therefore, it is advisable to consider using MHC-matched MSCs to mitigate such immune reactions in clinical application.

Despite both MSC-chondro and MSC-naïve prevented the proliferation of cytotoxic and helper T cells at all analyzed time-points compared to MSC-primed, their effects on Treg and B cells varied. Moreover, while MSC-primed induced a proliferative response in cytotoxic and helper T cells, also resulted in minimal activation of B cells and maximal proliferation of T regulatory cells.

MSC-primed cells have a greater capacity to induce Treg, particularly in the MHC-mismatched condition, whereas MSC-chondro may not be an appropriate choice for Treg induction

In our study, the results indicate that the effects of MSCs on Treg proliferation are complex and dependent on several factors, such as MHC matching, cell type, and time-points. An increase in Treg would be related to the immunosuppressive ability of MSCs, as these lymphocytes would help dampening the adaptive immune response and preventing rejection of foreign cells by the host (Kamm et al. 2021a). Overall, relative frequency and proliferation of Treg cells were consistently and significantly increased in MSC-primed compared to MSC-naïve and MSC-chondro in both allogenic co-cultures and time-points analyzed. Results also suggest that MSC-primed and MSC-naïve, especially in the MHC-mismatched groups, have a greater capacity to induce Treg, whereas MSC-chondro appears to have a less potent effect on Treg proliferation over time. However, the time-point of each administration also appears to play a role in Treg proliferation.

While Treg values remained high but similar along the time in the MSC-primed MHC-matched co-cultures, there were fluctuations over time in MHC-mismatched co-cultures. One week after the second administration (T5), a decreased Treg proliferation was observed compared to earlier time-points. This finding was accompanied by low CD8+ and CD4+ proliferation in T5, which may indicate that after a second administration, the MSC-primed MHC-mismatched were not as immunogenic as expected. In contrast, from three weeks after both MSC-primed administrations, the Treg response increased suggesting that this would be a later effect of MSCs.

Interestingly, in the MSC-naïve MHC-mismatched co-cultures the strongest Treg response was observed steadily over time after the second exposure compared to the

MHC-matched group. This suggests that the immunomodulatory effect of MSCs could be cumulative and increases with repeated exposure in MHC-mismatched conditions.

In addition, the Treg increase in both MSC-naïve and MSC-primed MHC-mismatched co-cultures aligns with the effort to increase the immune tolerance of both MSC types in MHC-mismatched setting, as an additional rise in helper and cytotoxic T cells was detected in both cases. The observed increase in Treg cells may potentially suppress T helper cell proliferation initially (Okeke y Uzonna 2019) in MSC-primed co-cultures, thereby exerting control over the immune response, but not in MSC-naïve ones.

In contrast, both allogenic MSC-chondro co-cultures showed the lowest Treg response throughout times. Furthermore, at the time-points when an increase in proliferation of helper and cytotoxic cells is observed, there does not appear to be a concurrent increase in Treg. This observation supports the notion that MSCs under different conditions employ distinct evasion mechanisms. In addition, it seems that MHC incompatibility would not promote such proliferation in Treg as we observed with the other types of MSCs along time-points analyzed. This could be attributed to the low immunogenic potential exhibited by the MSC-chondro, as well as their limited impact on Treg cell proliferation.

MSC-chondro and MSC-naïve promote the activation of B cells, whereas MSC-primed appears to have no stimulatory effect on them

For the population of B lymphocytes, which principal function is the production of antibodies against foreign antigens (Warrington et al. 2018), the MHC compatibility did not significantly affect the response of B cells, but the type of MSCs used did have an impact, with MSC-chondro and MSC-naïve tending to increase B cells response compared to MSC-primed. After re-exposure, an increase in B cells response was observed for MSC-chondro and MSC-naïve in both allogenic groups compared to the first exposure, suggesting that the effect of MSCs on B cells may be cumulative and influenced by the development of the humoral immune response.

However, MSC-primed showed the lowest B cell response over almost all time-points and even after the second administration. In agreement with our results, a previous study in humans MSCs (Luk et al. 2017) observed that MSC-primed inhibited the proliferation of B cells over MSCs-naïve. Moreover, MSC-primed induced the same levels of proliferation over time with no differences between the two allogenic groups, suggesting that repeated administrations would not induce B cell production by MSC-primed cells. In addition, although MSC-primed cells exhibit a high level of immunogenicity inducing the proliferation of both CD4+ and CD8+ T cells in a sustained manner, it seems that immunomodulatory properties of MSC-primed enable them to avoid triggering a humoral immune response. This suggests that the MSC-primed cells may have unique mechanisms to evade the host's immune system, which could be important for their therapeutic potential.

Before and after the first administration, both allogenic MSC-naïve groups showed similar proliferation rates for B cells, in agreement with previous studies where B cells

remained constant over the co-culture with allogenic MSCs (Kamm et al. 2021b). However, after repeated administration, MSC-naïve MHC-mismatched may not prevent B cell proliferation, which progressively increased over the course of the re-exposure. These findings would confirm that MHC incompatibility would make MSC-naïve more easily recognized after a second administration compared to MSC-chondro and MSC-primed, and that their immune-evasive mechanisms may not be sufficient.

On the other hand, MSC-chondro showed a consistently higher ability to induce B cell proliferation compared to MSC-naïve and MSC-primed, particularly in the first exposure for both allogenic setting. Overall, the pattern of B cell proliferation was similar between MHC-matched and MHC-mismatched co-cultures for MSC-chondro, except at T1 where there was a significant increase in proliferation for the MHC-mismatched setting. However, the increase observed in the MHC-mismatched group was not repeated after re-exposure. This suggests that the immunogenicity of MSC-chondro over B cells is not significantly affected by MHC matching along time. Only one study has investigated the capacity of chondrogenically induced equine allogeneic MSCs (ciMSCs) to activate CD3+ and CD138+ cells in the equine species, which showed no cellular immune response in co-cultures with ciMSCs and no observed immunomodulatory or immunogenic effect of ciMSCs (Van Hecke et al., 2021). However, it is important to note that the co-cultures were carried out for 5 days to a year and a half after treating 10 horses with ciMSCs, and the MHC-haplotype of the horses was not examined in allogenic co-cultures.

Overall, our results showed that differentiated MSCs did not provoke a marked proliferative response in resting lymphocytes, and it could suggest that MSC-chondro would not lose their regulatory ability and would not be recognized by the immune cells. However, MSC-chondro induced proliferation of B cells and showed the lowest ability to stimulate Tregs. In agreement with our results, a previous study in rat MSCs (Ryan et al. 2014) reported a significant increase in B cells when co-cultured with chondrogenically differentiated cells.

A possible explanation for why MSC-chondro induced the lowest proliferation of lymphocyte subpopulations but the higher increase in B cells could be that their immunomodulatory capacity was not sustained over time. Although MSC-chondro may go unnoticed by helper and cytotoxic lymphocytes after first administration, continuous re-exposure would activate B lymphocytes. However, while MHC incompatibility in MSC-chondro may not promote increased B cell proliferation after the second administration, it is possible that MSC-chondro were being targeted for destruction by cytotoxic lymphocytes. However, other possible explanations for these findings should be considered such as the ratio MSC-chondro:PBLs, the possible existence of undifferentiated MSCs in the MSC-chondro co-cultures and the impact of TGF-β3 on their immunogenic profile may have contributed to the prevent lymphocyte proliferation. These considerations were previously discussed in our earlier *in vitro* work (Cequier et al. 2022a), and their influence may have also contributed to the observed outcomes in this study.

Finally, in this study, the co-culture supernatants were analyzed to determine the presence of IFNγ, a pro-inflammatory cytokine produced by T lymphocytes associated with cell-mediated immunity. The secretion of IFNγ indicates the expansion of CD8+ or CD4+ effector and memory cells against donor MSCs (Berglund et al. 2017). However, some researchers suggest that the production of IFNγ can stimulate the regulatory ability of MSCs, leading to a decrease in immune cell proliferation (Clark et al. 2016; Cassano et al. 2018). The results presented in this study suggest that priming MSCs with pro-inflammatory cytokines prior to co-culture with PBLs can enhance their immunogenicity as measured by IFNg secretion, while PBLs co-cultured with MSC-chondro had the lowest IFNγ secretion. This could suggest further activation of lymphocytes in the presence of MSC-primed, as indicated by increased proliferation of CD8+ and CD4+ T cells in the study. However, it could also be considered the possible presence of residual exogenous IFNγ from the priming process. The secretion of IFNγ by lymphocytes in the presence of MSC-chondro supports the notion that equine MSCs maintain their regulatory properties and do not increase their immunogenicity after chondrogenic differentiation, as evidenced by the lowest CD8+ and CD4+ T cell proliferation.

Furthermore, the study found that the immunogenicity of the MSCs varied depending on whether the PBLs were MHC-matched or MHC-mismatched. MSC-primed cells induced significantly higher levels of IFNγ secretion in both MHC-matched and MHC-mismatched co-cultures compared to the other MSCs types and the positive control (MLR-mismatched), suggesting that priming can enhance the MSCs' ability to stimulate a T-cell response. Interestingly, after re-exposure to the MSCs, the IFNγ values in the MHC-matched co-cultures decreased compared to the first administration, while the MHC-mismatched co-cultures showed an increase in IFNγ secretion. This suggests that the MHC-mismatched response may have a memory effect that allows for a stronger immune response upon subsequent exposure.

Conclusions

Overall, these findings have important implications for the use of MSCs in cellular therapies and highlight the importance of carefully considering the cell type and culture conditions of MSCs when designing such therapies. Our finding indicated that the type of MSC used, and the degree of MHC-mismatch can have a significant impact on the immunogenic effects of equine MSCs. Further studies are needed to elucidate the mechanisms underlying the differential effects of MSCs on T and B cells proliferation to optimize their use in clinical applications.

Declarations

Ethics approval and consent to participate: not applicable

Consent for publication: not applicable

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MANUSCRITO VI

Allo-antibody production in response to repeated administration of equine mesenchymal stem cells (MSCs) in MHC-matched and MHC-mismatched recipients

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Abstract

Mesenchymal stem cells (MSCs) have the potential to be used to treat a wide range of equine pathologies, and their allogenic administration offers significant advantages over autologous therapies. However, MSCs are not truly immune-privileged but immune-evasive, and thus, their recognition and elimination by the immune system in the allogeneic setting should be considered. The balance between the immunomodulatory and immunogenic properties of MSCs is influenced by factors such as inflammation, chondrogenic differentiation, and compatibility with the major histocompatibility complex (MHC). These factors can either prevent or induce the development of immune memory mechanisms, which have implications for the clinical application of MSCs and may potentially limit their repeated administration.

The aim of this study was to assess the development of humoral immune response to equine MSCs, unmanipulated (MSC-naïve), pro-inflammatory primed (MSC-primed) and chondrogenically differentiated (MSC-chondro) by evaluating the production of cytotoxic allo-antibodies directed against donor's equine MHC after autologous, allogeneic MHC-matched and MHC-mismatched repeated administration. Equine MSC-naïve, MSC-primed or MSC-chondro were encapsulated in alginate hydrogels, and subcutaneously placed in either the donor horses (autologous) or in MHC-matched or MHC-mismatched recipient horses. The implants were removed after 1, 3, and 6 weeks, and the procedure was repeated in the same animals to evaluate the effect of a second administration. Serum samples were collected from each recipient before each administration and at each time point to assess antibody production by microcytotoxicity assays.

Autologous and allogeneic MHC-matched recipients did not develop antibodies against the MHC of the donor MSCs, regardless of having received MSC-naïve, MSC-primed or MSC-chondro. In contrast, MHC-mismatched horses developed allo-antibodies after the first administration and showed an increased antibodies production after the second

administration of the same MSCs. Although all three types of MSCs induced the development of a humoral immune response at MHC-mismatched receptors, MSC-chondro were the most recognized by the immune system.

The compatibility for MHC between donor and recipient is a key point to consider in order to avoiding the production of immune memory mechanisms against allogeneic MSCs, including antibody formation against donor's MHC. The immune response elicited against equine allogeneic MSCs and the factors influencing it must be further studied to develop more effective and safer cell therapies.

1 Introduction

Over the years, mesenchymal stem cells (MSCs) have captivated significant interest in the field of regenerative medicine in humans and various animal species, with a specific focus on their remarkable potential in the equine species. The therapeutic effectiveness of MSCs is now attributed primarily to their immunomodulatory properties, which involve both direct interactions between cells and signaling through paracrine mechanisms (Dias et al. 2019; Voga et al. 2020). The discovery of these properties has led to a major change in the previously held view of MSCs, which has increased the range of their therapeutic applications to inflammatory and immune-mediated conditions (Gao et al. 2016; Cequier et al. 2021; Prządka et al. 2021). Recognizing the profound implications of MSC immunomodulatory properties in treating inflammatory processes in horses, an increasing number of studies focus on investigating and understanding these immune-related aspects (Cassano et al. 2018; Caffi et al. 2020). Furthermore, the utilization of horses as an animal model in the realm of regenerative medicine research bestows significant value upon this species, as it plays a pivotal role in driving translational progress within the field (Colbath et al. 2017; Fortier et al. 2020; Ribitsch et al. 2020).

However, over the past 10 years, there has been intense debate about the safety and efficacy of allogeneic MSCs (Colbath et al. 2020b). The utilization of autologous MSCs presents several limitations, including the time-consuming process of isolation and culture before administration, as well as the potential complications arising from advanced age, genetic disorders, or concurrent illnesses of the individual (Carter-Arnold et al. 2014; Colbath et al. 2020a). To address these challenges, the establishment of allogeneic MSCs cell banks derived from healthy donors offers a valuable solution by providing readily available, well-characterized MSCs for early-stage administration in various pathologies. Additionally, the preservation of specific batches of characterized cells mitigates the variability associated with autologous cell use, facilitating a more homogeneous and expedited therapeutic approach due to reduced ex vivo expansion time (Cequier et al. 2021).

Although the use of allogeneic MSCs offer notable advantages, the potential recognition of MSCs by the immune system needs to be clarified. Initially, MSCs were considered immuno-privileged and unlikely to elicit an immune response. However, recent investigations have demonstrated that MSCs could be recognized by the immune system

of the recipient (Berglund et al. 2017). The recognition of allogeneic MSCs could have implications for the efficacy and safety of MSCs-based therapies, potentially limiting their repeated administration due to the development of humoral immune response (Rowland et al. 2021).

The recipient's immune response is influenced by different factors, including the inflammatory environment, the expression of major histocompatibility complex (MHC) class I and II antigens on MSCs, changes in MSCs following differentiation, MHC matching, and the tissue source of MSCs (Barrachina et al. 2018b; Broeckx et al. 2019; Caffi et al. 2020). In the context of equine MSCs, it has been observed that the administration of allogeneic MSCs to MHC-mismatched recipients can lead to the development of humoral immune responses and the production of allo-antibodies. In contrast, when MHC-matched recipients are involved, the immune system's interaction with the MSCs is comparable to that of autologous MSCs (Pezzanite et al. 2015; Berglund y Schnabel 2017; Rowland et al. 2021). Nevertheless, regardless of the MHC haplotype, the repeated administration of chondrogenic and tenogenic induced MSCs did not result in the production of allo-MSC antibodies (Van Hecke et al. 2021; Depuydt et al. 2022). However, other authors suggest that differentiation may affect the immunomodulatory properties of MSCs, rendering them unable to evade the immune system (Ryan et al. 2014; MacDonald y Barrett 2020).

In vitro pro-inflammatory priming of equine MSCs with proinflammatory cytokines like interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) can enhance their regulatory effect *in vivo* (Barrachina et al. 2018a). Nevertheless, repeated administration of pro-inflammatory primed MSCs may increase their immunogenicity through induced expression of MHC, rendering them more vulnerable to targeting by pre-existing antibodies. Nonetheless, partial MHC-compatibility between the donor and recipient may help mitigate the secondary humoral response after re-exposed to equine primed MSCs (Barrachina et al. 2020). However, there is limited knowledge regarding the immune response elicited against both MHC-matched and MHC-mismatched allogeneic equine MSCs, as well as the factors influencing this response, including inflammation and differentiation.

The objective of this study was to enhance our understanding of the immune response against allogeneic MSCs in horses by investigating humoral immune responses to equine MSCs in different states, including unmanipulated (MSC-naïve), pro-inflammatory stimulated (MSC-primed), and chondrogenically differentiated (MSC-chondro). This was achieved by assessing the production of cytotoxic allo-antibodies targeting the donor's MHC after repeated administrations of autologous and allogeneic MSCs, considering both MHC-matched and mismatched scenarios.

Equine MSC-naïve, MSC-primed or MSC-chondro were encapsulated in alginate scaffolds, and subcutaneously placed in either donor horses (autologous) or in MHC-matched or MHC-mismatched recipient horses. Each animal received three scaffolds with the same type of MSCs on one neck side that were removed after 1, 3 and 6 weeks. One month after removing the last scaffold, each animal was re-exposed to the same MSCs to

assess the development of immune memory mechanisms. Sera from all horses were collected at each time-point to assess the allo-antibody production by microcitotoxicity assays.

Our initial hypothesis was that repeated administration of equine MSCs would result in heightened immune recognition and increased production of allo-antibodies, with a more pronounced response in MSC-chondro recipients compared to MSC-primed recipients. Regarding the MHC compatibility, it was hypothesized that MHC-matched recipients would not develop allo-antibody production, while exposure to MHC-mismatched MSCs would lead to an increase in allo-antibody production.

2 Materials and Methods

2.1 Study design

Three horses were selected as MSCs donors due to their homozygous MHC haplotype, while 17 horses were selected as allogenic recipients (12 geldings, 7 mares and a stallion). For the MHC-matched group, 8 horses sharing one haplotype with one of the donors were selected, while the MHC-mismatched group consisted of 9 horses carrying different haplotypes from the donors.

Each group received allogenic equine MSCs under different conditions: MSC-naïve, MSC-primed and MSC-chondro. Additionally, each donor (n = 3), was utilized as an autologous control. Each horse received three alginate scaffolds containing 5×10^6 MSCs of the same type, which were placed subcutaneously on neck board. The three scaffolds were subsequently removed at different time-point (1, 3 and 6 weeks after implantation). One month following the removal of the last scaffold, each animal was re-exposed to the same type of MSCs (MSC-naïve, MSC-primed and MSC-chondro; MHC-matched or MHC-mismatched) on the opposite side of the neck to investigate the potential development of immune memory mechanisms under each condition.

In order to evaluate the humoral immune response, serum samples were obtained from each horse at specific time intervals. These time-points included: T0 (before the implantation of MSCs), T1 (one week after the placement of MSCs scaffolds), T2 (three weeks after the placement of MSCs scaffolds), T3 (six weeks after scaffold placement), T4 (one month after the removal of the last scaffold and just prior to re-exposure to the same MSCs on the opposite side of the neck), T5 (one week after the re-exposure), T6 (three weeks after the re-exposure), and T7 (six weeks after the re-exposure). Schematic timeline and methods are shown in **Figure 1**.

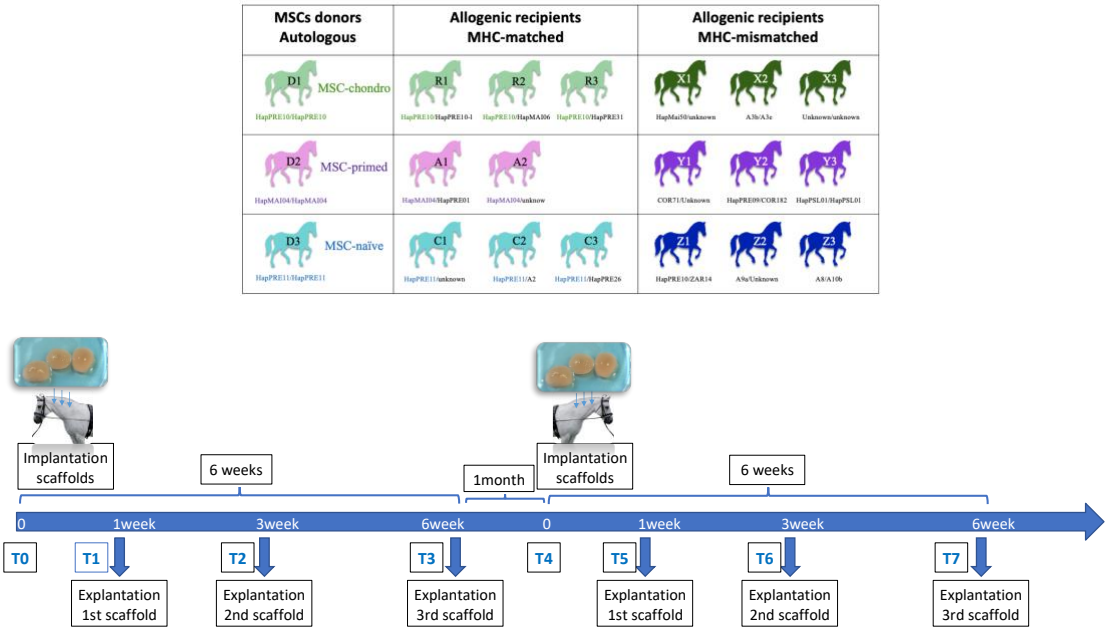


Figure 1. Schematic representation of the study design showing the equine MSCs donors and the MHC-matched and MHC-mismatched recipients. Equine MSC-naïve, MSC-primed or MSC-chondro were encapsulated in alginate hydrogels and three MSCs scaffolds with the same type of MSCs were subcutaneously placed in either the donor horses (autologous) or in MHC-matched or MHC-mismatched recipient horses. The scaffolds were removed at different time-point (1, 3 and 6 weeks) and one month following the removal of the last scaffold, each animal was re-exposed to the same type of MSCs. Sera collected from all recipients at different time-points were tested neat, 1:2 and 1:16 diluted against donor's MSCs as target cells using Microcytotoxicity assays.

2.2 Animal selection by MHC haplotyping

Twenty healthy horses aged between 2 to 12 years were selected based on their MHC haplotypes determined by microsatellite typing using a validated panel of 10 highly polymorphic intra-MHC regions, as previously described (Sadeghi et al. 2018; Barrachina et al. 2020). Definitive haplotypes were established for animals homozygous or with direct relatives (parent-offspring pairs or half-sibling groups) and the remaining animals were assigned with provisional haplotypes based on previously known ones reported in the bibliography (Rowland et al. 2018; Holmes et al. 2019; Cequier et al. 2022b).

In total, three groups of animals were selected for the study. Each group consisted of one homozygous horse for either the HapPRE10, HapPRE11, or HapMAI04 haplotype, and two or three heterozygous horses that shared one haplotype with the respective homozygous donor. Consequently, the homozygous horse within each group served as the MSCs donor due to its MHC compatibility with the heterozygous animals. In the MHC-mismatched group, a total of nine horses were chosen, each carrying haplotypes distinct from HapMAI04, HapPRE10, and HapPRE11 (**Figure 1**). The concept of utilizing homozygous individuals to match with various heterozygous individuals follows the same principle proposed for the establishment of haplo-banks of human induced pluripotent stem cells (iPSCs) (Sullivan et al. 2020).

All procedures involving animals were carried out under the Project License PI 15/16 approved by the in-house Advisory Ethics Committee for Animal Research from the University of Zaragoza. The care and use of animals were performed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63. All animals were kept on paddocks of the facilities of the Animal Research Service of the University of Zaragoza, with free access to water and fed with *ad libitum* grass hay.

2.3 Preparation of experimental MSCs for *in vivo* conditions: MSC-naïve, MSC-primed and MSC-chondro

Equine bone marrow MSCs were obtained and characterized, as previously described (Barrachina et al. 2017) as part of a previous study of our group (Cequier et al. 2022a). In brief, equine MSCs (n = 3) were isolated and expanded at 5,000 cells/cm^2 in conventional MSCs medium consisting in low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/mL streptomycin and 100 U/mL penicillin (all from Sigma–Aldrich) at 37 °C and 5% CO2.

The MSCs were then suspended at a concentration of 5 × 10^6 MSCs/ml in phosphate buffered saline (PBS) and combined in a 1:1 ratio with a 3% alginate solution. This resulted in a final concentration of 1.5% alginate in a total volume of 2 mL per scaffold as previously described (Watts et al. 2013). Scaffold polymerization was achieved as described earlier (de Vries-van Melle et al. 2014; Leijs et al. 2017; Santos et al. 2018) for 30 minutes into sterile 102 mM $CaCl_2$ (Sigma–Aldrich) at 37 °C in 5% CO2 conditions. For MSC-naïve and MSC-primed, scaffolds were cultured in conventional MSCs culture medium for 48 hours. For MSC-primed scaffolds, 12 hours before placement, 5 ng/mL of each pro-inflammatory cytokines TNFα and IFNγ (R&D Systems) were added to the conventional MSCs media as described earlier (Barrachina et al. 2018a). For MSC-chondro, chondrogenesis was induced after the encapsulation according to the methodology previously described (de Vries-van Melle et al. 2014; Ryan et al. 2014) for 21 days.

To ensure the removal of any xenogeneic antigens that might affect the immune response, all scaffolds (MSC-naïve, MSC-primed, and MSC-chondro) were washed twice with PBS and, 24 hours prior to placement, the FBS was substituted with autologous serum obtained from each recipient. This substitution aimed to render the MSCs devoid of xenogeneic antigens (Pezzanite et al. 2015). Subsequently, just before the intervention, scaffolds were again washed three times with PBS.

Autologous, MHC-matched and MHC-mismatched recipients received subcutaneous implantation of three alginate scaffolds containing the same type of MSCs (MSC-naïve, MSC-primed, or MSC-chondro) on the neck board (T0). The implants were subsequently removed at specific time intervals, namely 1, 3, and 6 weeks post-implantation (T1, T2, T3). After one month, each animal received a second administration of the same MSCs type on the contralateral neck board (T4). Subsequently, the implants were removed at

the same time intervals as before (T5, T6, T7). The purpose of this experimental design was to examine the impact of a secondary administration on the studied parameters.

In order to assess the humoral immune response, sequential collection of sera from all recipients was conducted prior to the extraction of each scaffold at the designated time points mentioned. The blood collection tubes were centrifugated at 1,200× g for 15 minutes and approximately 2-4 mL of serum per animal were obtained and stored at a temperature of −20 °C.

2.4 Preparation of target cells for microcytotoxicity assays

To perform the microcytotoxicity assays, cryopreserved MSCs obtained from the selected donors at passage 3 were thawed and cultured in conventional MSC culture medium at 37 °C and 5% CO2. Four days prior to conducting the microcytotoxicity assays, the serum component of the medium was substituted with a combination of 5% FBS and 5% natural horse serum (Pan-Biotech, Cat# P30-0701) tested for different equine diseases, free of xenogeneic antigens and previously decomplemented. After a 48-hour period, the medium was replaced with a fresh one containing only 10% equine serum for an additional 48 hours. Subsequently, the MSCs were detached from the plastic surface using 0.25% trypsin-EDTA (Sigma–Aldrich) and washed with PBS. The cell concentration was adjusted to 2×10^6 cells/mL in PBS to perform the microcytotoxicity assays immediately.

2.5 Microcytotoxicity assays

The microcytotoxicity dye exclusion assay, following established protocols (Schnabel et al. 2014; Berglund y Schnabel 2017; Barrachina et al. 2020), was conducted using equine MSCs as target cells to detect cytotoxic allo-antibodies against the MHC of the administered MSCs. The MSCs derived from each donor were tested against sera (neat, diluted 1:2 and 1:16) collected at the 7 designated time points from horses who received the corresponding MSCs as stated in the Study Design (**Figure 1**).

To establish a positive control, whereby cell death exceeding 80% could be observed, equine MSCs and serum obtained from a previous study of the group was used (Barrachina et al. 2018a, 2020). This positive control involved the mixing of sera collected from multiple animals, as it was determined that this specific combination resulted in a notably high percentage of cell death in the previously administered MSCs. Natural horse serum was used neat as a negative control in order to observe a cell death of less than 20%. Another control group was established where only cells from each donor were used to evaluate the MSCs in the absence of any serum challenge. This control enabled the assessment of MSCs in isolation, without any external serum influence.

Thawed sera from all horses were centrifuged at 12,000× g for 10 minutes at 4 °C and each serum sample was then evaluated undiluted as well as in serial dilutions of 1:2 and 1:16, using natural horse serum as the diluent. Subsequently, 1 µL of the MSCs suspension was incubated with the sera in Terasaki plates (One Lambda) under heavy mineral oil (Sigma–Aldrich). After 30 min RT incubation, 5 µL of rabbit complement

(One Lambda) was added and plates were incubated by an additional 1 hour at RT. Each well was stained with 2 µL of 5% Eosin Y dye (Sigma–Aldrich) for 5 minutes and fixed with 5 µL of 10% formalin (Sigma–Aldrich, pH 7.2–7.4) for 2 hours at 4 °C. For each condition (recipient, time, and serum dilution), as well as the control groups (positive, negative and cellular) three technical replicates were performed.

Within a timeframe of 12 hours, three authors (LB, AC, and EB) evaluated the percentage of death cells using an inverted phase contrast microscope (Nikon Eclipse TS100) at a magnification of x10. Live cells were identified by their refringent and rounded appearance, while dead cells exhibited a dark, flat, and irregular morphology. The cytotoxicity of each condition was based on the scoring system previously described (Pezzanite et al. 2015; Berglund y Schnabel 2017): grade 1 (<10% dead cells), grade 2 (10-19% dead cells), grade 4 (20-49% dead cells), grade 6 (50-80% dead cells), grade 8 (100% dead cells). Based on the analysis performed by these authors, cytotoxicity scores equal to or greater than 6 (indicating over 50% cell death) and scores equal to or lower than 2 (indicating less than 20% cell death) in neat serum were considered as evidence of substantial and insignificant presence of allo-antibodies in the sera, respectively. The average cytotoxicity score was calculated for each condition and used for subsequent statistical analyses.

2.6 Statistical analysis

Statistical analysis was carried out with the SPSS 26.0 software (SPSS, Inc). Interrater agreement was calculated with Cronbach's alpha for each type of target cells in each recipient group (MSCs-chondro MHC-matched and autologous, $\alpha = 0.948$; MSC-primed MHC-matched, $\alpha = 0.971$; MSC-naïve MHC-matched, $\alpha = 0.956$; MSC-chondro MHC-mismatched, $\alpha = 0.923$; MSC-primed MHC-mismatched, $\alpha = 0.919$; MSC-naïve MHC-mismatched, $\alpha = 0.920$), and thus, mean scores were calculated and used for subsequent statistical analysis. Descriptive analysis was performed by calculating overall percentage of animals with scores in neat sera over 6 (considered as significant antibody production) and below 2 (considered as non-significant antibody production) within each recipient group. Analytical statistics were performed to check differences in scores depending on the different study variables. The variables were "cell type" (MSC-naïve, MSC-primed and MSC-chondro), "recipients' group" (MHC-matched and MHC-mismatched) and "time-point" (eight categories: T0, T1, T2, T3, T4, T5, T6, T7) and "score" (three categories: score for neat sera, score for 1:2 diluted sera, score for 1:16 diluted sera). Score was set as the dependent variable and each other variable as factor to study differences as follows: (1) differences along time within each group of recipients for each type cells, (2) differences among types of target cells at each time-point within each group of recipients, (3) differences among recipient groups for each type of target cell at each time-point. Friedman test was performed for the first factor to study, Kruskal Wallis and Mann Whitney in the second and only Mann Whitney in the last one. Significance level was set at $p < 0.05$ for all analysis.

3 Results

The presence of cytotoxic allo-antibodies against equine MSCs (MSC-naïve, MSC-primed, MSC-chondro) administered to three groups of recipients (autologous, MHC-matched and MHC-mismatched) was assessed by microcytotoxicity assays confronting the serum of each receptor with the MSCs received. Autologous and allogeneic MHC-matched recipients did not develop relevant cytotoxic antibodies against the MHC of the MSCs donor, regardless of having received either of MSCs type (MSC-naïve, MSC-primed or MSC-chondro). Pre-existing antibodies were not detected, and all serum analysed along all time-points showed scores as 1 or 2, irrespective of the dilution tested. However, although no evidence of pre-existing antibodies was found in T0 in sera from animals receiving MHC-mismatched MSCs, higher scores were observed over time against the three MSCs types.

3.1 Heightened production of allo-antibodies following second administration in MHC-mismatched horses

Throughout the evaluated time-points, a progressive increase in cell death induced by allo-antibodies was observed among neat sera and dilution 1:2 of MHC-mismatched horses. Although 1:16 dilution scores followed the same trend, no significant changes were observed in sera scores along the time-points analysed (**Figure 2**). Conversely, in animals that received MHC-matched and autologous MSCs, no temporal escalation in cell death was observed in the recorded scores (**Figure 3**).

The scores obtained from neat sera in the MHC-mismatched recipients, regardless of the type of MSCs received, showed a significant increase compared to T0 at T5 (MSC-naïve and MSC-primed, $p < 0.05$; MSC-chondro, $p < 0.01$), T6 (MSC-primed and MSC-chondro, $p < 0.05$; MSC-naïve, $p < 0.01$), and T7 (MSC-primed and MSC-chondro, $p < 0.01$; MSC-naïve, $p < 0.05$) (**Figure 2A**). Furthermore, in MHC-mismatched horses administered with MSC-chondro and MSC-naïve, there was a significant increase in cell death at T5 ($p < 0.05$ in both cases), T6 (MSC-chondro, $p < 0.05$; MSC-naïve, $p < 0.01$), and T7 ($p < 0.05$ in both cases) when compared to T1. Although a similar trend was observed in MHC-mismatched horses receiving MSC-primed, the cell death increase was only statistically significant at T7 compared to T1 ($p < 0.01$) (**Figure 2A**).

Consistent patterns were observed in the serial dilutions of sera across the three groups of MHC-mismatched recipients, albeit with lower average scores. Overall, at the 1:2 dilution prior to the second exposure, there was a gradual increase in cell death observed across all time-points in the sera of MHC-mismatched horses (**Figure 2B**). However, this increase was only statically significant in sera of horses that received MSC-naïve at T3 and T4 compared to T0 ($p < 0.05$ in both time-points), as well as in sera recipients that received MSC-primed at T4 compared to both T0 and T1 ($p < 0.05$ in both time-points). However, in the case of MHC-mismatched horses administered with MSC-chondro, a notable increase in cell death was not observed until the second administration. This increase in cell death was statistically significant at T5 compared to T1, T2, and T3 ($p < 0.05$ at all time-points) (**Figure 2B**).

In addition, following the second administration, scores in 1:2 dilutions of sera obtained from MHC-mismatched recipients showed an increase compared to T0 along all time-points, as well as in undiluted sera. Notably, a significant increase in cell death compared to the pre-administration serum (T0) was observed at T5 (MSC-chondro, $p < 0.001$; MSC-primed, $p < 0.05$; MSC-naïve, $p < 0.01$) and T6 (MSC-chondro, $p < 0.05$; MSC-primed, $p < 0.01$; MSC-naïve, $p < 0.001$). Furthermore, an increase in cell death was also observed at T7 compared to T0 in the sera of horses that had received both MSC-chondro ($p < 0.01$) and MSC-primed ($p < 0.05$). Moreover, after the second administration, allogenic horses that received MSC-primed in MHC-mismatched setting significatively increased scores at T5, T6 and T7 compared to T1 (T5 and T7, $p < 0.05$; T6, $p < 0.01$) (**Figure 2B**). While horses that received MSC-naïve showed a significant score increase at T6 scores compared to T1 and T2 ($p < 0.05$ in both cases), there was also a significant decrease in cell death at T7 compared to T6 ($p < 0.05$), showing T7 scores like those obtained prior to the second administration (**Figure 2B**).

3.2 MHC-mismatched horses administered with MSC-chondro exhibited a higher production of allo-antibodies compared to horses that received MSC-naïve and MSC-primed

The analysis of cytotoxicity against donor-derived MSCs (MSC-naïve, MSC-primed, or MSC-chondro) previously administered in the MHC-mismatched group showed a higher percentage of cell death associated with the specific MSC type received at punctual time-points. Overall, this trend showed a higher percentage of cell death in MSCs obtained from the MSC-chondro donor compared to MSCs from MSC-naïve and MSC-primed donors (**Figure 2**). However, in the MHC-matched group, the recorded scores were in the range of 1 to 1.25 and no significant differences were found between the MSCs type received (**Figure 3**).

Significantly higher scores were observed at T1 in sera from horses who received MSC-chondro in MHC-mismatched setting compared to those who received MSC-naïve in both 1:2 ($p < 0.05$; **Figure 2B**) and 1:16 ($p < 0.05$; **Figure 2C**) dilutions. Furthermore, after the second administration, a higher cell death increased was also observed for MSC-chondro over MSC-naïve at T5 in neat ($p < 0.05$, **Figure 2A**), 1:2 ($p < 0.05$; **Figure 2B**), and 1:16 ($p < 0.05$; **Figure 2C**) dilutions as well as at T7 in neat ($p < 0.05$; **Figure 2A**) and 1:2 ($p < 0.05$; **Figure 2B**) dilutions. In addition, an increase in cytotoxicity of MSC-chondro compared to MSC-primed was also observed at T5 in neat sera ($p < 0.05$; **Figure 2A**). Regarding 1:2 dilution, it was also observed a cell death increase at T2 and T7 in MSC-primed compared to MSC-naïve ($p < 0.05$ in both cases; **Figure 2B**) but not in neat sera.

3.3 Horses receiving MSCs in MHC-mismatched combinations exhibited a consistently higher production of allo-antibodies compared to MHC-matched horses across all tested time-points

When comparing cytotoxic scores between MHC-matched and MHC-mismatched groups at each time-point, a consistent pattern was observed, indicating a general tendency for lower scores in the sera of allogenic and MHC-matched recipients regardless the MSCs type received (**Figure 3**). Notably, in the MSC-chondro group, a significant increase in cell death was observed in all MHC-mismatched recipients across all time-points and dilutions analysed, ($p < 0.05$ in all time-points; **Figure 3A**, **3D** and **3G**).

While a cell death increase was observed in the MSC-naïve groups when comparing MHC-mismatched to MHC-matched sera, this increase demonstrated statistical significance from T1 onwards in both neat ($p < 0.05$; **Figure 3C**) sera and 1:2 dilution ($p < 0.05$; **Figure 3F**). Furthermore, from T2 to T7, this increase remained significant across all three tested dilutions ($p < 0.05$ for all dilutions and time-points; **Figure 3C**, **3F** and **3I**). In the case of MSC-primed groups, it was observed that MHC-mismatched sera produced higher scores of cell death compared to MHC-matched sera at all analysed time-points. However, despite the absence of statistically significant differences, all the analysed significances had a *p*-value of less than 0.01 (**Figure 3B**, **3E** and **3H**).

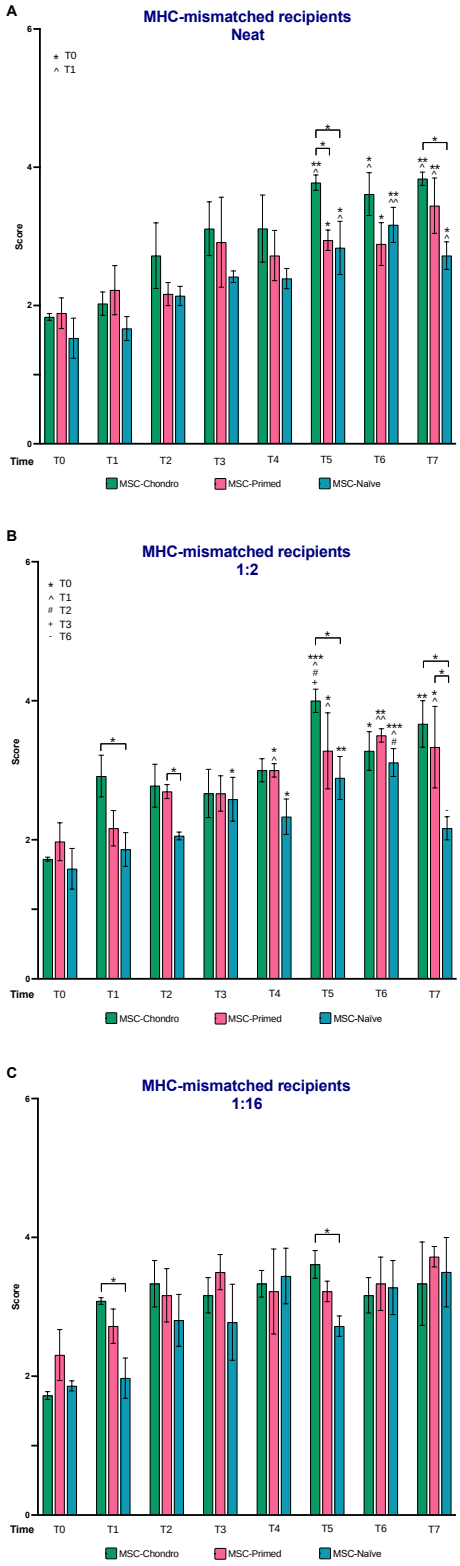


Figure 2. Evolution of cytotoxic scores along the time in each MHC-mismatched recipient group comparing the different MSC types administered. Mean ± S.E.M. of cytotoxic scores (Y axis) assigned to neat (**A**), 1:2 diluted (**B**), and 1:16 diluted sera (**C**) MSC-chondro (green bars), MSC-primed (pink bars) and MSC-naïve (blue bars) recipients along the time (X axis; T0, pre-administration of corresponding MSCs; T1, 1 week after first MSC administration; T2, 3 weeks after first MSC administration; T3, 6 weeks after first MSC administration; T4, just before the second MSC administration; T5, 1 week after second MSC administration; T6, 3 weeks after second MSC administration; T7, 6 weeks after second MSC administration). Significant differences between time-points compared with T0, T1, T2, T3 and T6 are respectively represented by asterisk (*), circumflex (^), hashes (#), cross (+) and hyphen (–) above the corresponding bar (*, #, ^, +, –, $p < 0.05$; **, ^^, $p < 0.01$; ***, $p < 0.001$). Significant differences between different target cells at one particular time are represented by a squared line with an asterisk (* $p < 0.05$).

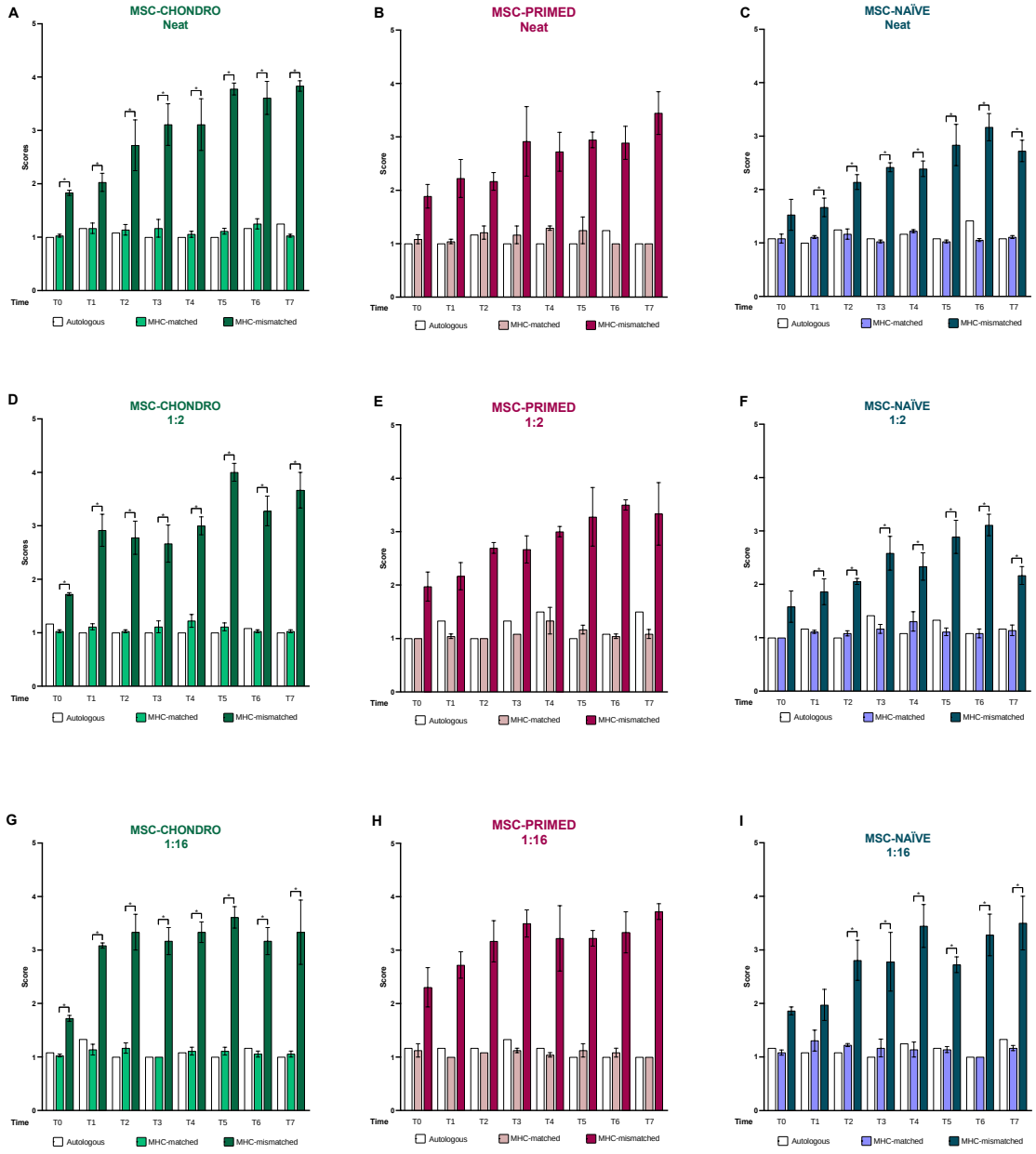


Figure 3. Evolution of cytotoxic scores along the time comparing the three recipients' groups (autologous, MHC-matched and MHC-mismatched). Mean ± S.E.M. of cytotoxic scores (Y axis) assigned to neat sera (top row; **A**, **B**, **C**), 1:2 diluted sera (middle row; **D**, **E**, **F**) and 1:16 diluted sera (bottom row; **G**, **H**, **I**) from recipients of MSC-chondro (green bars, left column; **A**, **D**, **G**), MSC-primed (pink bars, middle column; **B**, **E**, **H**) and MSC-naïve (blue bars, right column, **C**, **F**, **I**) along the time (X axis; T0, pre-administration of corresponding MSCs; T1, 1 week after first MSCs administration; T2, 3 weeks after first MSCs administration, T3, 6 weeks after first MSC administration, T4, just before the second MSC administration; T5, 1 week after second MSCs administration; T6, 3 weeks after second MSCs administration, T7, 6 weeks after second MSCs administration). Asterisks (*) point out statistically significant differences (* $p < 0.05$) between recipients' groups at one particular time-point.

4 Discussion

The findings of this investigation demonstrate that local application of allogeneic equine MSCs can trigger the generation of allo-antibodies with cytotoxic capacity targeted at the MHC molecules of the MSC donor. To the best of authors' knowledge, this is the first study assessing the development of humoral immune responses subsequent to repeated administration of distinct MSCs types in both MHC-matched and MHC-mismatched horses. Our results highlight the presence of an immune memory mechanism that may restrict the repeated administration of MSCs. It is noteworthy that the occurrence of a secondary humoral immune response was observed exclusively in the MHC-mismatched group after the second administration of the same MSCs, while it was not observed in MHC-matched or autologous recipients. Additionally, the study results indicate that the choice of MSCs type (MSC-naïve, MSC-primed, or MSC-chondro) should be considered in an MHC-mismatched scenario involving repeated administrations, whereas this consideration may not be as significant in autologous or MHC-matched settings.

Before delving into further discussion regarding our findings, it is crucial to acknowledge the limitations of this study. The sample size utilized in the presented experiments is limited due to the challenges inherent in working with large animal species like horse (Ribitsch et al. 2020), and particularly small in the MSC-primed administered to MHC-matched group (n = 2). This lower sample size in comparison to the other allogeneic groups might have hindered the detection of additional significant differences within this group, despite its alignment with the observed trends in other allogeneic MHC-matched groups. Additionally, it is important to note that autologous group consisted of only three horses, as they were involved solely for control purposes as previously described (Pezzanite et al. 2015; Berglund y Schnabel 2017).

This study also has a limitation regarding the type of MSCs used in the microcytotoxicity assays. While these MSCs originated from the same donor and passage as those administered *in vivo*, they were not of the same cell type as the ones received by the animals. Although the research team had previously conducted microcytotoxicity assays using MSC-naïve and MSC-primed as target cells (Barrachina et al. 2020), performing these assays with MSC-chondro presented several challenges and difficulties. The microcytotoxicity assay protocol requires exposing a defined number of MSCs to the serum obtained from the recipients. However, due to the distinctive morphology acquired by MSCs after differentiating into chondrocytes over a two-week period, accurate counting becomes unfeasible. Furthermore, distinguishing between live and dead MSC-chondrocytes poses a challenge as they exhibit vastly different morphologies, thereby impeding the appropriate assignment of scores. Additionally, the aim of microcytotoxicity assays is to detect allo-antibodies targeting the donor's MHC and previous studies in equine specie showed that microcytotoxicity assays yielded comparable allo-antibody production whether MSCs or peripheral blood leukocytes (PBLs) were used as target cells (Berglund y Schnabel 2017).

Previous investigations in horses have provided supporting evidence indicating that no production of antibodies occurred following repeated administrations of autologous

MSCs (Rowland et al. 2021; Rosa et al. 2022). Moreover, several authors have consistently reported the absence of humoral immune responses when donor and recipient were MHC-matched (Pezzanite et al. 2015; Berglund y Schnabel 2017; Rowland et al. 2021). In addition, these same studies assessed immune response development in MHC-mismatched horses, and in all cases, allo-antibodies were generated, regardless of whether the horses received one or two administrations of MSCs. However, the generation of allo-antibodies occurred at different time points.

Under the conditions of this study, there was a gradual but limited increase in allo-antibody production following the first administration in all MHC-mismatched groups. Overall, after the second MSCs administration at T5, the scores exhibited a significant increase compared to the first exposure, reaching the highest peak recorded in this investigation and maintaining elevated levels throughout the remaining time period analysed.

Previous research conducted in horses reported an earlier onset of allo-antibody production against MHC-mismatched MSCs. Specifically, these studies reported the onset of allo-antibody production within a timeframe of 7 days after the first administration of MHC-mismatched MSCs (Berglund y Schnabel 2017; Barrachina et al. 2020; Rowland et al. 2021; Rosa et al. 2022). Other studies documented the appearance of allo-antibodies approximately 14 days post-administration, with a range of 7 to 14 days and the antibody peak production was observed between 2 and 3 weeks after first administration of allogeneic MSCs (Pezzanite et al. 2015). Additionally, Owens et al. (2016) reported that the development of antibodies against equine allo-MSCs could occur within 3-4 weeks after multiple MSCs injections, although these antibodies tended to decrease over time in most of the horses analysed.

Consistent with previous investigations, our study demonstrated the progression increase of cytotoxic allo-antibodies over the analysed time-points in the sera of all MHC-mismatched recipients after the first administration but with a significant increase after the re-exposure, suggesting the development of both primary and secondary humoral responses. The increased cell death observed after re-exposure to allogenic MHC-mismatched MSCs indicates the development of an adaptive immune response to MSCs, as the response observed from T1 to T4 aligns with innate immune reactions *in vivo*. Interestingly, this dynamic was observed irrespective of the specific cell type administered in MHC-mismatched recipients, although horses receiving MSC-chondro exhibited the most pronounced and rapid increase in antibody production upon re-exposure at T5, T6 and T7.

However, recent investigations have assessed the humoral immune response in horses following repeated administration of allogeneic tenogenic primed MSCs (tpMSCs) and chondrogenic induced MSCs (ciMSCs) (Van Hecke et al. 2021; Depuydt et al. 2022). Both studies reported no presence of antibodies against ciMSCs or tpMSCs, indicating the absence of humoral response development after multiple injections. Although the flow cytometric crossmatch assay primarily detects antibodies against MSCs rather than donor-specific anti-MHC antibodies, the disagreement with earlier studies could be

attributed to variances in MSCs sources, MSC differentiation methodologies and the specific time-points analysed. Assessing the progression of the humoral response over time and considering the impact of each administration enables the identification of the onset, peak, and subsequent decline of antibody production. Consistent with findings from other researchers (Rowland et al. 2021), the sustained production of allo-antibodies may not persist in the MSC-naïve group at the long-term follow-up, as observed in our study at T7 compared to the previous time-points analysed. In addition, the low antibody production observed following repeated administration of allogeneic MSCs-naïve in two MHC-mismatched recipients highlights the importance of considering the potential inter-individual variability in the humoral response to MSCs among horses (Pezzanite et al. 2015).

Under the conditions of this study, the MHC-mismatched group that received MSC-naïve developed comparatively lower antibody production across multiple time points analysed. Furthermore, according to our initial hypothesis, MSC-primed administration to the MHC-mismatched group would result in a lesser humoral response compared to the MSC-chondro groups, and this hypothesis was confirmed following the second administration.

In the equine species, only one prior investigation, conducted by our research team, analysed the humoral response following two administrations of MSC-primed cells in recipients with MHC-halfmatched and MHC-mismatched profiles (Barrachina et al. 2020). The increase of immunomodulatory properties resulting from pro-inflammatory priming may have attenuated the development of a secondary humoral response in MHC-halfmatched recipients, presenting a potential advantage for allogeneic therapy. Nevertheless, an increased percentage of cell death was noted in the MHC-mismatched groups following each administration of MSC-primed cells, indicating a potential acceleration in the development of both primary and secondary humoral responses compared to the MSC-naïve group (Barrachina et al. 2020).

Based on the aforementioned findings, our present study demonstrated that MHC-mismatched recipients who received MSC-primed developed higher allo-antibody production compared to those who received MSC-naïve after the second administration. This finding suggests that the increased expression of MHC molecules on the surface of MSC-primed may contribute to an accelerated humoral response in MHC-mismatched recipients. Nevertheless, it is important to highlight that allogeneic MSCs have shown the capability to induce the production of allo-antibodies, even when they did not express MHC class II molecules on their surface (Pezzanite et al. 2015). Therefore, it is proposed that future studies incorporate allogeneic MHC-matched recipients as potential control groups to optimize the use of allogenic MSCs therapies.

Furthermore, a recent study investigated the repeated intra-articular administration of equine allogeneic MSCs in healthy recipients and in recipients with induced acute experimental synovitis (Rosa et al. 2022). Despite the potential increase of MHC II expression on MSCs in the induced lesion group due to inflammation, both the induced lesion group and the healthy group developed similar and elevated allo-antibody scores.

In spite of the similarity in scores between MHC-mismatched groups observed in our study following the initial administration, it is important to highlight that the recorded cell death after the first exposure ranged from 10-49%. Furthermore, after the second administration, the recorded scores remained lower than those reported in previous studies (Rowland et al. 2021; Rosa et al. 2022), with the highest scores reaching approximately 4, corresponding to 20-49% of cell death. The observed low scores may be attributed to the protective effect of alginate scaffolds utilized for MSCs administration (Leijs et al. 2017), which could have limited and delayed the recognition of MSCs by the host immune system. Additionally, Rowland et al. 2021 observed that A2 haplotype horses produced lower cell death scores compared to horses with other haplotypes following the second administration, despite all horses were MHC-mismatched to MSCs donors. While the observed variation could potentially be attributed to individual differences among animals, it is noteworthy that the MHC haplotype of both the donor and recipient, even in an MHC-mismatched scenario, may have conferred a degree of protection to the MSCs, rendering them less susceptible to immune targeting compared to MSCs with different haplotypes.

Furthermore, similarly to previous studies investigating allo-antibody production in different serum dilutions, including neat serum, 1:2 dilution, and 1:16 dilution, a decrease in scores that was directly proportional to the dilution analysed was observed (Berglund y Schnabel 2017; Rosa et al. 2022). However, it should be noted that when a 1:16 dilution of sera was analysed, certain differences in the results were not discernible, suggesting that higher dilutions of sera may render some results less visible.

In conclusion, these finding underscore the importance of MHC compatibility between the donor and recipient to mitigate the development of a humoral immune response that could compromise repeated allogenic MSCs therapies. Additionally, the repeated administration of different types of MSCs (MSC-naïve, MSC-primed, or MSC-chondro) did not impact the development of humoral immune responses in MHC-matched recipient horses. In contrast, in the MHC-mismatched groups, while different types of MSCs induced comparable lower primary humoral responses after first administration, MSC-chondro might be more easily recognized when repeatedly administered compared to MSC-primed and MSC-naïve. Future *in vivo* investigations are warranted to elucidate the implications of humoral immune development on the efficacy and safety of allogeneic therapies. Further research is also needed to investigate additional factors that contribute to evading the humoral immune response and MSCs recognition following repeated administrations in MHC-mismatched scenarios.

5 Ethics statement

The animal study was reviewed and approved by Advisory Ethics Committee for Animal Research from the University of Zaragoza (Project License PI 15/16).

6 Author Contributions

All authors (A.C., E.B., A.R., A.V., S.F., I.F., P.Z., M.B.S., C.R., F.J.V. and L.B.) substantially contributed to the conception and design of the study. A.C., L.B., E.B. and I.F. were responsible for acquisition of data. A.C., L.B., F.J.V., E.B. and C.R. were responsible for analysis and interpretation of data. A.R., A.V., S.F., M.B.S., E.B, I.F. and F.J.V. provided technical support. L.B. A.R., F.J.V. and C.R. provided important conceptual guidance. C.R. and P.Z. was responsible for obtaining funding. A.C. drafted the article and all authors (E.B., A.R., A.V., S.F., I.F., P.Z., M.B.S., C.R., F.J.V. and L.B.) revised the manuscript for important intellectual content. All authors (A.C., E.B., A.R., A.V., S.F., I.F., P.Z., M.B.S., C.R., F.J.V. and L.B.) approved the final submitted version of the article and agree to be accountable for all aspects of the work in ensuring that accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors have read and agreed to the published version of the manuscript.

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8 Abbreviations

ciMSCs: chondrogenic induced MSCs; DMEM: Dulbecco's Modified Eagle's Medium; FBS: Fetal bovine serum; IFNγ: Interferon gamma; iPSCs: Induced pluripotent stem cells; MHC: Major histocompatibility complex; MSC: Mesenchymal stem cell; PBL: Peripheral blood lymphocytes; PBS: Phosphate buffered saline; tpMSCs: Tenogenic primed MSCs; TNFα: Tumor necrosis factor alpha.

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DISCUSIÓN GENERAL

DISCUSIÓN GENERAL

A continuación, y antes de exponer las Conclusiones obtenidas, se procederá a realizar una breve discusión en conjunto de los seis Manuscritos presentados en la presente Tesis Doctoral.

La últimos avances han demostrado que las MSCs no solo ejercen su acción terapéutica a través de mecanismos de integración y diferenciación, sino que tiene especial relevancia sus propiedades paracrinas (Voga et al. 2020). Este cambio de paradigma ha impulsado la investigación sobre la aplicación de las MSCs en un número creciente de patologías, tanto en la especie equina como en la humana, tal y como se muestra en el Manuscrito I. En los resultados recogidos en este Manuscrito se destaca que además del sistema locomotor, las MSCs equinas resultan ser de interés para el tratamiento de numerosas patologías oftálmicas, respiratorias, reproductivas, tegumentarias, metabólicas y neurológicas. Asimismo, se destacan la uveítis recurrente equina, las enfermedades de la piel con procesos fibroproliferativos y el asma como posibles modelos de enfermedad para la investigación, debido a su semejanza con dichas afecciones en la especie humana.

Las propiedades inmunomoduladoras e inmunogénicas de las MSCs equinas desempeñan un papel crucial en su eficacia terapéutica y en su capacidad para evitar ser detectadas por el sistema inmunitario del receptor durante su administración alogénica (Voga et al. 2020). Además, dichas propiedades están fuertemente influenciadas por el entorno en el que se encuentran las MSCs, y su respuesta a dicho entorno puede mejorar su potencia induciendo una mayor capacidad reguladora o comprometer su seguridad y eficacia al ser reconocidas y eliminadas por la respuesta inmunitaria desencadenada (Berglund et al. 2017b).

En los resultados recogidos en el Manuscrito II de la presente Tesis Doctoral se valoró cómo la inflamación, la diferenciación y la compatibilidad para el MHC podían influir en las propiedades inmunológicas de las MSCs equinas *in vitro*. Estudios previos que habían evaluado la capacidad de las MSCs equinas estimuladas con citoquinas proinflamatorias para inhibir la proliferación de linfocitos alogénicos demostraron que dicha estimulación aumentaba este efecto inmunomodulador en comparación con las MSC-SE (Cassano et al. 2018b; Caffi et al. 2020). En los resultados recogidos en el Manuscrito II también se observó que la estimulación de las MSCs inducía la disminución de la proliferación de linfocitos T citotóxicos, T colaboradores y linfocitos B, por lo que dicha estimulación activaría su potencial regulador.

Sin embargo, las MSC-E, sobre todo condiciones MHC-incompatibles, aunque indujeron una respuesta proliferativa en las células T citotóxicas y colaboradoras, no activaron las células B. Asimismo, la mayor inmunogenicidad de las MSC-E estaría asociada al aumento de la expresión del MHC y estos resultados indicarían que la inflamación aumentaría el potencial inmunogénico de las MSCs y facilitaría su reconocimiento por

el sistema inmunitario del receptor a través de la inducción de la expresión del MHC. Es importante destacar que, contrariamente a la hipótesis inicial planteada, las MSCs equinas no perdieron su capacidad reguladora ni aumentaron su inmunogenicidad tras la diferenciación condrogénica. Sin embargo, las MSC-C mostraron una capacidad reducida de estimular las células T reguladoras y fueron las que más estimularon la proliferación de linfocitos B en comparación a las MSC-SE y MSC-E. Estudios previos en otras especies habían señalado que las MSCs murinas y equinas diferenciadas condrogénicamente mostraban un potencial inmunomodulador disminuido (Ryan et al. 2014; Van Hecke et al. 2021), en desacuerdo con los resultados recogidos en el Manuscrito II.

Comprender cómo las MSCs responden a estos estímulos es crucial para mejorar la eficacia de las terapias celulares tanto en medicina veterinaria como en medicina humana, pero todavía existe un conocimiento limitado sobre cómo las células del sistema inmune pueden influir en las MSCs equinas y qué implicaciones terapéuticas podría tener dicha influencia. Los resultados recogidos en el Manuscrito III mostraron que el cocultivo de MSCs equinas con linfocitos activados inducía un aumento de la expresión de genes que codifican moléculas inmunomoduladoras, así como un aumento de la secreción de dichas moléculas, especialmente en el caso de las MSC-E, sugiriendo que un entorno inflamatorio podría inducir un perfil regulador en las MSCs equinas.

Sin embargo, también se observó una activación moderada del perfil inmunogénico de las MSCs equinas, más marcada en el caso de las MSC-E cocultivadas con linfocitos MHC-incompatibles, lo que podría aumentar su capacidad de desencadenar una respuesta del sistema inmune. Cabe destacar que la mayor expresión de marcadores inmunogénicos parecía ir acompañada de una mayor activación del perfil regulador, lo que podría equilibrar la balanza entre ambas propiedades en las MSCs equinas.

Además, tanto la estimulación con citoquinas proinflamatorias como el cocultivo con linfocitos activados fueron capaces de inducir el perfil regulador de las MSCs equinas y, aunque la acción de ambos estímulos pareció ser aditiva en términos de la expresión de marcadores inmunomoduladores, también se observó un potencial efecto aditivo en relación con los marcadores de inmunogenicidad. Aunque dicha sobreexpresión podría facilitar el aloreconocimiento de las MSCs extrañas, la activación de los linfocitos contribuiría a facilitar su escape inmunitario al inducir el perfil regulador de las MSCs. Esta posible explicación está en consonancia con el concepto de equilibrio inmunomodulación-inmunogenicidad (Ankrum et al. 2014; Berglund et al. 2017b), según el cual las MSCs son capaces de evadir la respuesta inmunitaria equilibrando sus capacidades para suprimirla y activarla.

Resulta de gran importancia esclarecer qué sucede con el perfil inmunomodulador e inmunogénico de las MSCs tras haber sido expuestas al sistema inmune del caballo, algo que no había sido estudiado en la especie equina por la dificultad que supone recuperar las células una vez administradas. Dicha limitación se superó encapsulando las MSCs en

hidrogeles de alginato que permitieron su recuperación y posterior análisis, llevando a cabo el Manuscrito IV, cuyo objetivo fue evaluar el perfil inmunomodulador e inmunogénico de las MSC-SE, MSC-E y MSC-C en caballos MHC-compatibles y MHC-incompatibles, así como evaluar la respuesta inmunitaria local de los receptores a los diferentes tipos de MSCs tras dos administraciones subcutáneas en las tablas del cuello.

Las MSC-C y las MSC-SE mostraron mayores perfiles inmunomoduladores a lo largo de los diferentes tiempos estudiados, lo que indicaría un mayor potencial terapéutico de estos tipos de MSCs. Además, las MSC-C tras ser administradas tanto a caballos MHC-compatibles como MHC-incompatibles conservaron sus propiedades inmunomoduladoras, contrariamente a estudios previos en la especie equina (Van Hecke et al. 2021) pero en consonancia con los resultados obtenidos en el Manuscrito II. Además, al evaluar los parámetros histológicos en los grupos alogénicos a lo largo del tiempo, se observó que la compatibilidad para el MHC podía desempeñar un papel importante en la atenuación de la respuesta inmune local inducida por MSCs, particularmente con el uso de MSC-SE y MSC-C en entornos MHC-compatible.

Asimismo, las MSC-SE administradas a caballos MHC-incompatibles mostraron un mayor perfil inmunogénico y un menor perfil inmunomodulador junto a una mayor respuesta inmunitaria local en comparación con el grupo MHC-compatible tras ambas administraciones. Estos resultados coinciden con los observados en estudios previos en los que tras la administración intraarticular de MSC-SE en caballos MHC-incompatibles se observó una mayor respuesta inmunitaria humoral y local en comparación con los MHC-compatibles (Rowland et al. 2021). Los resultados obtenidos resaltan la importancia de considerar la compatibilidad para el MHC entre donante y receptor en el momento de aplicar terapias con MSCs equinas de este tipo.

A pesar de los resultados previos *in vitro* que evidenciaban un incremento en la expresión de *COX2*, *iNOS2* e *IL6* en las MSC-E (Cassano et al. 2018a; Caffi et al. 2020; Connard et al. 2021; Lee et al. 2021), los resultados presentados en el Manuscrito IV revelaron que tras la administración de MSC-E en ambos grupos alogénicos, no se observaba el aumento sostenido de la expresión de dichos genes inmunomoduladores, resultados que contradicen los hallazgos previos obtenidos en los ensayos *in vitro* descritos en los Manuscritos II y III. Sin embargo, sí que se mantuvo el perfil inmunogénico más elevado en las MSC-E en comparación con los otros tipos de MSCs estudiados, coincidiendo con los resultados observados en los Manuscritos II y III.

Los resultados obtenidos en el Manuscrito IV sugieren que el efecto inmunomodulador del tratamiento con citoquinas proinflamatorias podría no mantenerse a largo plazo, prevaleciendo en su lugar el efecto inmunogénico comprometiendo las capacidades inmunoevasivas de estas células. Asimismo, la limitada capacidad inmunomoduladora de las MSC-E podría deberse a la ausencia de un entorno inflamatorio adecuado, tal como se ha descrito en situaciones de lesión por investigaciones previas (Barrachina et al. 2018a). Además, los resultados del análisis histológico mostraron que el

reclutamiento linfocitario fue precoz y mayor en los caballos que recibieron MSC-E, lo que estaría en concordancia con la baja expresión de los genes que codifican para moléculas inmunomoduladoras responsables de inhibir la proliferación de células T por parte de las MSCs equinas (Carrade Holt et al. 2014), así como al aumento de las propiedades inmunogénicas.

En el Manuscrito V se valoró la respuesta inmune celular sistémica y la generación de respuesta celular de memoria reexponiendo *in vitro* los PBLs de los distintos receptores al mismo tipo de MSCs que recibió de su donante (MSC-SE, MSC-E o MSC-C) tras recibir dos administraciones locales de cada tipo de MSCs *in vivo*.

En todos los tiempos analizados, tras la primera y segunda administración, y en ambos grupos alogénicos las MSC-E indujeron la proliferación de los linfocitos colaboradores y citotóxicos. En cambio, las MSC-C y las MSC-SE no indujeron dicha proliferación tras su primera administración. Además, todos los tipos de MSCs reexpuestas a PBLs MHC-incompatibles tendieron a inducir un aumento de la proliferación de linfocitos T, pero este efecto fue más marcado en las MSC-E en casi todos los tiempos y subpoblaciones linfocitarias, así como en las MSC-SE tras la segunda administración.

Sin embargo, las MSC-E mostraron una mayor capacidad para inducir la proliferación de los linfocitos T reguladores a lo largo de todos los tiempos de estudio, sobre todo, en la condición MHC-incompatible, coincidiendo con los resultados observados en el Manuscrito II. Cabe destacar que las MSC-C y las MSC-SE en los cocultivos MHC-incompatibles promovieron la activación de las células B, mientras que las MSC-E fueron las que menor efecto tuvieron sobre ellas.

Estos resultados indicarían que, si bien las MSC-E mostraron una mayor inmunogenicidad al promover la proliferación sostenida de células T colaboradoras y citotóxicas, sus propiedades inmunomoduladoras les permitirían evitar la inducción de una respuesta inmune humoral. Asimismo, los resultados respaldan investigaciones previas que señalaron que las MSC-E pueden poseer mecanismos singulares para evadir el sistema inmunológico del receptor, lo cual podría ser de gran relevancia para su potencial terapéutico (Luk et al. 2017; Barrachina et al. 2018a).

En general, los resultados recogidos en el Manuscrito V, de la misma manera que los del Manuscrito II mostraron que las MSCs diferenciadas no inducían una marcada respuesta proliferativa en los linfocitos de los receptores, por lo que se corroboraría que las MSC-C no perderían su capacidad reguladora. La menor inmunogenicidad mostrada por las MSC-C en los cocultivos MHC-incompatibles en comparación con los otros tipos celulares concuerda con los hallazgos observados en la especie equina en relación a la eficacia y seguridad de estas MSCs tras administraciones repetidas (Van Hecke et al. 2021). No obstante, sería necesario determinar si los efectos terapéuticos de las MSC-C tras su administración *in vivo* mejorarían con la utilización de donantes MHC-compatibles.

Asimismo, como se había también observado en los resultados recogidos en el Manuscrito II, las MSC-C cocultivadas con PBLs MHC-incompatibles fueron las que mayor proliferación de linfocitos B indujeron y a su vez menor influencia para estimular las T reguladoras tuvieron. Una posible explicación de por qué las MSC-C indujeron la menor proliferación de subpoblaciones linfocitarias pero el mayor aumento de linfocitos B podría ser debida a que su capacidad inmunomoduladora *in vivo* no se mantuvo en el tiempo y por ello podrían ser más fácilmente reconocidas al reexponerlas a los PBLs *in vitro*.

En el Manuscrito VI se recogen los resultados de la valoración de la producción de aloanticuerpos citotóxicos frente a los distintos tipos de MSCs administradas (MSC-SE, MSC-E, MSC-C) en las diferentes condiciones (receptores autólogos, MHC-compatibles, MHC-incompatibles). Los resultados de este manuscrito indicaron que la administración local de MSCs equinas alogénicas podía inducir la producción de aloanticuerpos, lo que supone un mecanismo de memoria inmune que podría limitar su uso repetido. Esta producción de aloanticuerpos podría prevenirse si el donante y el receptor fueran MHC-compatibles, ya que solo se detectaron niveles de citotoxicidad relevantes (>20%) en receptores MHC-incompatibles. En consonancia con estos resultados, en estudios recientes en la especie equina tampoco se detectaron anticuerpos citotóxicos en los caballos que recibieron MSCs MHC-compatibles, pero los niveles de anticuerpos aumentaron notablemente tras la segunda inyección de MSCs MHC-incompatibles (Rowland et al. 2021).

La ausencia de desarrollo de anticuerpos citotóxicos frente a las MSCs en receptores MHC-compatibles indicaría la importancia de considerar la compatibilidad del MHC y la respuesta inmunitaria humoral en la terapia alogénica con MSCs (Pezzanite et al. 2015). Asimismo, aunque la compatibilidad del MHC entre donante y receptor podría ser de gran importancia en la administración de MSCs alogénicas, parece que el tipo de MSCs administradas no influye en esta respuesta. Sin embargo, la generación de aloanticuerpos frente a las MSC-SE y MSC-E podría contribuir a explicar por qué en algunos estudios previos *in vivo* el efecto terapéutico no se mantuvo a largo plazo (Barrachina et al. 2020).

Los resultados obtenidos en los 6 Manuscritos de esta Tesis Doctoral enfatizan la importancia de llevar a cabo investigaciones *in vivo* con el fin de comprender en detalle las complejas interacciones entre las MSCs equinas y el sistema inmunitario del receptor. Es necesario llevar a cabo más investigaciones para explorar otros factores y mecanismos que puedan contribuir a la capacidad de evasión inmunitaria de las MSCs equinas, y de esta manera, optimizar su potencial terapéutico. Esto permitirá ampliar el conocimiento sobre las MSCs y su aplicación clínica en el futuro para el desarrollo de terapias celulares efectivas y seguras.

VI. CONCLUSIONES / CONCLUSIONS

VI. CONCLUSIONES

En las condiciones de nuestros estudios y con base a los resultados obtenidos, pueden establecerse las siguientes conclusiones:

1. El avance en el conocimiento de las propiedades inmunomoduladoras de las MSCs equinas ha promovido la investigación en patologías oftálmicas, neurológicas, respiratorias y cutáneas. Se ha propuesto el caballo como modelo animal para el estudio de la uveítis recurrente, las afecciones fibroproliferativas de la piel y el asma en la especie humana.

2. La estimulación de las MSCs equinas mediante citoquinas proinflamatorias (TNFα e IFNɣ) activa su potencial inmunomodulador *in vitro*, pero también induce la expresión de moléculas inmunogénicas, sobre todo en condiciones de incompatibilidad para el MHC, hecho que podría facilitar su reconocimiento inmune cuando fueran utilizadas en terapias alogénicas.

3. El efecto inmunomodulador de la estimulación con citoquinas proinflamatorias observado *in vitro* no se conserva *in vivo*, pero prevalece el perfil inmunogénico comprometiendo la capacidad inmunoevasiva de las MSCs estimuladas.

4. Tras inducir la diferenciación de las MSCs equinas a condrocito, éstas no pierden sus propiedades inmunomoduladoras ni aumentan las inmunogénicas *in vitro*, aunque la diferenciación puede favorecer el desarrollo de mecanismos de memoria inmunológica.

5. Las MSCs diferenciadas a condrocito conservan sus propiedades inmunomoduladoras tras ser administradas tanto en caballos compatibles como incompatibles para el MHC, pero la compatibilidad para el MHC atenúa la respuesta inmune local inducida.

6. Tras la administración repetida de MSCs sin estimular en caballos incompatibles para el MHC, las MSCs desarrollan un perfil más inmunogénico y menos inmunomodulador, que se relacionaría con una mayor respuesta inmunitaria local en comparación con la administración de MSCs compatibles.

7. Las condiciones en que se administran las MSCs incompatibles producen diferente respuesta celular: mientras que la exposición inflamatoria promueve la activación de linfocitos T, las MSCs sin estimular y la diferenciación condrogénica activan en mayor medida a los linfocitos B, favoreciendo el desarrollo de una respuesta celular de memoria.

8. La administración de MSCs compatibles para el MHC no induce producción de aloanticuerpos, independientemente de las condiciones a las que se han sometido las MSCs. Por el contrario, en receptores incompatibles, sí que se induce una respuesta humoral que además se intensifica después de una segunda administración, siendo las MSCs diferenciadas las que inducen mayores niveles de aloanticuerpos.

CONCLUSIONS

Under the conditions of our studies and based on the obtained results, the following conclusions can be drawn:

1. Advances in understanding the immunomodulatory properties of equine MSCs have driven research in ophthalmic, neurological, respiratory, and cutaneous pathologies. Horses have been proposed as an animal model for studying recurrent uveitis, fibroproliferative skin disorders, and asthma in humans.

2. Stimulation of equine MSCs with proinflammatory cytokines (TNFα and IFNɣ) activates their immunomodulatory potential *in vitro*, but it also induces the expression of immunogenic molecules, especially under conditions of MHC incompatibility. This could facilitate their immune recognition when used in allogeneic therapies.

3. The immunomodulatory effect observed *in vitro* after stimulation with proinflammatory cytokines is not preserved *in vivo*, but the immunogenic profile prevails, compromising the immune evasive ability of stimulated MSCs.

4. After inducing the differentiation of equine MSCs into chondrocytes, they do not lose their immunomodulatory properties or increase their immunogenicity *in vitro*, although differentiation may promote the development of immunological memory mechanisms.

5. Mesenchymal stem cells differentiated into chondrocytes retain their immunomodulatory properties when administered in horses MHC-matched or MHC-mismatched, but MHC compatibility attenuates the induced local immune response.

6. After repeated administration of unstimulated MSCs in MHC-mismatched horses, MSCs develop a more immunogenic and less immunomodulatory profile, which is related to a greater local immune response compared to the administration of matched MSCs.

7. The conditions under which MHC-mismatched MSCs are administered lead to different cellular responses: while inflammatory exposure promotes the activation of T lymphocytes, unstimulated MSCs and chondrogenic differentiation more strongly activate B lymphocytes, favoring the development of a cellular memory response.

8. Administration of MHC-matched MSCs does not induce the production of alloantibodies, regardless of the conditions to which the MSCs have been subjected. In contrast, in MHC-mismatched recipients, a humoral response is induced, which intensifies after a second administration, with differentiated MSCs inducing the highest levels of alloantibodies.

VII. ANEXOS

Anexo I: Informe aprobación de la Comisión Ética Asesora para la Experimentación Animal de la Universidad de Zaragoza

Comisión ética asesora para la experimentación animal
Universidad Zaragoza

COMISIÓN ÉTICA ASESORA PARA LA EXPERIMENTACIÓN ANIMAL
REGISTRO DE SALIDA
Nº 328 Fecha 23/06/16
UNIVERSIDAD DE ZARAGOZA

Ref. PI15/16

CONTROL DE BUENA PRÁCTICA EN EXPERIMENTACIÓN ANIMAL

Título del procedimiento de experimentación:

Optimización del uso de MSCs alogénicas en el tratamiento de patologías articulares equinas: equilibrio inmunomodulación-inmunogenicidad

Título del proyecto en el que se incluye:

Optimización del uso de MSCs alogénicas en el tratamiento de patologías articulares equinas: equilibrio inmunomodulación-inmunogenicidad

Tipo de proyecto: II **Centro de realización:** ES 50 297 0012 006 (SAEA) y OTROS: HV

Investigador responsable: Francisco José Vázquez Bringas

Duración: 3 años **Fecha estimada de inicio del procedimiento:** Enero, 2017

Animales que implica:

especie (s): Caballos, asnos e híbridos (*Equidae*)

peculiaridades: Adultos (4-15 años), distintos sexos y razas

número: 24

Severidad: Leve **Evaluación retrospectiva:** NO

Fecha de presentación: 8 de abril de 2016

Reunida la Comisión Ética Asesora para la Experimentación Animal* el día 18 de mayo de 2016, y una vez revisada la documentación disponible en relación al procedimiento de experimentación descrito, considera:

QUE EL PROCEDIMIENTO CUMPLE LOS PRINCIPIOS ÉTICOS Y DE PROTECCIÓN DE LOS ANIMALES UTILIZADOS PARA EXPERIMENTACIÓN QUE SE HA IMPUESTO LA UNIVERSIDAD DE ZARAGOZA, Y SE ADAPTA A LA NORMATIVA VIGENTE (R.D. 53/2013). Por todo ello, se emite **informe FAVORABLE****.

Esta evaluación será válida únicamente para ESTE PROCEDIMIENTO y durante un periodo de tiempo de cinco años, salvo modificación sustancial del procedimiento presentado. La realización del procedimiento queda supeditada igualmente a la disponibilidad de las instalaciones necesarias en el momento en que se plantee la realización del mismo.

*Órgano habilitado para realizar la evaluación y evaluación retrospectiva (Resolución de 6 de agosto de 2013 de la Dirección General de Alimentación y Fomento Agroalimentario del Gobierno de Aragón).

****Observaciones:** Precisa autorización del órgano competente para destino final de los animales (Art. 30): Adopción.

EL SECRETARIO DE LA COMISIÓN

Jorge Palacio Liesa

EL PRESIDENTE DE LA COMISIÓN

Luis Miguel García Vinuesa

Domingo Miral, s/n, 50009 Zaragoza
Tel. +34 976 76 28 47
cea@unizar.es / http://cea.unizar.es

En Zaragoza, a 27 de mayo de 2016

La Dirección General de Alimentación y Fomento Agroalimentario del Gobierno de Aragón, a la vista de la evaluación emitida por el órgano habilitado, autoriza la realización del proyecto en los términos descritos en la solicitud.

Enrique Novales Allué
Director General de Alimentación y Fomento Agroalimentario

unizar.es

Comisión ética asesora para la experimentación animal
Universidad Zaragoza

Ref. PI15/16

MODIFICACIÓN DE PROCEDIMIENTO

Título procedimiento de experimentación:

Optimización del uso de MSCs alogénicas en el tratamiento de patologías articulares equinas: equilibrio inmunomodulación-inmunogenicidad

Título del proyecto en el que se incluye:

Optimización del uso de MSCs alogénicas en el tratamiento de patologías articulares equinas: equilibrio inmunomodulación-inmunogenicidad

Investigador responsable en la U.Z.: Francisco José Vázquez Bringas

Animales que implica:

especie (s): Caballos, asnos e híbridos (*Equidae*)

peculiaridades: Adultos (4-15 años), distintos sexos y razas

número: 24

Fecha de presentación: 22 de junio de 2021

Reunida la Comisión Ética Asesora para la Experimentación Animal* el día 30 de junio de 2021, y una vez revisada la documentación disponible en relación a las modificaciones del procedimiento de experimentación descrito (PI15/16), considera:

QUE EL PROCEDIMIENTO CUMPLE LOS PRINCIPIOS ÉTICOS Y DE PROTECCIÓN DE LOS ANIMALES UTILIZADOS PARA EXPERIMENTACIÓN QUE SE HA IMPUESTO LA UNIVERSIDAD DE ZARAGOZA, Y SE ADAPTA A LA NORMATIVA VIGENTE (R.D. 53/2013).

Por todo ello, se emite **informe FAVORABLE****.

Esta evaluación será válida únicamente para ESTE PROCEDIMIENTO y durante el periodo de tiempo establecido en la evaluación inicial. La realización del procedimiento queda supeditada igualmente a la disponibilidad de las instalaciones necesarias en el momento en que se plantee la realización del mismo.

*Órgano habilitado para realizar la evaluación y evaluación retrospectiva (Resolución de 6 de agosto de 2013 de la Dirección General de Alimentación y Fomento Agroalimentario del Gobierno de Aragón).

****Observaciones:** 1. Reducir el grupo control acelular de 3 a 1 animal.
2. Reutilización de caballo de procedimientos docentes.

En Zaragoza, a la fecha de la firma.

EL SECRETARIO DE LA COMISIÓN
Jorge Palacio Liesa

LA PRESIDENTA DE LA COMISIÓN
Rosa María Bolea Bailo

[Firmado electrónicamente y con autenticidad contrastable según el artículo 27.3.c) de la Ley 39/2015]

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JORGE IGNACIO PALACIO LIESA	Secretario de la Comisión Ética Asesora para la Experimentación Animal	01/07/2021 14:23:00
ROSA MARIA BOLEA BAILO	Presidenta de la Comisión Ética Asesora para la Experimentación Animal	02/07/2021 10:08:00

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ANEXO II: ÍNDICE DE TABLAS

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Anexo IV: Material Suplementario Manuscrito II

Supplementary material 1

Characterization of equine bone marrow derived mesenchymal stem cells (BM-MSCs).

1. Methodology

After their isolation, cells were characterized at passage 3 by the expression of defined markers and their ability to differentiate into adipocytes, osteoblasts and chondrocytes. Expression of positive markers CD90, CD105, CD44 and CD73 and lack of expression of the negative markers CD34 and CD45 was evaluated by real time quantitative polymerase reaction (RT-qPCR), and the surface expression of CD90 and CD105 was also studied by flow cytometry. In addition, surface expression of MHC-I and MHC-II was studied by flow cytometry in both MSC-naïve and MSC-primed since inflammatory exposure can induce changes in MHC expression.

1.1 Gene expression (RT-qPCR):

Isolation of mRNA and complementary DNA (cDNA) retrotranscription were performed using Cell-to-cDNA II (Ambion) according to the manufacturer's instructions and RT-qPCR reactions were performed and monitored with a QuantStudio3 Real Time PCR System device (Applied Biosystems). All reactions were carried out in a total volume of 10 µL with 2 µL of cDNA as the template and Fast SYBR Green Master Mix (Applied Biosystems). Amplification was performed in triplicate for each sample as follows: 20'' at 95 °C for initial activation, followed by 40 cycles consisting of 3''/95 °C and 30''/60 °C and a dissociation curve protocol run after every PCR reaction. The levels of gene expression were determined by the comparative Ct method. Normalization factor was calculated as the geometric mean of the quantity of two housekeeping genes, GAPDH and B2M. Genes analysed, and corresponding primer sequences were previously designed by our group [13] and are presented in Table S1.1.

Table S1.1. Primers used for gene expression analysis by RT-qPCR.

Gene	*Accession number*	*Primer sequence (5'–3')*	*Amplicon size (bp)*
		Housekeeping	
GAPDH	NM_001163856	F: GGCAAGTTCCATGGCACAGT R: CACAACATATTCAGCACCAGCAT	128
B2M	NM_001082502.2	F: TCGTCCTGCTCGGGCTACT R: ATTCTCTGCTGGGTGACGTGA	102
		Characterization cell surface markers	
		Mesenchymal cell markers	
CD90	EU881920	F: TGCGAACTCCGCCTCTCT R: GCTTATGCCCTCGCACTTG	93
CD105	XM_001500078	F: GACGGAAAATGTGGTCAGTAATGA R: GCGAGAGGCTCTCCGTGTT	100
CD44	NM 001085435	F: CCCACGGATCTGAAACAAGTG R: TTCTGGAATTTGAGGTCTCCGTAT	95
CD73	XM 001500115	F: GGGATTGTTGGATACACTTCAAAAG R: GCTGCAACGCAGTGATTTCA	90
		Hematopoietic markers	
CD34	XM_001491596	F: CACTAAACCCTCTACATCATTTTCTCCTA R: GGCAGATACCTTGAGTCAATTTCA	150
CD45	AY_114350	F: TGATTCCCAGAAATGACCATGTA R: ACATTTTGGGCTTGTCCTGTAAC	100

GenBank accession numbers of the sequences used for primers design. Primers (F: Forward and R: Reverse) and length of the amplicon in base pair (bp). Genes were grouped in agreement with the functions and implications of encoded molecules. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; B2M, beta-2 microglobulin; CD90, Thy-1; CD105, endoglin; CD44, hyaluronate receptor; CD73; CD34, haematopoietic marker; CD45, haematopoietic marker.

1.2 Surface markers expression (Flow cytometry):

Briefly, the cells were suspended in PBS/2 mM EDTA at 10^6cells/mL. 50 µL aliquots of cells were transferred to FACS tubes and separately incubated for 15 min at 4 °C in the dark with mouse anti-human monoclonal antibodies for CD90-PE (clone 5E10, BDPharmingen), CD105-FITC (clone SN6, R&D Systems), HLA-ABC-FITC (Beckman Coulter) and HLA-DR-APC (Immunostep). Anti-horse reactivity of antibodies was previously tested [13,29]. Subsequently, cells were washed with PBS (Gibco), diluted in 500 µL of PBS/2 mM EDTA and analysed with the Gallios flow cytometer (Beckman Coulter). Sytox Blue was used to gate live cells. Gating strategy was performed as described in [29] and data were analysed with FCS Express 7 Flow software (De Novo Software).

1.3 Tri-lineage differentiation:

The induction of differentiation into the osteogenic, adipogenic and chondrogenic lineages was performed by using specific media and successful differentiation was checked using specific staining and gene expression of lineage markers as previously described [15,17]. Each differentiation condition was assayed in triplicate for each donor (n=3), including non-differentiated controls cultured with regular media. For osteogenic, adipogenic and chondrogenic differentiation, cells were stained with 2 % Alizarin Red stain (pH 4.6), 0.3 % Oil-Red stain and 1 % Alcian Blue stains, respectively.

2. Results

2.1 Gene expression and surface markers expression:

Gene expression results showed that isolated MSCs from the three horses expressed CD90, CD105 and CD73 and did not express CD45, according to that established by the International Society for Cellular Therapy as minimal criteria for defining human MSCs [28]. However, CD34 expression was also observed in spite of being considered a negative marker (Figure S1.1). Nevertheless, phenotype variability among species has been described and CD34 gene expression has been previously observed in equine MSCs [27]. These MSCs were also positive for the typical MSC surface markers CD105 and CD90 determined by flow cytometry (Figure S1.2).

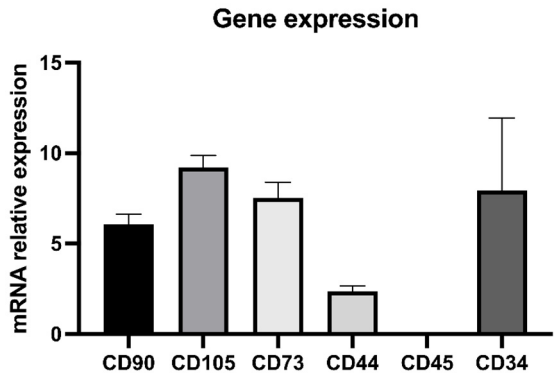


Figure S1.1. Data obtained from characterization assays using RT-qPCR are presented as mean ± S.E.M (n=3) of the mRNA relative expression of CD90, CD105, CD73, CD44, CD34 and CD45.

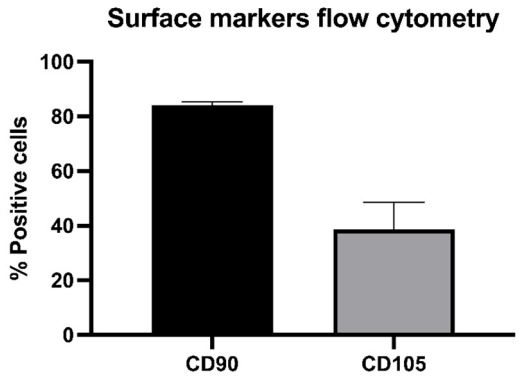


Figure S1.2. Mean ± S.E.M (n=3) of the percentage of positive cells for the surface markers CD90 and CD105 studied by flow cytometry.

2.2 Tri-lineage differentiation:

The tri-lineage differentiation ability was confirmed in equine MSCs (Figure S1.3). Specific staining for each differentiation assay resulted in calcium deposits for osteogenic differentiation (Figure S1.3.A), lipid droplets formation for adipogenic induction (Figure S1.3.B), and lacunae formation and proteoglycans production for chondrogenic differentiation (Figure S1.3.C). Mesenchymal stem cells used as control did not spontaneously differentiate (Figure S1.3.D).

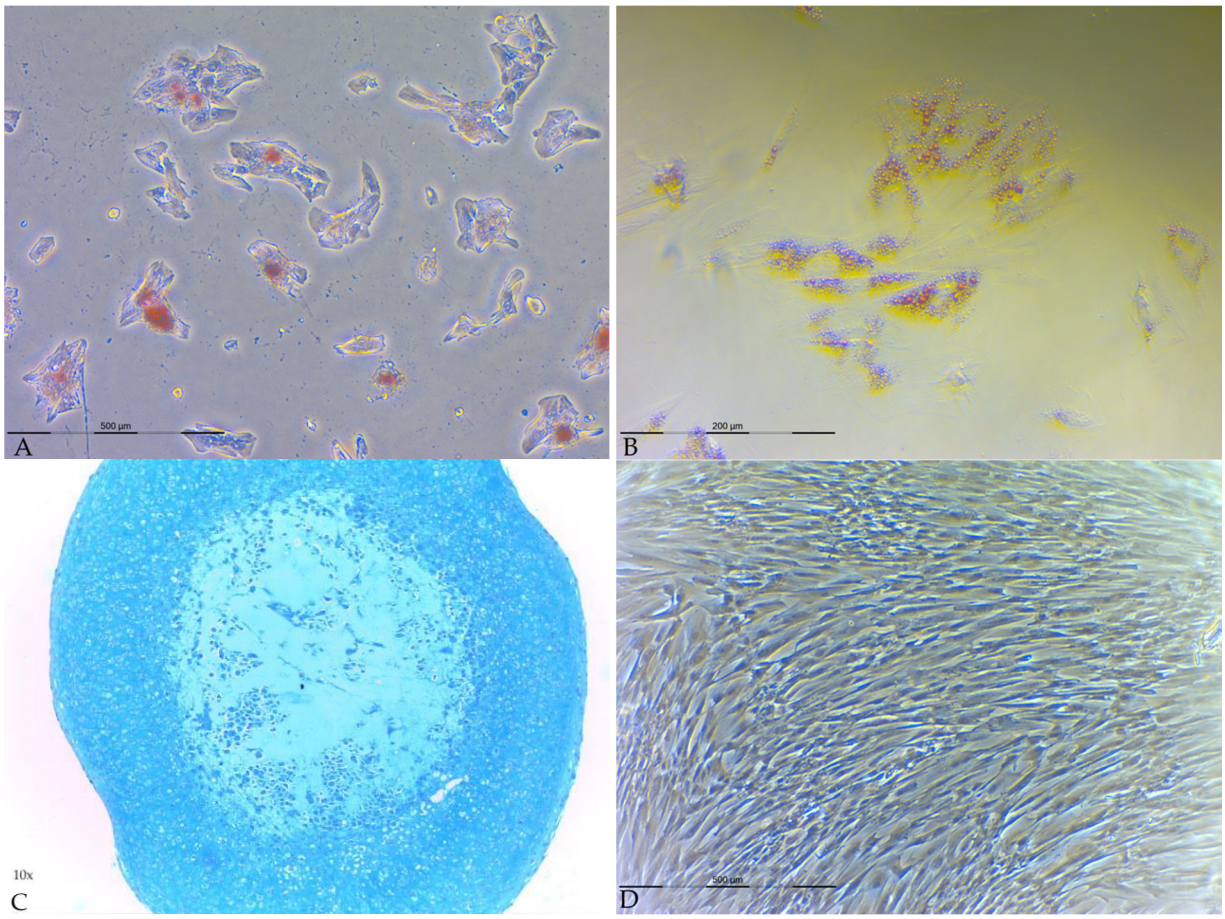


Figure S1.3. Results from the differentiation assays: calcium deposits stained by Alizarin Red in the cells undergoing osteogenesis (A), lipid droplets stained by Oil-O Red in the adipogenesis (B), proteoglycan rich extracellular matrix stained by Alcian Blue with lacunae formation after chondrogenic induction in 3D (pellet) culture (C) and undifferentiated control (D).

Supplementary Material 2

Table S2.1. Antibodies used in panel 1 for flow cytometry assays

	Antibody	Host	Target species	Clone	Type and isotype	Conjugated and Fluorochrome	Dilution	Gallios cytometer channel	Target cells	References
PANEL 1	**Pan B-cells** MCA1899 Bio-Rad	Mouse	Anti-horse	CVS36	Monoclonal IgG1	Unconjugated (Surface staining)	1:100	-	B lymphocytes	[40,45]
	Alexafluor™ 700 Goat anti-Mouse IgG Secondary Antibody A-21036 Invitrogen	Goat	Anti-mouse	-	Policlonal IgG H+L	Alexa Fluor™ 700 Secondary antibody for anti-Pan B-cells	1:200	FL-7		
	CD21 561357 BD Pharmigen	Mouse	Anti-human	B-ly4	Monoclonal IgG1	APC (Surface staining)	1:40	FL-6	B lymphocytes	[44,45]
	CD3 MCA1477 Bio-Rad	Rat	Anti-human (reacts with horse, bovine, pig and more)	CD3-12	Monoclonal IgG1	Unconjugated (Intracellular staining)	1:100	-	T lymphocyte	[4,34,41]
	PE Mouse anti-Rat IgG1 Secondary Antibody 12-4812-82 Invitrogen	Mouse	Anti-rat	R1-12D10	Monoclonal IgG1	PE Secondary antibody for anti-CD3	1:100	FL-2		

Table S2.2. Antibodies used in panel 2 for flow cytometry assays

	Antibody	Host	Target species	Clone	Type and isotype	Conjugated and Fluorochrome	Dilution	Gallios cytometer channel	Target cells	References
PANEL 2	**CD4** MCA1078 Bio-Rad	Mouse	Anti-horse	CVS4	Monoclonal IgG1	Unconjugated (Surface staining)	1:200		Helper T cells	[4,32,41,43,45]
	APC/Cyanine7 Rat anti-mouse IgG1 Secondary Antibody 406619 BioLegend	Rat	Anti-mouse	RMG1-1	Monoclonal IgG1	APC/Cyanine7 tandem Secondary antibody for anti-CD4	1:200	FL-8		
	CD25/IL-2R AF-223 R&D Systems	Goat	Anti-human	-	Polyclonal IgG	Unconjugated (Surface staining)	1:50		Regulatory T cells	[42,44,46,48]
	Alexa Fluor® 647 Affini Pure Donkey Anti-Goat IgG (H+L) 705-605-003 Jackson Immuno Research	Donkey	Anti-goat	-	Polyclonal IgG (H+L)	Alexa Fluor 647 Secondary antibody for anti-CD25:	1:400	FL-6		[44]
	CD8 MCA2385 Bio-Rad	Mouse	Anti-horse	CVS8	Monoclonal IgG1	RPE (Surface staining)	1:5 (H2Od)	FL-2	Cytotoxic T cells	[45]

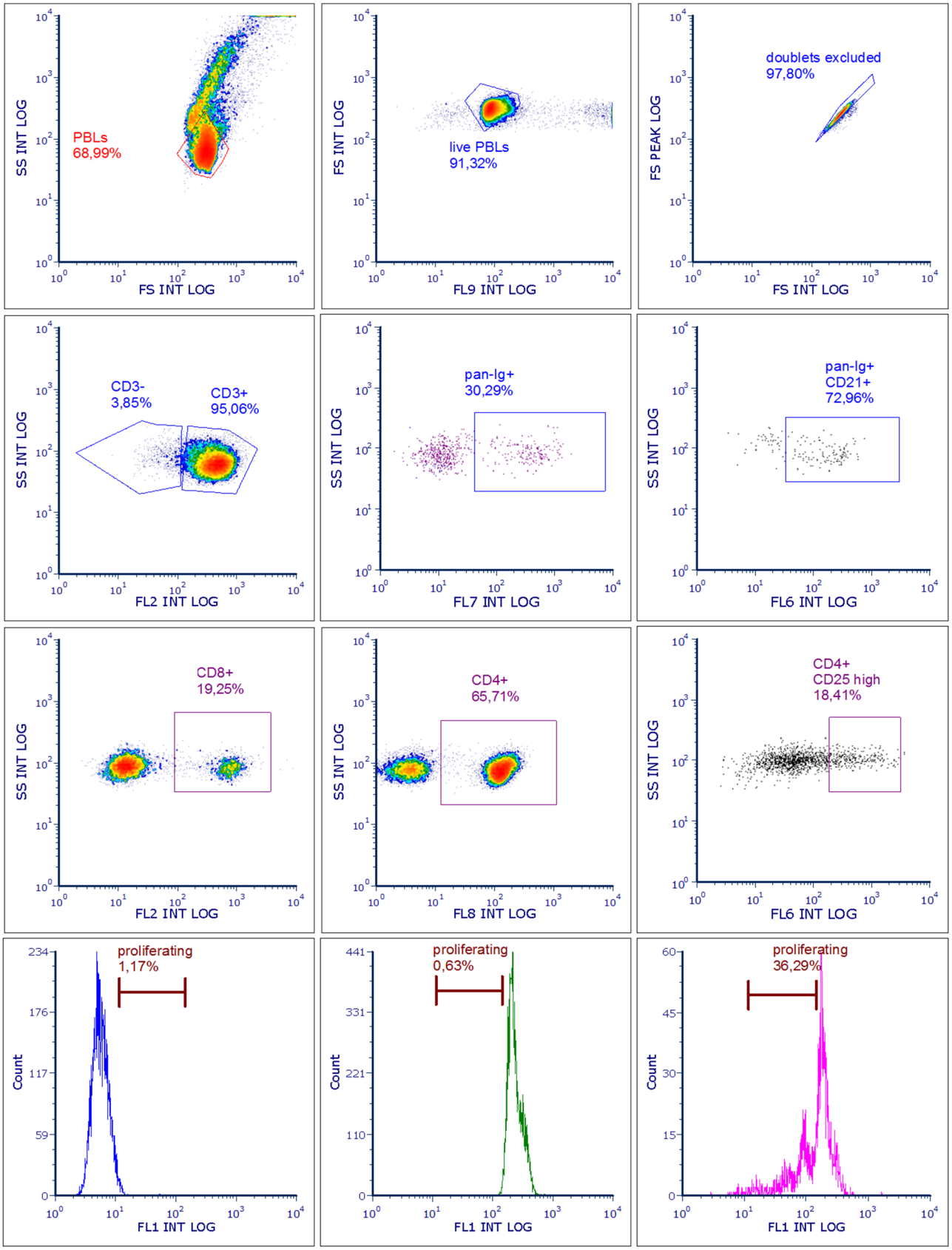


Figure S2.1. Flow cytometry gating strategy. First row shows how the population of lymphocytes (PBLs) was gated in the forward and side scatter (FSC x SSC) plot, and dead cells (FSC x FL9) and doublets (FS int x FS peak) were subsequently excluded. Second row represents gating of subpopulations analysed in panel 1: T cells as CD3+ (FL2) and B cells as CD3-/pan-Ig+/CD21+ (FL2/FL7/FL6). Third row presents gating for populations in panel 2: cytotoxic and helper T cells were gated as CD8+/CD4- and CD4+/CD8-, respectively (FL2/FL8), and regulatory T cells (Treg) were gated as CD4+/CD25^{high} (FL8/FL6). Fourth row: to study the proliferation of each subpopulation, CFSE dilution was assessed in FL1 using unstimulated and unstained PBLs to define the autofluorescence (left, blue histogram) and unstimulated and CFSE-labelled PBLs to set the non-proliferating population (middle, green histogram). Histogram in the right (pink) represents PBLs in the proliferating gate.

Anexo V: Material Suplementario Manuscrito III

Supplementary Material

Table S1. List of major histocompatibility complex (MHC) microsatellite haplotypes identified in the horses enrolled in the study.

	MHC class I		MHC class III		MHC class II						
Microsatellite loci	UMNJH-38	COR110	ABGe9019	UMNe65	ABGe9030	EQMHC 1	COR112	COR113	UM011	COR114	
Horses ID											**Haplotype**
D1	165	221	301	261	215	190	262	270	179	241	HapPRE10
	165	221	301	261	215	190	262	270	179	241	HapPRE10
R1	165	221	301	261	215	190	262	270	179	241	HapPRE10
	156	215	301	261	215	190	262	270	179	241	HapPRE10-like
R2	165	221	301	261	215	190	262	270	179	241	HapPRE10
	156	221	320	250	219	190	254	270	172	249	HapMAI06
R3	165	221	301	261	215	190	262	270	179	241	HapPRE10
	156	207	318	263	215	184	262	260	172	243	HapPRE31
D2	156	217	312	261	205	194	258	260	169	243	HapMAI04
	156	217	312	261	205	194	258	260	169	243	HapMAI04
A1	156	217	312	261	205	194	258	260	169	243	HapMAI04
	156	205	305	253	205	194	266	268	174	234	HapPRE01
A2	156	217	312	261	205	194	258	260	169	243	HapMAI04
	156	207	312	263	211	192	264	270	172	249	Unknown1
D3	156	221	314	259	215	190	262	272	169	255	HapPRE11
	156	221	314	259	215	190	262	272	169	255	HapPRE11
C1	156	221	314	259	215	190	262	272	169	255	HapPRE11
	156	221	312	261	205	190	262	270	180	245	Unknown2
C2	156	221	314	259	215	190	262	272	169	255	HapPRE11
	156	211	301	259	209	192	262	268	174	234	A2*
C3	156	221	314	259	215	190	262	272	169	255	HapPRE11
	156	207	314	261	215	190	262	270	180	247	HapPRE26

MHC homozygous horses are indicated in bold. Asterisks indicate haplotypes that have been previously identified in other horse breeds (17).

Supplementary Material

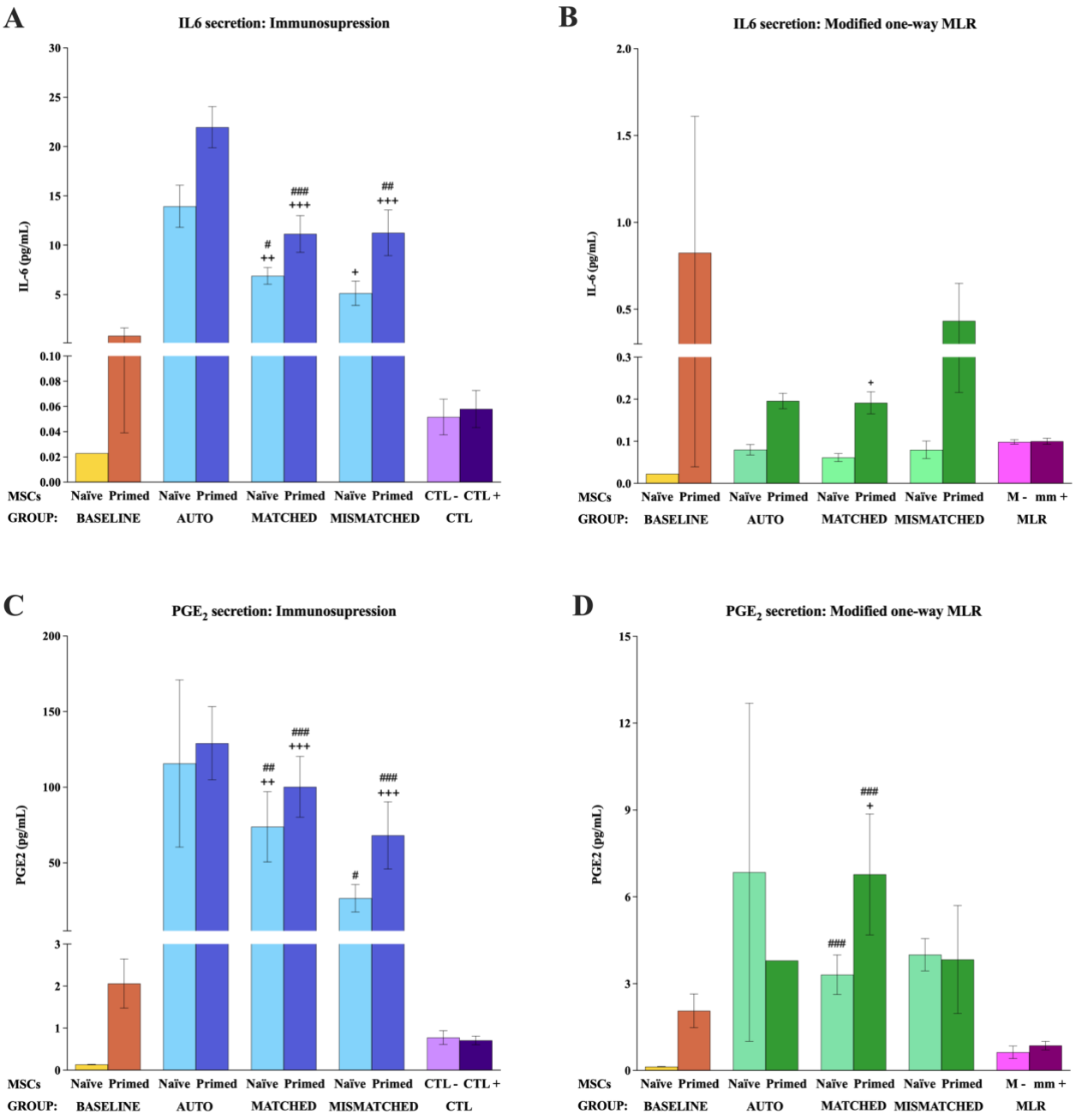


Figure S1. Significant differences over controls are presented for interleukin 6 (IL6) and prostaglandin E2 (PGE_2) secretion. The graphs in this figure are modifications of those presented in Figure 2. C and D (IL6) and Figure 3. C and D (PGE_2) to include the concentration of the mediators found in the supernatant of the controls, and to show the significant differences of the experimental conditions over these controls. These results are not presented in the main figures as the mediators' secretion by lymphocyte controls is not relevant for the study and was just assessed to corroborate that the mesenchymal stem cells (MSCs) were the main contributors to this secretion. We considered that presenting multiple comparison in the same figure would prevent highlighting the most relevant findings for the study, so the comparison over controls is separately presented in this supplementary material. Data are presented as Mean ± S.E.M of the concentration (pg/mL) of

IL6 (**A**, **B**) and PGE_2 (**C**, **D**) found in the supernatants of equine MSCs unstimulated (MSC-naïve) or cytokine primed (MSC-primed) before (baseline) and after their co-culture with autologous (n = 3) or allogeneic matched (n = 8) or mismatched (n = 7) lymphocytes, either activated (**A**, **C**; immunosuppression assays) or resting (**B**, **D**; modified one-way mixed lymphocyte reactions, MLR). In immunosuppression assays, lymphocytes from all donors were cultured alone as positive (phytohemagglutin isoform P activation; CTL+, dark purple bar) and negative (unstimulated; CTL-, light purple bar) controls. In modified one-way MLRs, positive and negative controls were set by establishing, respectively, matched (MLR M-, light pink bar) and mismatched (MLR mm+, dark pink bar) classic MLRs using responder PBLs from each donor. Significant differences over the positive controls are presented by '+' (+ = $p < 0.05$; ++ = $p < 0.01$; +++ = $p < 0.001$) and over the negative controls as '#' (# = $p < 0.05$; ## = $p < 0.01$; ### = $p < 0.001$) placed above the corresponding bars of experimental conditions.

Anexo VI: Material Suplementario Manuscrito IV

Resource 1: Considerations taken into account prior to *in vivo* analyses

Prior to any *in vivo* assay, different parameters were assessed, and certain techniques were optimized which the authors consider should be mentioned. Considerations included assessing the viability of MSCs at different times once encapsulated in the alginate gels and corroborating that the MSCs were able to differentiate into chondrocytes within the implant. Subsequently, implant placement and removal and histological assessment of the implants were also optimized.

1.1 Viability of equine BM-MSCs in the alginate scaffold

Mesenchymal stem cells viability in the alginate scaffold was assessed at 24 hours and 14 days after encapsulation using Viability/Cytotoxicity Assay Kit for Animal Live & Dead Cells (Biotium). Briefly, scaffolds were washed twice PBS and incubated 30 min, 37 °C, 5% CO_2 with 2 µM ethidium homodimer-3 (necrotic marker measuring nucleus membrane integrity) and 1 µM calcein AM (viability marker measuring intracellular esterase activity) to stain dead cells in red and live cells in green, respectively (Trouche *et al.*, 2010; Koh *et al.*, 2017; Swioklo *et al.*, 2017).

Dead cells control were obtained by treating scaffolds with 70% ethanol for 10 min prior to fluorescent live/dead staining (Ceccaldi *et al.*, 2012). Unstained scaffolds containing cells and stained empty scaffolds (acellular) were used to evaluate autofluorescence. Viability and cell distribution were examined using a confocal spectral fluorescence microscope (Zeiss LSM 880 Airyscan).

1.2 Chondrogenic differentiation

For MSC-chondro, chondrogenesis was induced in a three-dimensional alginate matrix system after encapsulation (de Vries-van Melle *et al.*, 2014; Ryan *et al.*, 2014). After polymerization, constructs were washed three times in PBS as described above and, subsequently, once with incomplete chondrogenic differentiation medium (ICM) consisting of high-glucose DMEM (Sigma-Aldrich) supplemented with 10% FBS, 100U/ml penicillin and 100 µg/ml streptomycin, 2mM L-glutamine, 1% insulin, transferrin and selenium supplements solution (ITS+ premix, Beckton Dickinson), 1 mM sodium pyruvate (Gibco), 100 nM dexamethasone, 50 µg/mL ascorbic acid-2-phosphate and 40 µg/mL proline (Sigma-Aldrich). Two milliliters of complete chondrogenic medium (CCM) supplemented with 10 ng/mL TGFβ3 (Peprotech) were used per construct in 12-well plates. All plates were incubated at 37 ºC and 5% CO_2 for three weeks, with medium changed every other day. Constructs containing MSCs but cultured with regular culture medium described above as undifferentiated controls. Differentiation to chondrocyte in classical pellet was also carried out following the methodology previously described. After 21 days, histology and reverse transcription polymerase chain reaction (RT-qPCR) analyses were performed to confirm chondrogenic differentiation following the methodology previously described (Barrachina et al., 2018).

For gene expression assessment of the chondrogenic markers Collagen type II alpha I (*COL2A1*) and Aggrecan (*ACAN*) by RT-qPCR, the methodology explained in the main

manuscript was followed. Increased expression of both markers was observed in both alginate-differentiated MSCs and conventional pellet-differentiated MSCs compared to undifferentiated MSCs (**Fig. 1.2.A**)

For histology, scaffolds were fixed in 4% paraformaldehyde (PFA) in PBS (Olderøy et al., 2014; Reppel et al., 2015; Xu et al., 2015; Zhang et al., 2015), embedded in paraffin, and cut into 5-mm sections. The sections were hydrated with increasing gradients of alcohols and stained with H&E or 3% Alcian Blue dyes to assess lacunae formation and the proteoglycan content of the extracellular matrix formed. Then, sections were rinsed with distilled water, dehydrated with decreasing amounts of alcohol, and mounted (Barrachina et al., 2018). **Figure 1.2.B** shows the differentiation to chondrocyte in alginate compared to undifferentiated alginate implants.

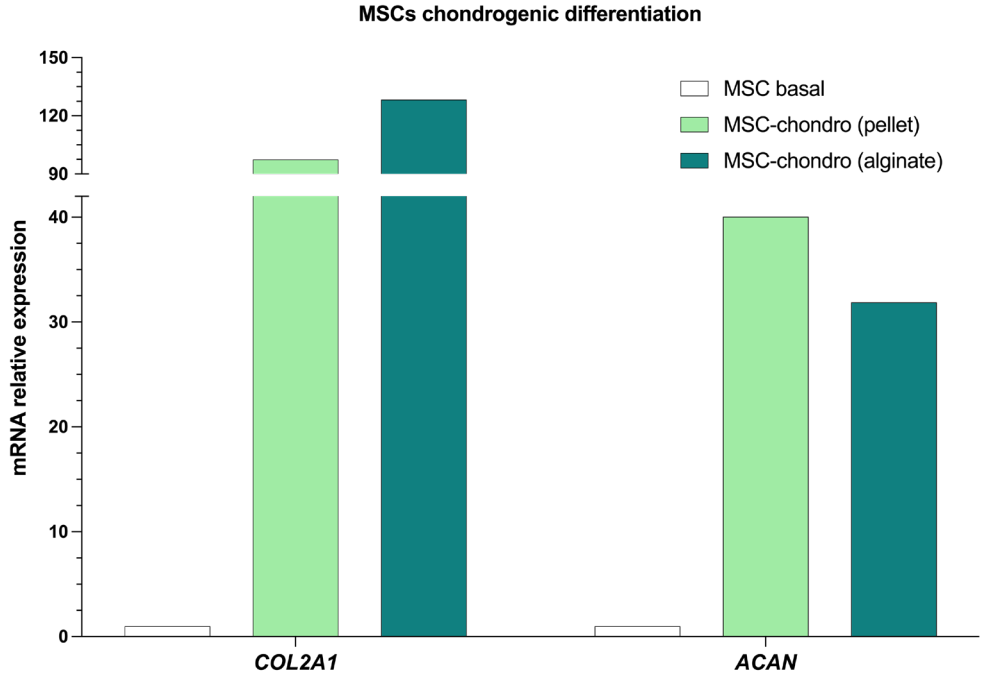


Fig. 1.2.A Data obtained from chondrogenic comparation assays using RT-qPCR are presented as mean ± S.E.M (n = 3) of the mRNA relative expression of Collagen type II alpha I (*COL2A1*) and Aggrecan (*ACAN*).

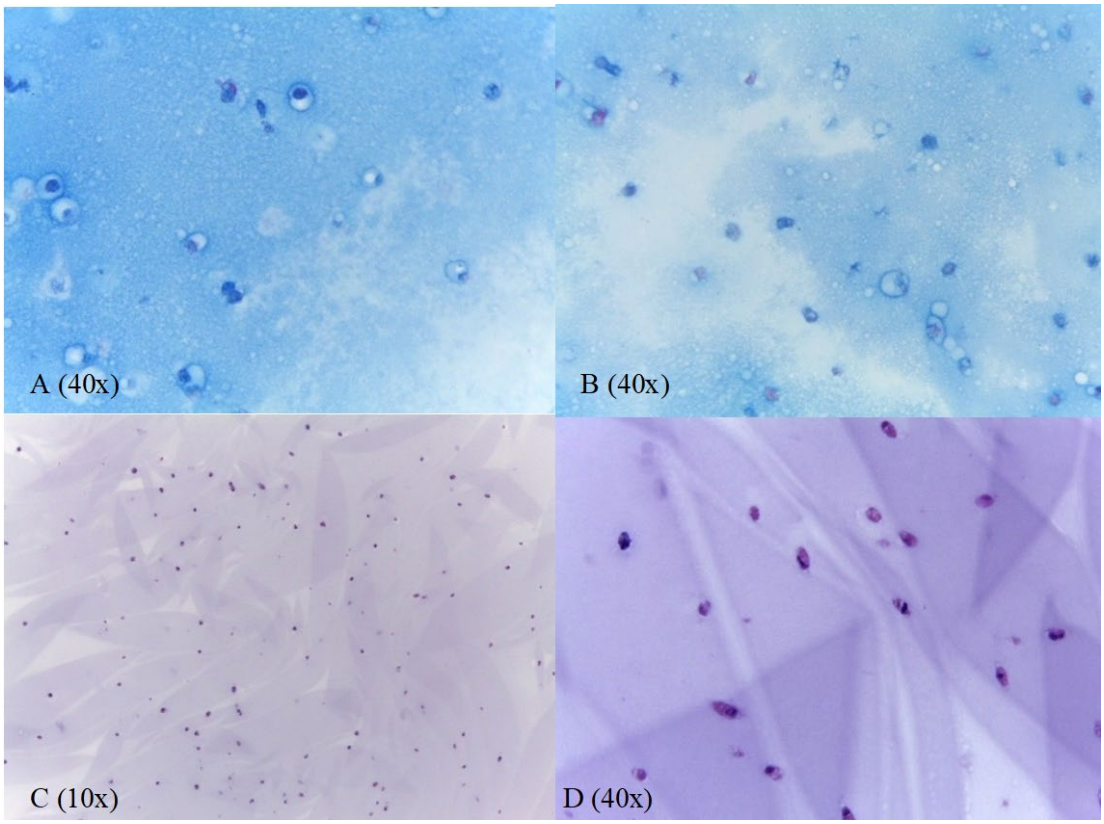


Figure 1.2.B Results from the differentiation assays: proteoglycan rich extracellular matrix stained by Alcian Blue with lacunae formation after chondrogenic induction in 3D alginate culture (A, B) and undifferentiated control stained by H&E (C, D).

1.3 Green fluorescent protein

In order to optimize scaffolds placement and removal in addition to histological assessment of the implants, a pilot test was carried out on a horse administered with GFP-labelled MSC-naïve.

Equine MSCs were genetically modified using a Moloney Murine Leukemia Virus (MMLV)-based retrovirus containing a GFP expression vector, which was generously provided by Professor A. Raya from CMRB. Briefly, Phoenix-AMPHO cells (ATCC® CRL3213 ™) were seeded at 450.000 cells/cm^2 in a 100 mm culture dish a day before transfection. The cells were incubated overnight at 37°C with 5% CO_2 in DMEM high glucose supplemented with 10% FBS and 1% penicillin and streptomycin.

To produce the viral particles, the media were replaced, and a transfection mix composed of 900 µL of 9 µg MMLV-GFP, 27 µL of FuGENE 6 Transfection Reagent (Promega), and Opti-MEM Reduced Serum Medium was added on top of the cells. The plates were then incubated at 37°C with 5% CO2 overnight. After incubation, viral supernatant was collected at 48 and 72 hours, passed through a 0.45 µm cellulose acetate filter and subsequently mixed with 5 µg/mL Polybrene (Sigma-Aldrich).

Equine MSCs were seeded in 100 mm dishes at a density of 5 × 10^5 cells per dish, one day before transduction. Viral supernatant was added to each dish, and the dishes were then centrifuged at 750 x g for 45 minutes at 32 °C. Following centrifugation, the MSCs

were incubated overnight at 32 °C with 5% CO_2 to allow for efficient transduction. The next day, the transduction process was repeated to enhance the viral uptake by the MSCs. As a negative control, dishes without the GFP expression vector were included. On day 4, the transduced cells were examined for GFP expression using fluorescent microscopy. The presence of GFP fluorescence indicated successful transduction of the MSCs. The GFP-expressing MSCs were further expanded in culture media and subsequently cryopreserved for future use.

In order to optimize implant placement and subsequent histological assessment of the MSCs after administration, 5 million GFP-MSCs were encapsulated in alginate hydrogels following the methodology described in the main manuscript. These implants were placed in a pilot horse and removed at the aforementioned times (1 week, 3 weeks and 6 weeks). Implants were assessed by GFP and DAPI fluorescence microscopy (**Fig 1.3**).

In all three analyzed scaffolds, it was observed that the GFP-MSCs remained within the implants and maintained their fluorescence after administration. Initially, the original plan was to conduct all in vivo experiments using GFP-labelled MSCs. However, due to the varying efficacy of transduction depending on the MSCs donor, unlabeled MSCs were administered instead. Moreover, the use of unlabeled MSCs was preferred to avoid potential alterations in the immunomodulatory capacity of equine MSCs caused by GFP transduction, which could introduce a variable factor.

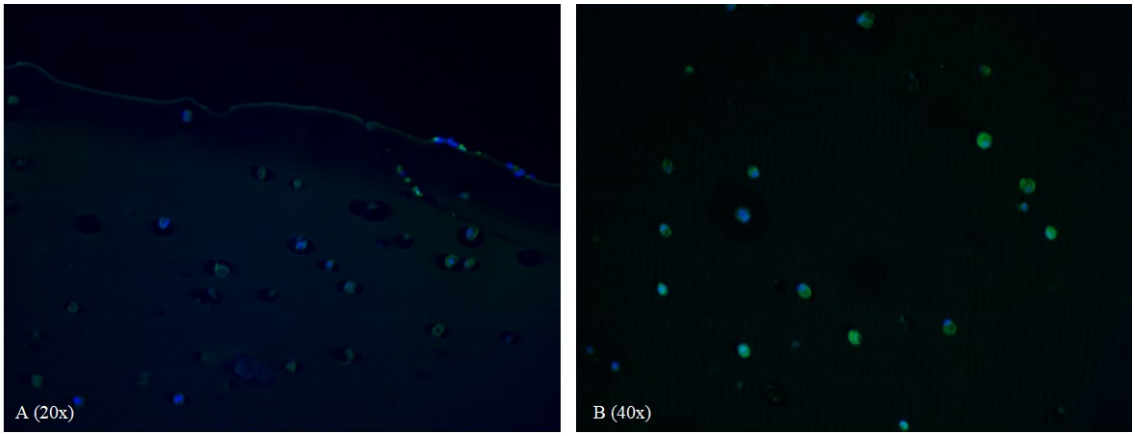


Fig. 1.3 Fluorescent imaging of GFP and DAPI in GFP-labelled MSCs encapsulated in alginate scaffolds recovered from the pilot horse. The outline of the alginate with the MSCs inside (A) and the MSCs at higher magnification inside the scaffold (B) are presented.

Resource 2: Ultrasound assessment of alginate scaffolds

After scaffold placement, an ultrasound examination (ATL Ultrasound HDI-3500) of the site was performed to check that the placement technique had been carried out properly. In addition, on the scheduled day of removal of each scaffold (first, second and third), prior to the surgical procedure, another ultrasound examination of the scaffold site was performed. These ultrasound examinations were very useful for monitoring the condition of the scaffolds and the surrounding tissue, and also for locating the scaffolds prior to their removal. Above all, it was useful for locating them at the 6-week times (T3 and T6) when the scaffolds were barely visible from the outside. A series of representative ultrasound images of the different scaffolds are shown below (**Fig. 2.1** and **Fig. 2.2**).

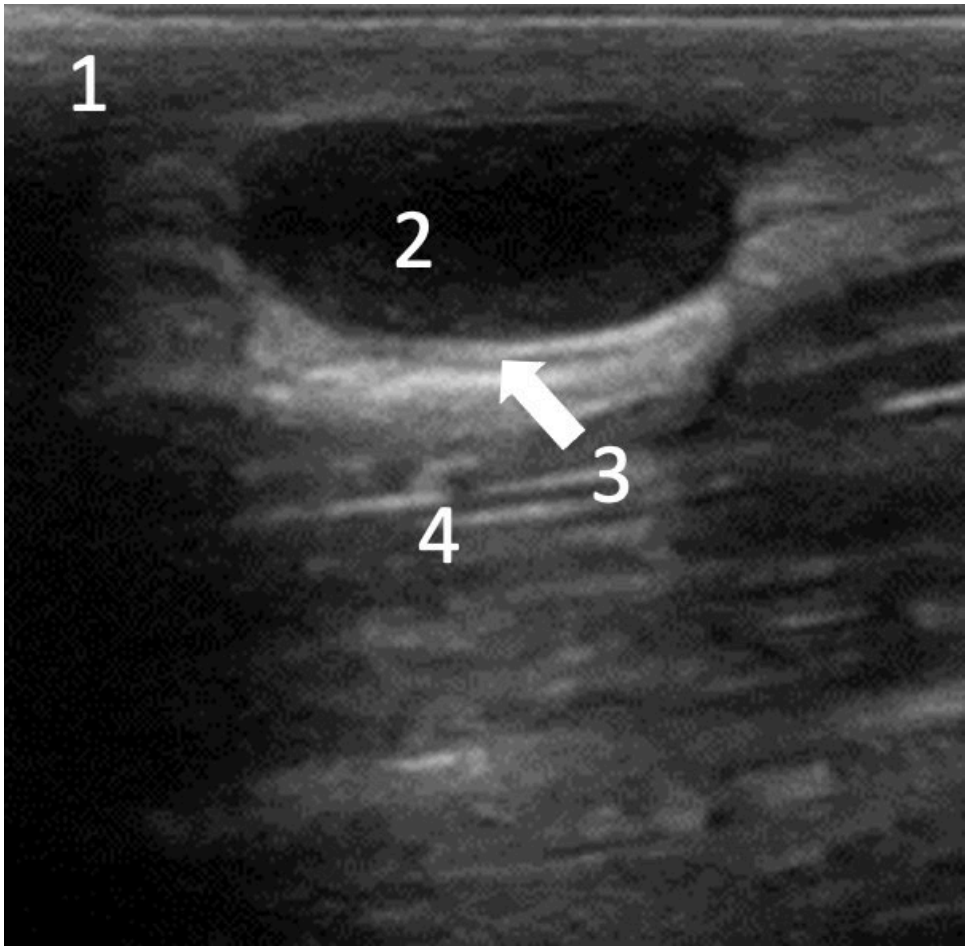


Fig 2.1 Ultrasound image of a scaffold showing skin and subcutaneous tissue (1), oval anechoic structure corresponding to the scaffold (2), hyperechoic structure surrounding the scaffold corresponding to the fibrous tissue capsule (3) and muscular structure (4) corresponding to the rhomboid serratus or trapezius muscles depending on the scaffold in question (first, second or third).

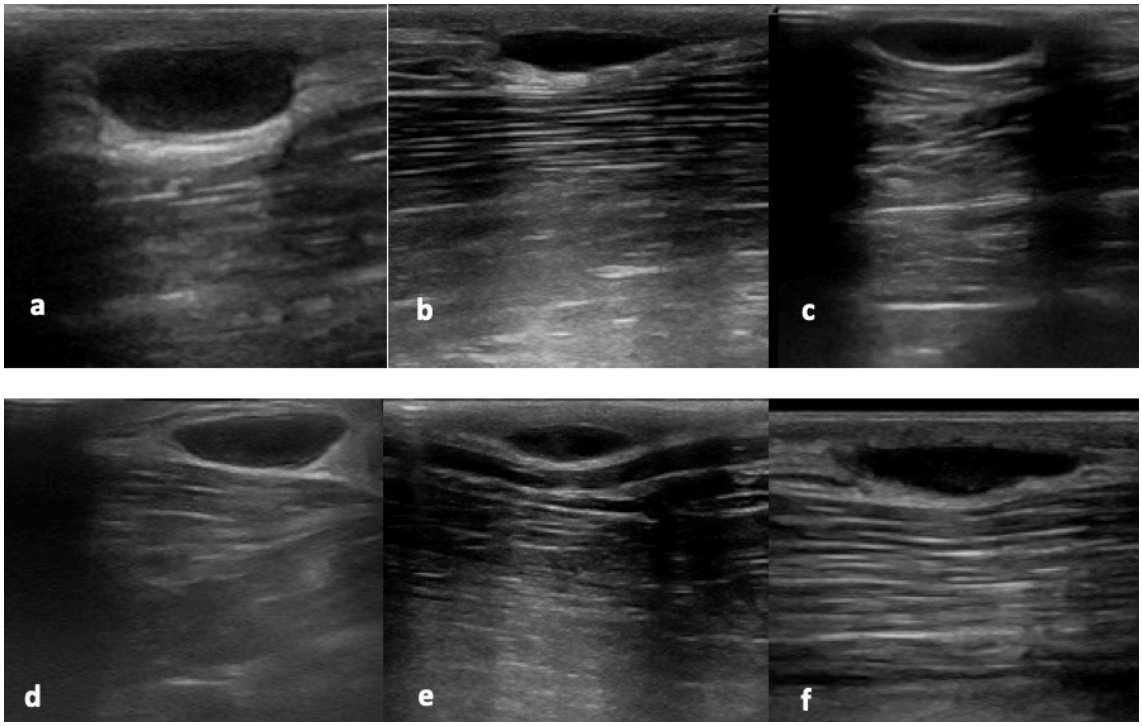


Fig. 2.2 Six different ultrasound images of different scaffolds can be observed, showing different scaffolds sizes and thicknesses of the capsules surrounding them. Note in the case of image f that it corresponds to a fractured scaffold, hence its irregular shape and a large perimetral hyperechoic fibrous capsule surrounding it; a) T5 scaffold (3 weeks after second MSCs administration, second scaffold) from one of the MHC-matched horses that received MSC-chondro; b) T5 scaffold (3 weeks after second MSCs administration, second scaffold) from one of the MHC-matched horses that received MSC-primed; c) T4 scaffold (1 week after second MSCs administration, first scaffold) from one of the MHC-matched horses that received MSC-naïve; d) T4 scaffold (1 week after second MSCs administration, first scaffold) from one of the MHC-matched horses that received MSC-chondro; e) T3 scaffold (6 weeks after first MSCs administration, third scaffold) from one of the MHC-matched horses that received MSC-chondro; f) T6 scaffold (6 weeks after second MSCs administration, third scaffold) from one of the MHC-matched horses that received MSC-chondro.

Resource 3: Histopathology scoring systems

The histological scoring system was based on a subjective ordinal scale. All histological preparations were analyzed by a blinded observer. Entire H&E sections were scanned using a 3D Scan Pannoramic Histech scanner (3D Histech P-1000 scanner), at 40x resolution, obtaining whole slide images. Images were analyzed using SlideViewer 2.6 software (3D Histech kft.), which provides a detailed morphometric analysis with precise measurements of different histological parameters at high resolution.

Table 3.1 Histopathology scoring

Lymphocytes	0	No lymphocytes along the section
	1	Occasional lymphocytes along the section
	2	Mild presence of lymphocytes with small foci
	3	Diffuse presence of lymphocytes throughout the section
Polymorphonuclear cells (PMN)	0	Absence of PMN along the section
	1	Occasional PMN along the section
	2	Mild presence of PMN with small foci
	3	Diffuse presence of PMN throughout the section
Eosinophils	0	Absence of Eosinophils along the section
	1	Occasional Eosinophils along the section
	2	Mild presence of Eosinophils with small foci
	3	Diffuse presence of Eosinophils throughout the section
Histiocytes	0	Absence of Histiocytes along the section
	1	Occasional Histiocytes along the section
	2	Mild presence of Histiocytes with small foci
	3	Diffuse presence of Histiocytes throughout the section
Vascular proliferation	0	Absence of vascular proliferation
	1	Slight and focal vascular proliferation
	2	Moderate vascular proliferation
	3	Prominent and diffuse vascular proliferation throughout the section
Fibroblast proliferation	0	Absence of fibroblast proliferation
	1	Slight and focal fibroblast proliferation
	2	Moderate fibroblast proliferation
	3	Prominent and diffuse fibroblast proliferation throughout the section

Fibrosis	0	Absence of fibrosis
	1	Slight and focal fibrosis
	2	Moderate fibrosis
	3	Prominent and diffuse fibrosis throughout the section
Hemorrhage	0	Absence of hemorrhage
	1	Slight and focal hemorrhage
	2	Moderate hemorrhage
	3	Prominent and diffuse hemorrhage throughout the section
Amount of the scaffold	0	Absence of implant
	1	Superficial depot below the internal capsule
	2	Implant involving less than half of the wall
	3	Implant involving more than half of the wall
Multinucleated cells	0	Absence of multinucleated cells around implant
	1	Occasional multinucleated cells around implant
	2	Moderate multinucleated cells around implant
	3	Several multinucleated cells around implant
Synovial metaplasia	0	Absence of synovial metaplasia
	1	Slight and focal synovial metaplasia along the capsule
	2	Moderate synovial metaplasia along the capsule
	3	Diffuse synovial metaplasia along the capsule
Fat necrosis	0	Absence of fat necrosis
	1	Slight and focal fat necrosis with occasional necrotic adipocytes
	2	Small clusters of necrotic adipocytes
	3	Large clusters of necrotic adipocytes
Hemosiderin	0	Absence of hemosiderin
	1	Focal and isolated depots of pigment
	2	Small clusters of pigment
	3	Large depots of pigment
Fat	0	Absence of fat in the histologic section
	1	Slight and focal presence of fat
	2	Moderate presence of fat, less than 50% of the section
	3	Presence of fat, more than 50% of the section
Muscular fascia	0	Absence
	1	Presence

Representative images of the assignment of scores for the different parameters assessed are shown. It is important to note that the scores obtained in each of the samples were normalized to the acellular control (value 1).

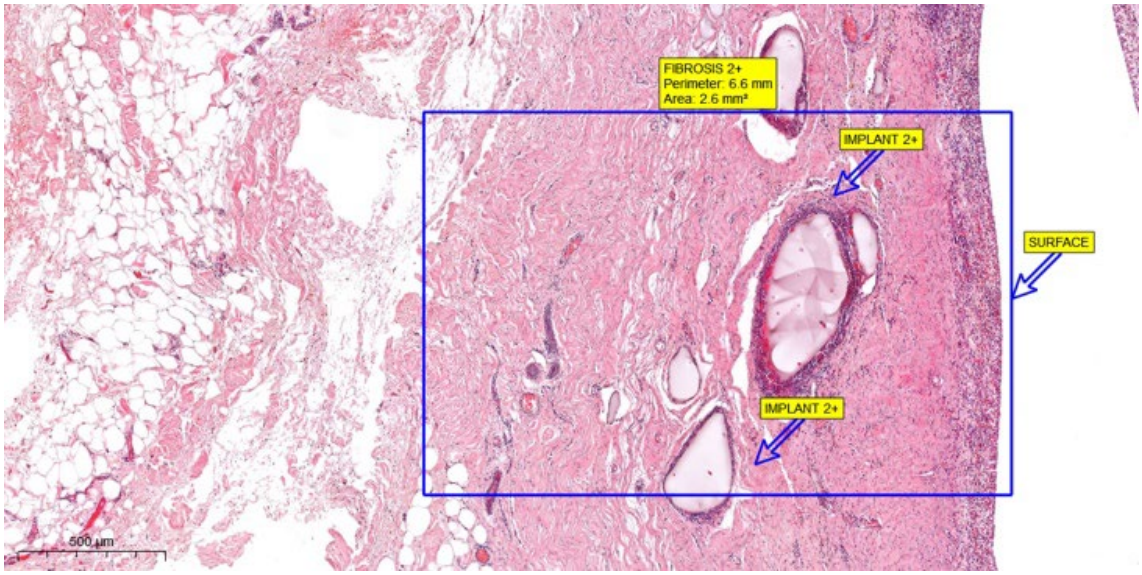


Fig. 3.1 Histological preparation of the capsule recovered at T6 (6 weeks after second MSCs administration, sixth scaffold) from one of the MHC-matched horses that received MSC-primed.

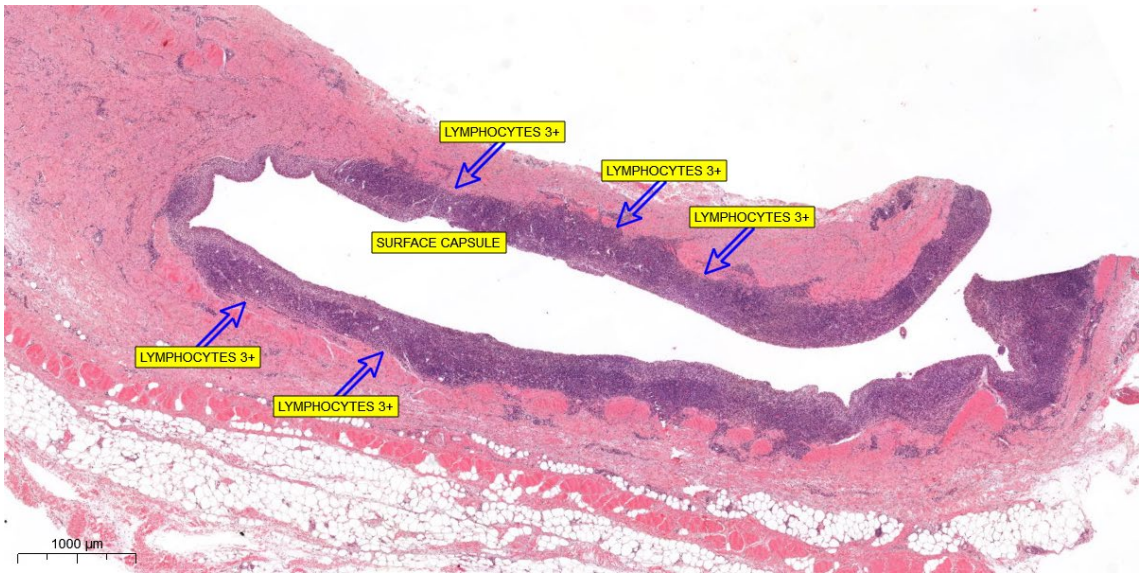


Fig. 3.2 Histological preparation of the capsule recovered at T2 (3 weeks after first MSCs administration, second scaffold) from one of the MHC-matched horses that received MSC-chondro.

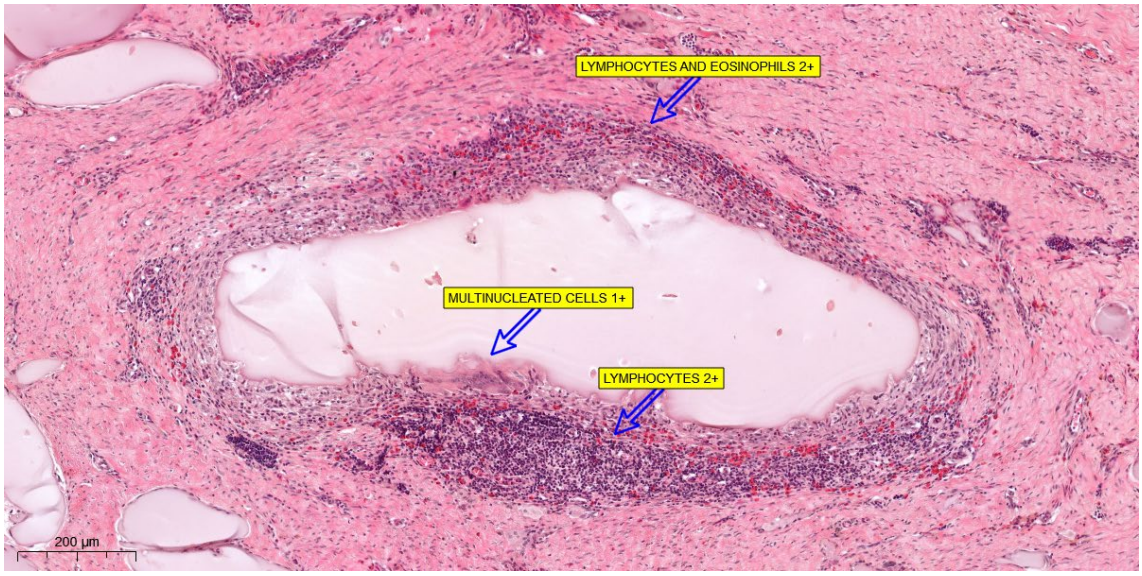


Fig. 3.3 Histological preparation of the capsule recovered at T6 (6 week after second MSCs administration, sixth scaffold) from one of the MHC-mismatched horses that received MSC-naïve.

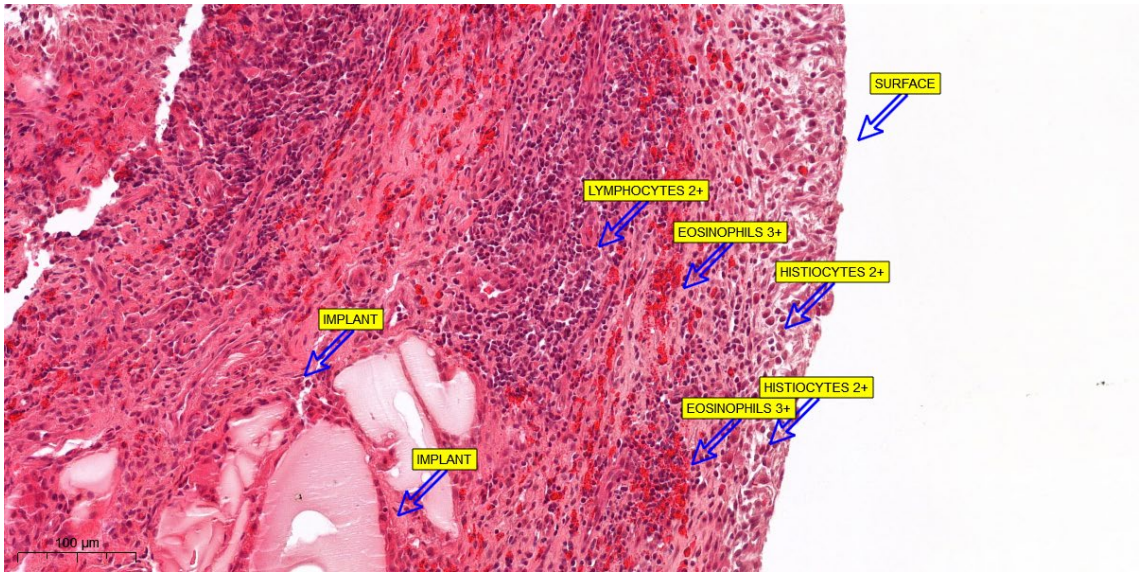


Fig. 3.4 Histological preparation of the capsule recovered at T3 (6 week after first MSCs administration, third scaffold) from one of the MHC-mismatched horses that received MSC-naïve.

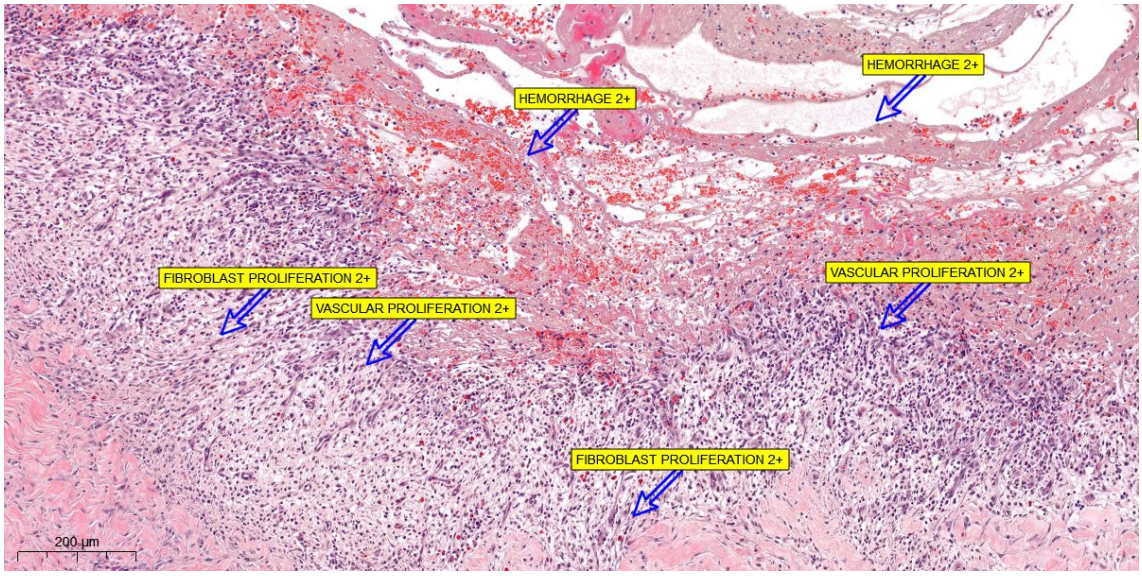


Fig. 3.5 Histological preparation of the capsule recovered at T4 (1 week after second MSCs administration, fourth scaffold) from one of the MHC-matched horses that received MSC-chondro.

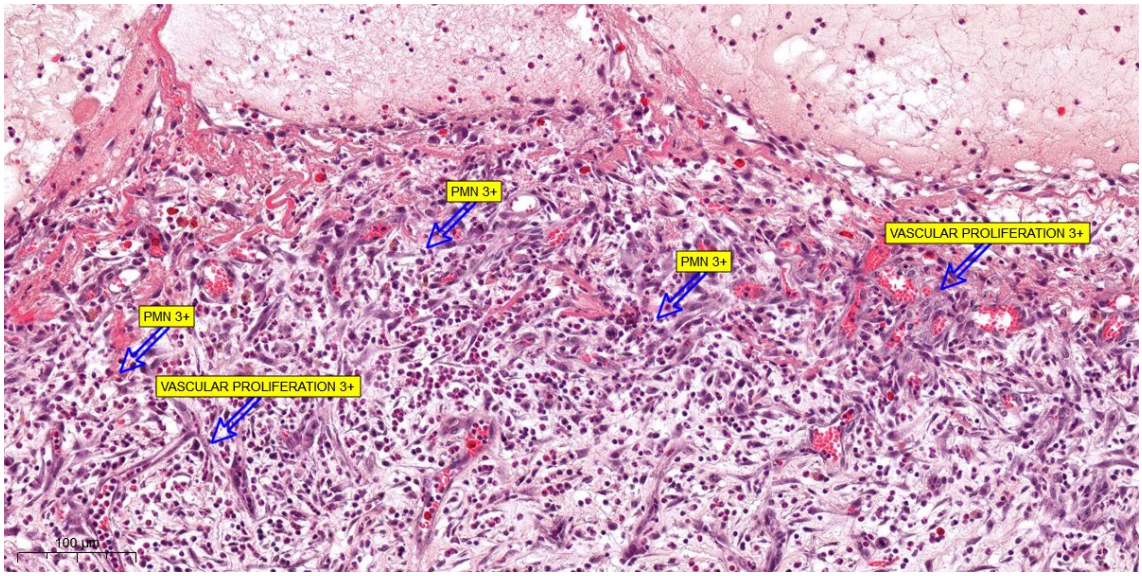


Fig. 3.6 Histological preparation of the capsule recovered at T4 (1 week after second MSCs administration (fourth scaffold) from one of the MHC-mismatched horses that received MSC-chondro.

Resource 4: Graphical representation of gene expression and histology scores

Graphical representation of gene expression of markers related to the immunomodulation and immunogenicity of mesenchymal stem cells (MSCs)

Significant differences represented are the same ones explained along the principal manuscript between groups (MHC-matched and MHC-mismatched) and over time-points: T1, 1 week after first MSCs administration (first scaffold); T2, 3 weeks after first MSCs administration (second scaffold), T3, 6 weeks after first MSC administration (third scaffold), T4, week after second MSCs administration (fourth scaffold); T5, 3 weeks after second MSCs administration (fifth scaffold), T6, 6 weeks after second MSCs administration (sixth scaffold). It is important to note that the scores obtained in each of the samples were normalized to the acellular control (value 1).

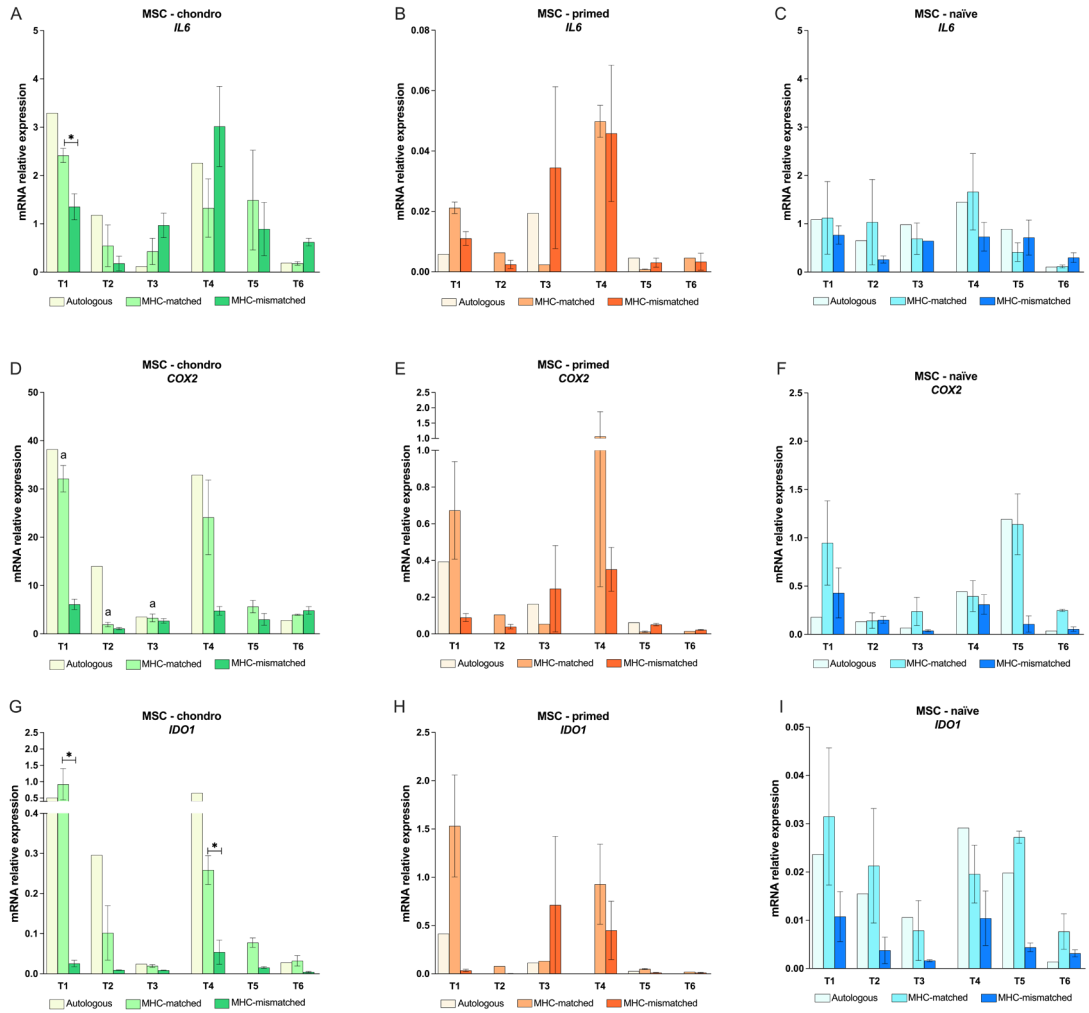


Fig. 4.1 Changes in interleukin 6 (*IL6*) (A, B, C), cyclooxygenase 2 (*COX2*) (D, E, F) and indoleamine 2,3-dioxygenase 1 (*IDO1*) (G, H, I) expression in equine MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars) following administration to autologous, MHC-matched and MHC-mismatched horses. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using each baseline MSCs type as reference sample (value 1). Significant differences between groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$). Significant differences between time-points compared to T1 are represented by lower case letter [a] ([a], $p < 0.05$).

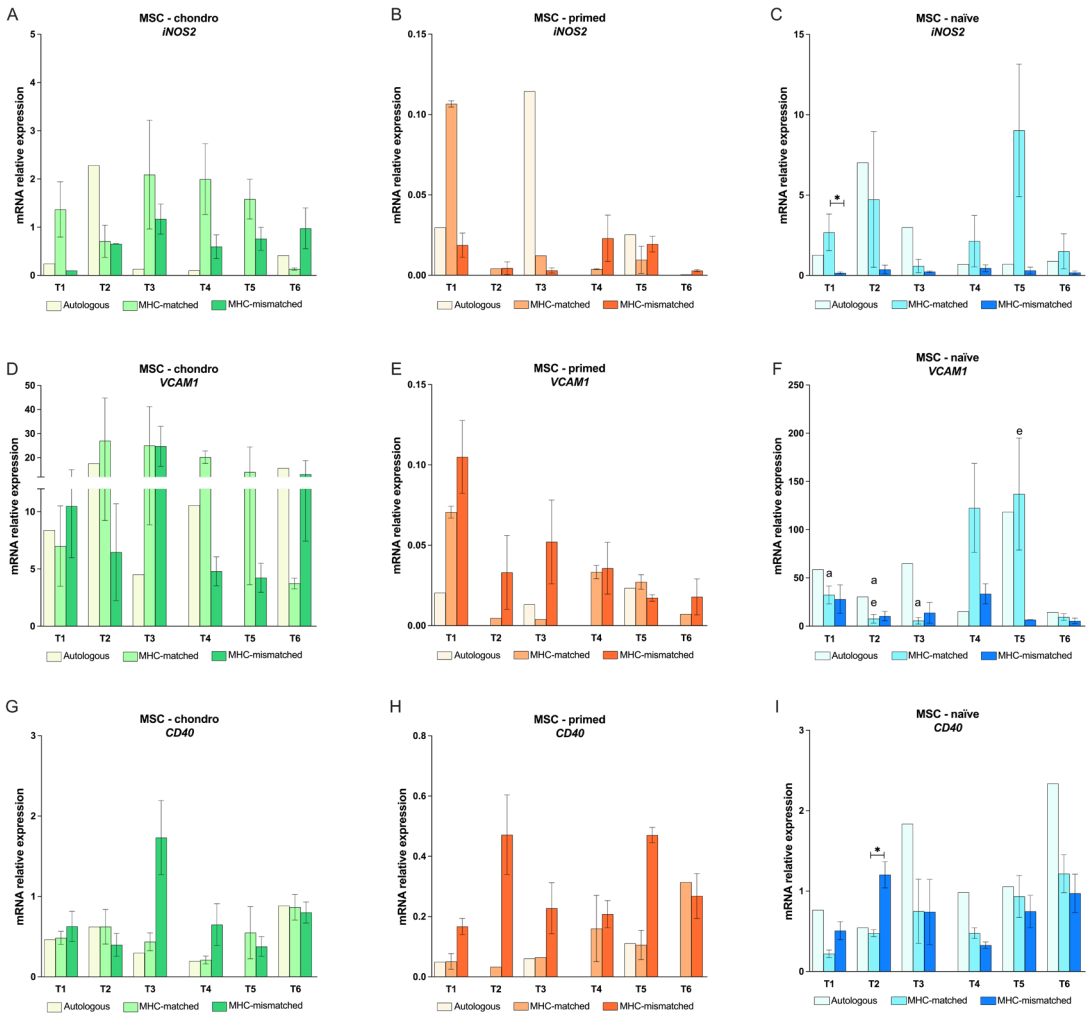


Fig. 4.2 Changes in inducible nitric oxide synthase 2 (*iNOS2*) (A, B, C), vascular cell adhesion molecule 1 (*VCAM1*) (D, E, F) and costimulatory molecule *CD40* (G, H, I) expression in equine MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars) following administration to autologous, MHC-matched and MHC-mismatched horses. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using each baseline MSCs type as reference sample (value 1). Significant differences between groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$). Significant differences between time-points compared to T1 and T5 are represented by lower case letter [a], [e] ([a], [e], $p < 0.05$).

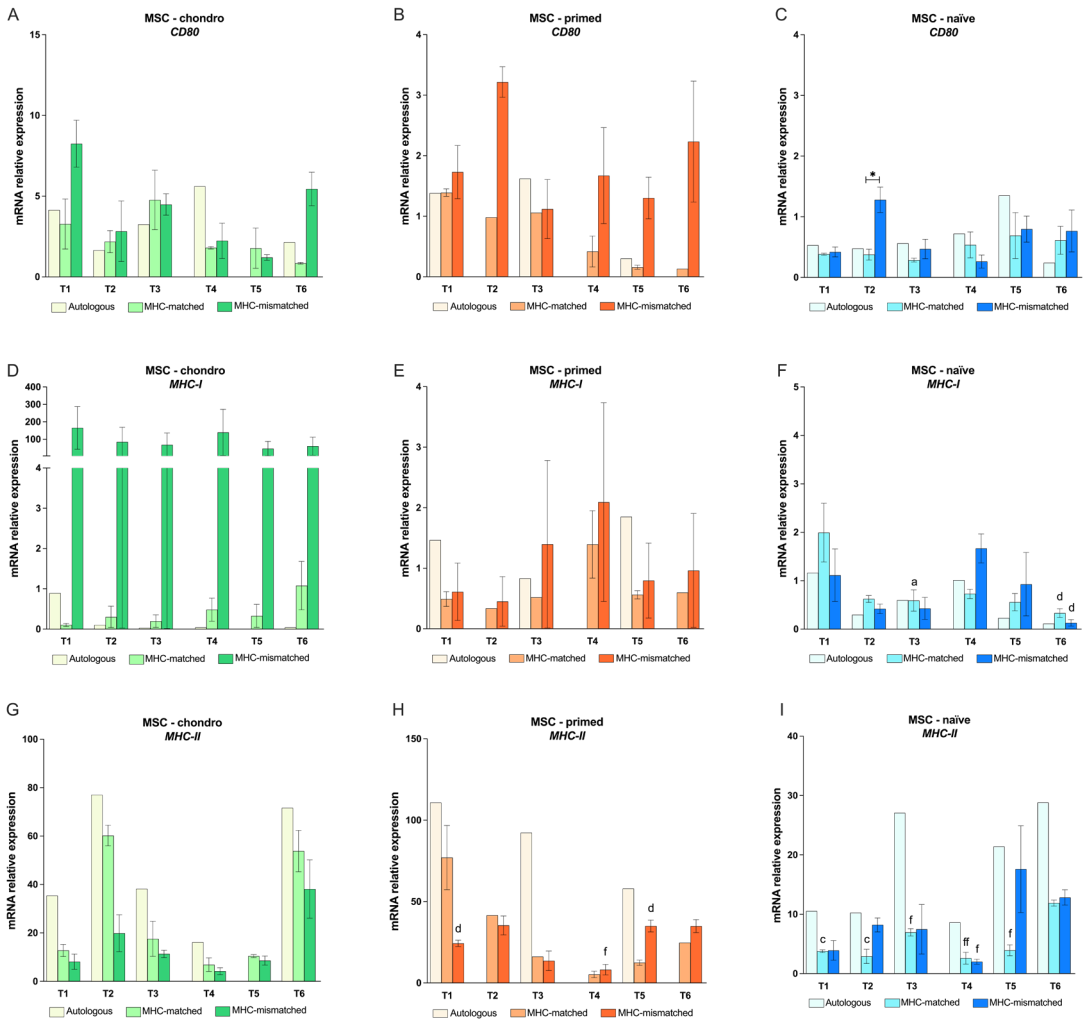


Fig. 4.3 Changes in costimulatory molecule *CD80* (A, B, C), *MHC-I* (D, E, F) and *MHC-II* (G, H, I) expression in equine MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars) following administration to autologous, MHC-matched and MHC-mismatched horses. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using each baseline MSCs type as reference sample (value 1). Significant differences between groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$). Significant differences between time-points compared to T1, T3, T4 and T6 are represented by lower case letter ^{a}, ^{c}, ^{d} and ^{f}, respectively (^{a}, ^{c}, ^{d}, ^{f}, $p < 0.05$).

Graphical representation of histology scores for assessing local immune response to mesenchymal stem cells (MSCs)

Significant differences represented are the same ones explained along the principal manuscript between groups (MHC-matched and MHC-mismatched) and over time-points: T1, 1 week after first MSCs administration (first scaffold); T2, 3 weeks after first MSCs administration (second scaffold), T3, 6 weeks after first MSC administration (third scaffold), T4, week after second MSCs administration (fourth scaffold); T5, 3 weeks after second MSCs administration (fifth scaffold), T6, 6 weeks after second MSCs administration (sixth scaffold).

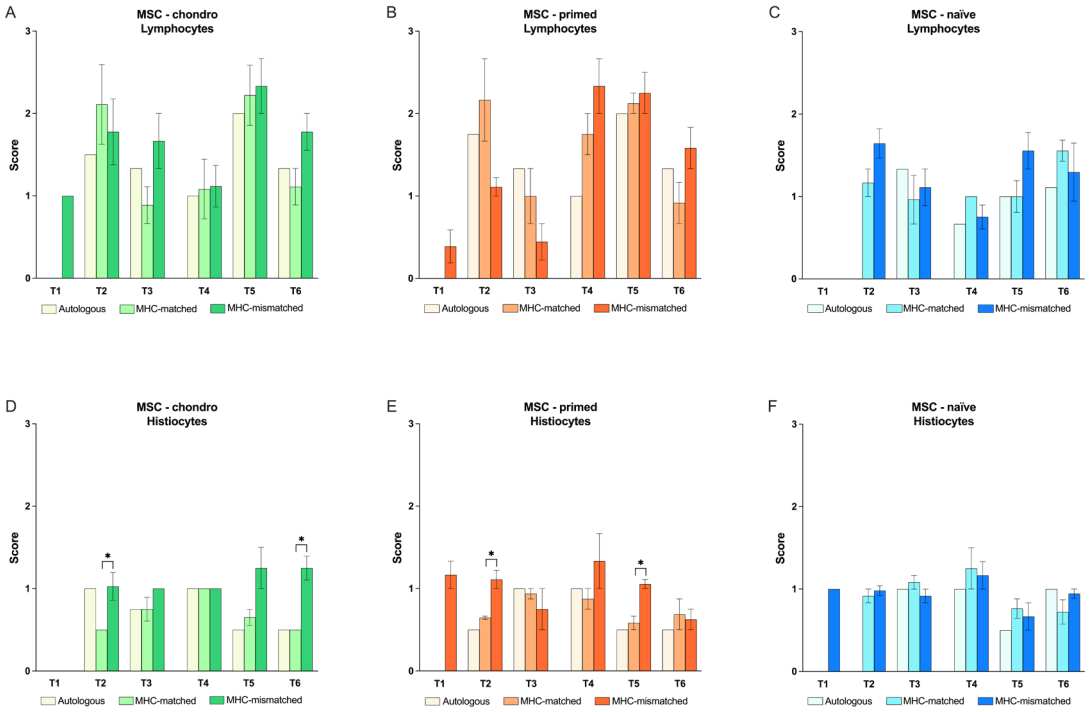


Fig. 4.4 Evolution of lymphocytes (A, B, C) and histiocytes (D, E, F) scores along the time in autologous, MHC-matched and MHC-mismatched horses following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Scores are normalized over acellular control (value 1) and represented as mean ± S.E.M. Significant differences between groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$).

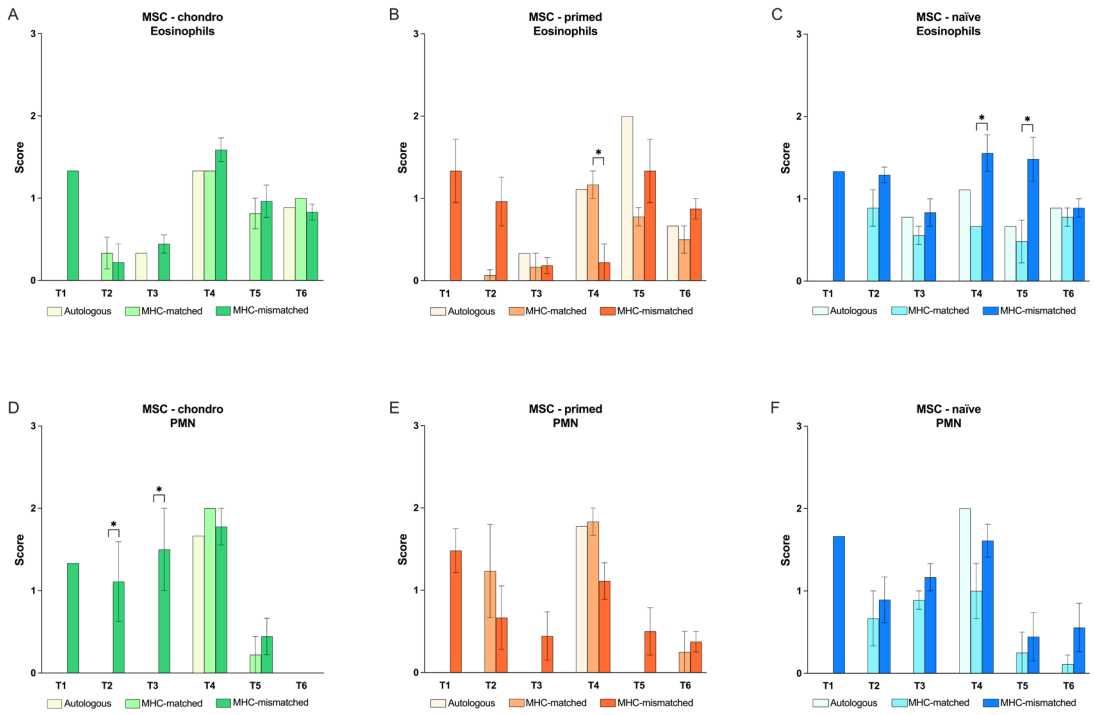


Fig. 4.5 Evolution of eosinophils (A, B, C) and polymorphonuclear (PMN) cells (D, E, F) scores along the time in autologous, MHC-matched and MHC-mismatched horses following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Scores are normalized over acellular control (value 1) and represented as mean ± S.E.M. Significant differences between groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$).

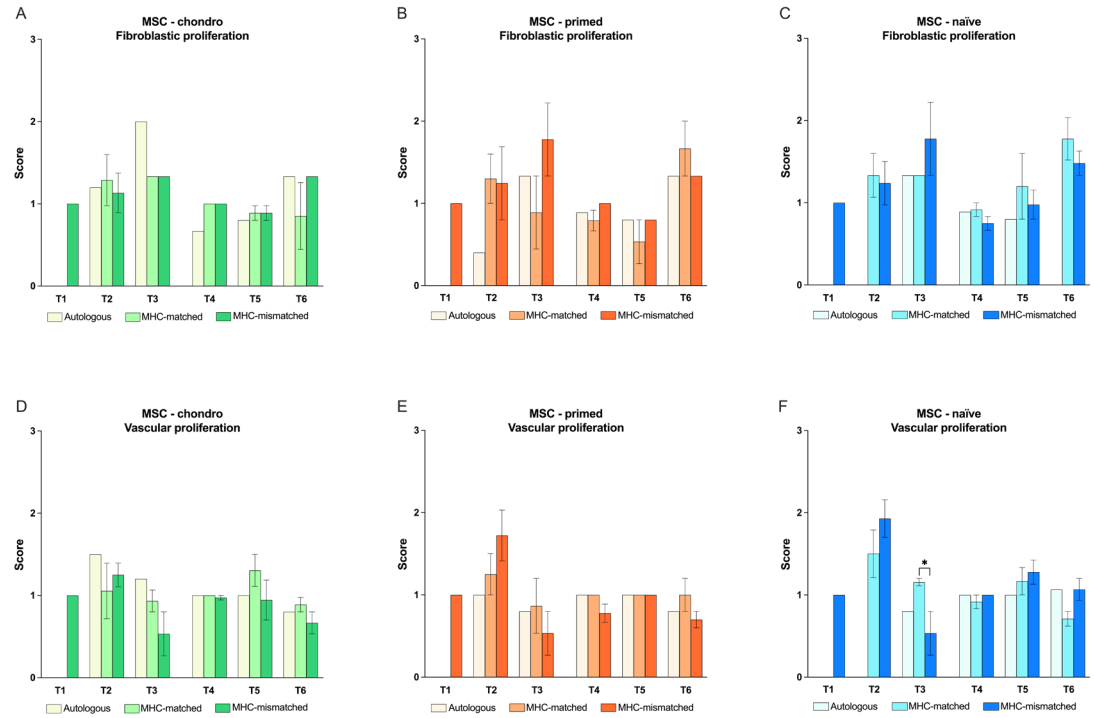


Fig. 4.6 Evolution of fibroblastic proliferation (A, B, C) and vascular proliferation (D, E, F) scores along the time in autologous, MHC-matched and MHC-mismatched horses following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Scores are normalized over acellular control (value 1) and represented as mean ± S.E.M. Significant differences between groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$).

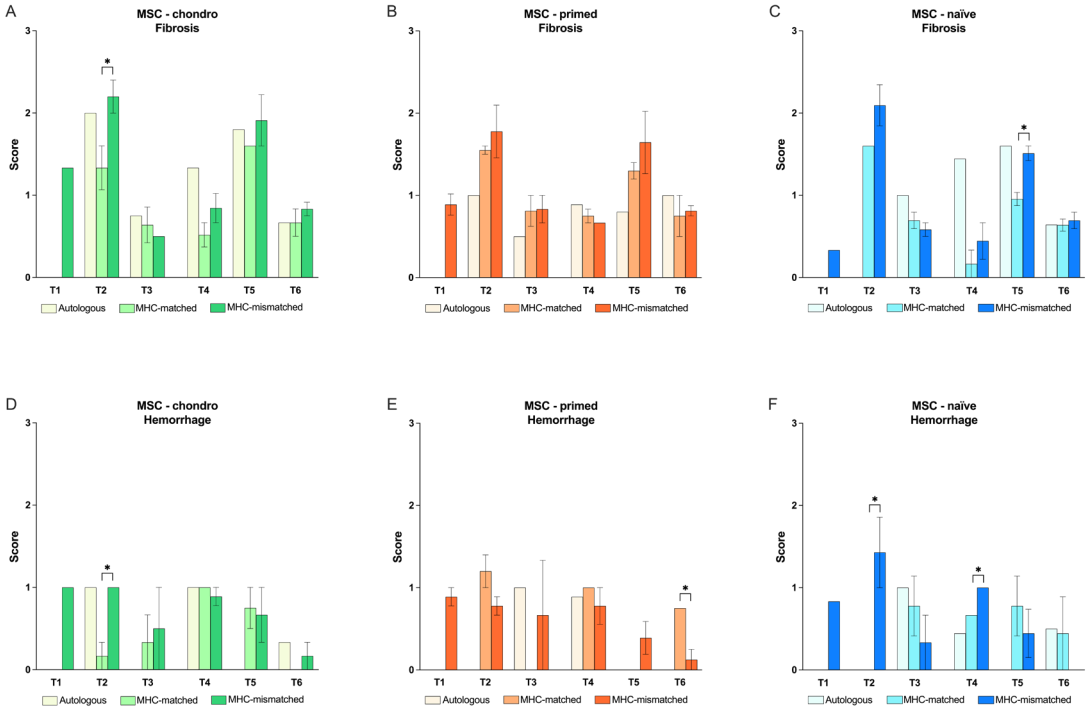


Fig. 4.7 Evolution of fibrosis (A, B, C) and hemorrhage (D, E, F) scores along the time in autologous, MHC-matched and MHC-mismatched horses following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Scores are normalized over acellular control (value 1) and represented as mean ± S.E.M. Significant differences between groups at one time-point are represented by a squared line with an asterisk (*, $p < 0.05$).

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