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MHC compatibility influences the interaction between different types of equine mesenchymal stem/stromal cells and the local immune response

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ABSTRACT

The allogeneic administration of equine mesenchymal stem/stromal cells (MSCs) has numerous advantages over autologous therapy, but their interactions with the patient's immune system need to be further elucidated. These interactions can be influenced by factors such as the compatibility between donor-receptor for the major histocompatibility complex (MHC) and by the MHC expression levels, which can change under different conditions like inflammatory exposure and chondrogeneic differentiation. In this study, we evaluated the local immune response induced by chondrogeneically differentiated (MSC-chondro), pro-inflammatory primed (MSC-primed) and basal (MSC-naïve) MSCs, and how this response changes the immunomodulatory and immunogenic profiles of MSCs *in vivo*. Equine MSCs were embedded in alginate scaffolds and subcutaneously implanted into autologous, MHC-matched and MHC-mismatched horses. Scaffolds were recovered at different time-points for histologic and gene expression analyses, and the procedure was repeated to assess the effect of a second administration. Our results suggest that MHC-compatibility may play a key role in attenuating the local immune response induced by MSCs, which may be related to the upregulation of immunomodulatory genes in the three MSC types *in vivo*. In contrast, when MSCs were administered into MHC-mismatched horses, expression of immunogenic genes was higher across all MSC conditions. Therefore, the conditions in which MSCs are administered may not affect the long-term local immune response, but MHC-matched administration would favour the immune evasion of MSCs, thus being advisable especially when repeated MSC administrations are required. Comprehensively investigating the *in vivo* immune response against equine allogeneic MSCs is crucial for advancing veterinary cell therapies.

1. Introduction

The therapeutic interest in mesenchymal stem/stromal cells (MSCs) is greatly increasing for several pathologies, including musculoskeletal injuries such as those affecting the horse. Owed to their sportive role, horses pose remarkable value as both patients and translational models, especially for joint pathologies (Ribitsch et al., 2021). Numerous studies have shown that MSC regulatory properties, particularly immunomodulation, are mainly responsible for their therapeutic effects, involving both direct cell-to-cell contact and contact-independent paracrine signalling (Hillmann et al., 2019). Furthermore, the MSC immunomodulatory activity is also critical to facilitate their escape from immune recognition when administered allogeneically (Voga et al., 2020). The use of allogeneic cell therapy presents important advantages as it is based on banking thoroughly characterized MSCs to make them more widely available and with higher standards (Berglund et al., 2017; Colbath et al., 2020). However, MSCs may be recognized and rejected by the immune system after their allogeneic administration (Ankrum et al.,

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2014), so these cells are no longer considered as immune privileged but rather immune evasive. This term has been coined based on both the ability of MSCs to regulate the immune response, and their generally low expression of the major histocompatibility complex (MHC), which facilitates MSC escape from immune recognition. However, both the immunomodulatory potential and the immunogenic profile of MSCs are variable between individuals, tissue sources, and MSC environment or status (Cassano et al., 2018; De Schauwer et al., 2014).

In this regard, the importance of MHC compatibility between donor and recipient is increasingly acknowledged. Several studies in the horse describe the generation of immunological memory against MHC molecules, which may have an impact on repeated administration of MSCs (Berglund et al., 2017; Rowland et al., 2021). While the MHC haplotype is an intrinsic factor to each individual that cannot be modified, there are other factors that can change the inherent immune properties of MSCs. Different studies have shown how exposure of MSCs to an inflammatory environment (priming) can increase their immunomodulatory properties by increasing the expression of different molecules such as interleukin (*IL6*), cyclooxygenase 2 (*COX2*), indoleamine 2,3-dioxygenase 1 (*IDO1*) or inducible nitric oxide synthase (*iNOS2*) (Barrachina et al., 2017; Caffi et al., 2020). However, MSC priming can also increase the expression of immunogenic markers like *MHC-I* and *MHC-II*, which may have implications for allogeneic MSC administration (Barrachina et al., 2017). The expression of these molecules after MSC differentiation is also increasingly studied, since MSC chondrogeneic induction is another strategy proposed to develop treatments for joint pathologies. Previous studies suggested that chondrogeneic differentiation increases immunogenicity and reduces immunomodulatory capacities of MSCs (Ryan et al., 2014). However, more recent *in vitro* and *in vivo* studies in horses have not observed an increase in MSC immunogenicity after their chondrogeneic differentiation, although the impact on their immunomodulatory properties remains debated (Cequier et al., 2022a; Van Hecke et al., 2021).

To maximize MSC clinical effects, ideally their immunomodulatory properties should outweigh their immunogenic potential. However, the exact mechanisms determining the immune profile of MSCs are not fully understood. This is pivotal to elucidate which factors, and in which direction, can modify the balance between immunomodulation and immunogenicity in MSCs. Importantly, this balance is not static but can change depending on the stimuli received by MSCs. Actually, the immune response elicited by the MSCs themselves might further stimulate them, acting as a feedback loop. For example, under certain circumstances, MSCs can activate T cells and induce their secretion of interferon gamma (IFNγ), a cytokine that can both activate the immune regulatory properties of MSCs and increase their MHC expression (Barrachina et al., 2017; Cequier et al., 2022a). However, it is currently unknown to what extent the immune response observed in the recipient leads to changes in the immune profile of MSCs *in vivo*.

In this study, we aimed at shining light into the interplay between MSC properties and the immune response that they elicit *in vivo* in the horse. We hypothesized, first, that different factors for MSC administration (namely MHC compatibility, chondrogeneic differentiation and inflammatory exposure) would result in different levels of immune response, and second, that such immune response would affect the immunomodulatory and immunogenic profiles of equine MSCs *in vivo*. Specifically, we hypothesized that MSC pro-inflammatory priming would enhance their ability to modulate the recipient's local immune response, thus maintaining their immunomodulatory properties after administration. Conversely, we hypothesized that chondrogeneically differentiated MSCs would induce a stronger local immune response, leading to reduced expression of immunomodulatory molecules and increased expression of immunogenic markers. Additionally, we hypothesized that MHC incompatibility would further increase the local immune reaction elicited by any type of MSCs, and thus would further change their immune profile. To test these hypotheses, we focused on the study of the local immune response for two reasons: first, local MSC administration is the most common in equine clinics; and second, it is more feasible to recover locally administered MSCs for analysis.

There has been limited *in vivo* study in horses about the local immune response triggered after the administration of MSCs, as well as about which changes undergo these cells after their administration, mostly because of the difficulty in recovering the cells once administered to the animal. Alginate hydrogels are a good option for encapsulating MSCs (Ho et al., 2022) and can allow retrieving the scaffolds containing the cells from the animals. Importantly, the alginate cannot be degraded by mammalian cells as they lack the necessary enzymes, and the structure of the alginate allows for the exchange of nutrients and metabolites. This feature makes alginate an excellent option to study the interaction between the animal's local immune response and the MSCs (Watts et al., 2013).

To achieve the goals of this study, we used a system in which equine MSCs chondrogeneically differentiated (MSC-chondro), pro-inflammatory primed (MSC-primed), or in basal conditions (MSC-naïve), were encapsulated in alginate hydrogels and implanted subcutaneously into MHC-matched or mismatched recipients. Such scaffolds were recovered at different time-points to analyse both the local immune response in the adjacent tissue and the gene expression of the MSCs retained within the scaffold. The administration was repeated to also explore the effect of immune memory mechanisms in a second administration of MSCs.

2. Material and methods

2.1. Study design

Equine bone marrow-derived MSCs were administered twice to horses that were either MHC-matched or MHC-mismatched with the MSC donors, and the local immune response as well as the changes in MSC gene expression profile were analysed over time. Three horses, each one homozygous for a specific MHC haplotype (HapPRE10/HapPRE10, HapPRE11/HapPRE11, and HapMAI04/HapMAI04), were chosen as MSC donors (Cequier et al., 2024). The recipient horses were divided into two groups based on their MHC compatibility with the donors. The MHC-matched group consisted of eight heterozygous horses sharing one haplotype with one of the donors: three HapPRE10 heterozygotes, three HapPRE11 heterozygotes, and two HapMAI04 heterozygotes. The MHC-mismatched group included nine horses with haplotypes different from HapPRE10, HapPRE11, and HapMAI04. Within each MHC-matched or MHC mismatched group, the recipient horses were further divided into three subgroups of 2–3 animals. Each subgroup received one of the following types of MSCs: chondrogeneically differentiated MSCs (MSC-chondro), pro-inflammatory primed MSCs (MSC-primed) or basal MSCs (MSC-naïve). Additionally, the three homozygous donor horses acted as autologous controls receiving one of the three MSC conditions, and one separate animal acted as sham operated control (Fig. 1A).

Each animal simultaneously received three alginate hydrogel scaffolds, with each scaffold containing 5×10^6 MSCs of the corresponding specific condition (MSC-chondro, MSC-primed or MSC-naïve). The scaffolds were placed in subcutaneous pockets created in the neck. This method was chosen to keep the MSCs located at a specific anatomical site, reducing the risk of migration, and to allow for the retrieval of the scaffolds at different time-points. Three acellular scaffolds were placed in the sham-operated control horse.

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 MSC immune regulatory and immunogenicity profiles were evaluated. One month after the removal of the last scaffold, each horse was re-exposed to the same MSC condition (MSC-chondro, MSC-primed or MSC-naïve; MHC-matched or MHC-mismatched) using the contralateral neck side. The scaffolds were removed at the same time-points to study the effect of a second

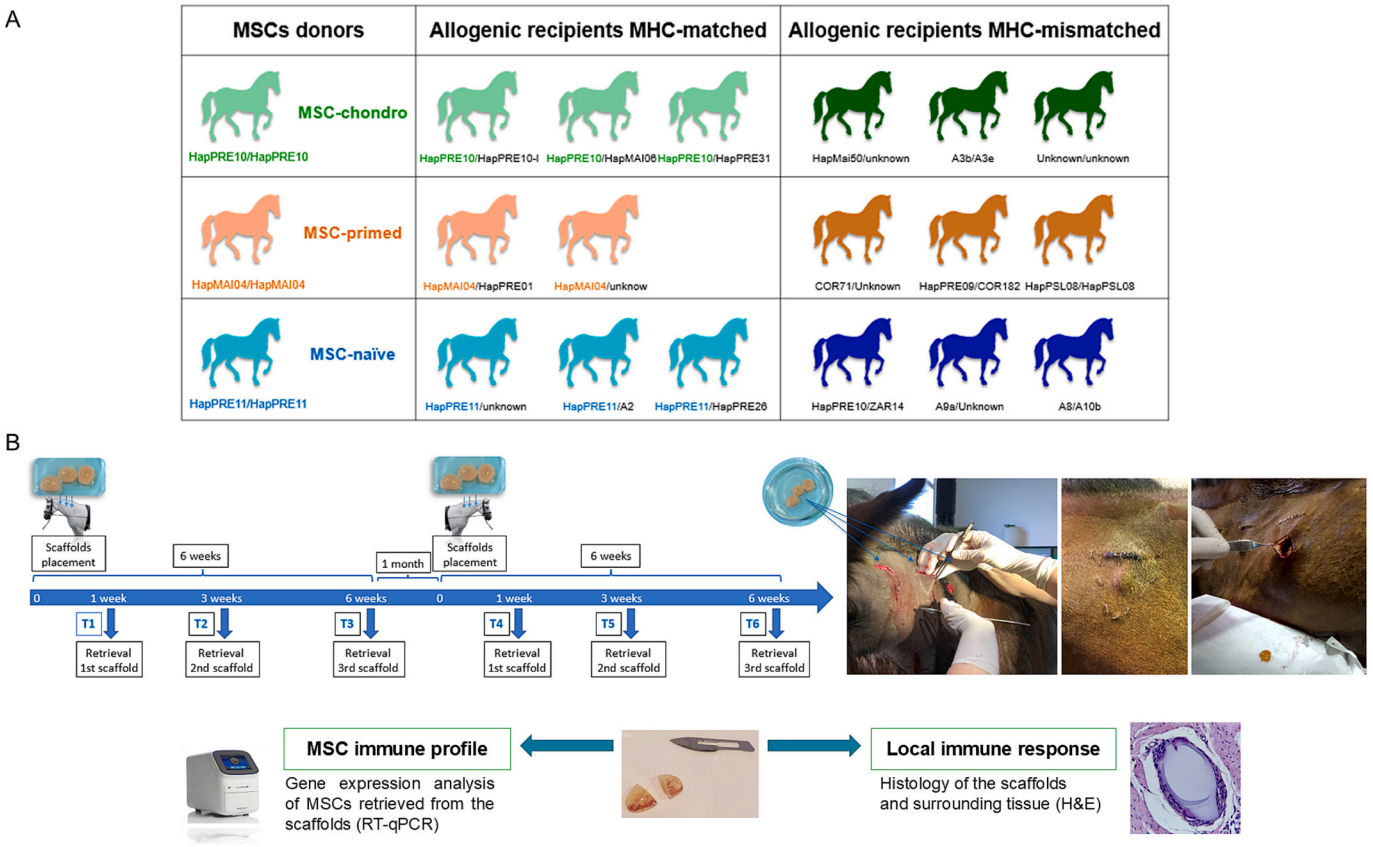

Fig. 1. Study design. (A) Groups of recipient horses (Autologous -also acted as donors-, allogeneic MHC-matched and allogeneic MHC-mismatched) and types of MSCs received (MSC-chondro, MSC-primed and MSC-naïve). The MHC haplotypes of each individual are indicated. (B) Schematic representation of the study design and of the subcutaneous placement and retrieval of the scaffolds at different times. Legend: H&E: haematoxylin-eosin; RT-qPCR: Real time quantitative polymerase chain reaction.

administration (Fig. 1B).

2.2. Animal selection by MHC-haplotyping

Twenty healthy horses, age ranging from 2 to 12 years (including 12 geldings, 7 mares, and 1 stallion), were chosen according to their MHC haplotype. These horses had no prior history of MSC treatments or transfusions. Their MHC haplotypes were determined by microsatellite typing using a validated panel of 10 markers, corresponding to highly polymorphic regions within the MHC genes (Barrachina et al., 2020; Sadeghi et al., 2018). Haplotype assignment in these animals was performed in a previous study from our group, in which full information about methodology and haplotypes is openly accessible (Cequier et al., 2024). In addition to these 20 animals, a 12-year-old gelding of unknown haplotype was used as sham-operated control, receiving acellular alginate scaffolds to account for the local reaction inherent to introducing a foreign body.

All procedures involving animals were approved by the Ethical Advisory Committee for Animal Experimentation of the University of Zaragoza (PI-15/16). The care and use of animals were performed accordingly with the Spanish Policy for Animal Protection RD118/2021, which meets the European Union Directive 2010/63. All animals were kept on paddocks in the facilities of the Animal Research Service of the University of Zaragoza, with free access to water and grass hay.

2.3. Preparation of MSCs for in vivo administration: MSC-chondro, MSC-primed and MSC-naïve

Equine bone marrow derived MSCs were obtained and characterized as part of a previous study conducted by our group (Cequier et al., 2022a), following the methods earlier described (Barrachina et al., 2017). In brief, MSCs from three donors were isolated and expanded at a density of 5000 cells/cm^2 using a conventional MSC medium consisting of low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % foetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/mL streptomycin, and 100 U/mL penicillin (all from Sigma–Aldrich) at 37 °C, 5 % CO2 and 90 % relative humidity. Full information on the characterization of equine MSCs used in this study is openly available in Cequier et al., 2022a. Mesenchymal stem/stromal cells were used between passage two to four in this study.

Alginate (ultrapure low-viscosity 67 % guluronate, UPLVG; NovaMatrix, FMC Corporation) was diluted at 3 % in phosphate buffered saline (PBS, Gibco, Thermo Fisher) by stirring for 1 h at 55 °C 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 with trypsin-EDTA 0.25 % (Sigma–Aldrich), cells were suspended at 5 × 10^6 MSCs/mL in PBS and mixed 1:1 with 3 % alginate solution for a final concentration of 1.5 % alginate in a 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 minor modifications. To make each implant, 2 mL of the MSC-alginate mix were transferred with a syringe into a concave custom-made device, which was immediately immersed into a sterile 102 mM $CaCl_2$ (Sigma–Aldrich) solution for 5 min until polymerization was apparent. Then, 1 mL 102 mM $CaCl_2$ solution was added to the bottom of 12-well plates and scaffolds were transferred to wells, covering each one with an additional 1 mL 102 mM $CaCl_2$ solution to completely submerge the constructs. Acellular implants were made following the same methodology but only PBS was used instead of the MSC suspension. All scaffolds were incubated 30 min, 37 °C and 5 %

CO_2, after which remaining $CaCl_2$ solution was removed, and constructs were washed three times with PBS.

For MSC-naïve and MSC-primed, scaffolds were maintained in conventional MSC culture medium for 48 h to allow the cells adjusting to the new conditions. In addition, for MSC-primed scaffolds, 12 h prior to their *in vivo* placement, 5 ng/mL of tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) (R&D Systems) were added to the conventional MSC media according to previous reports (Barrachina et al., 2018a). For MSC-chondro, chondrogenesis was induced in the MSCs embedded in the scaffold according to the methodology explained in Supplementary material 1.**2** by using 10 ng/mL of transforming growth factor beta-3 (TGFβ-3) (PeproTech) for 21 days. One extra scaffold for each MSC type was prepared in the same way and directly snap-frozen at −80 °C for later use as baseline reference in gene expression analysis (MSCs prior to administration). In addition, for all the MSC types (MSC-chondro, MSC-primed and MSC-naïve), the FBS in the media was replaced by 10 % autologous serum from each recipient during the 24 h prior to the *in vivo* placement of the scaffolds, in order to remove xenogeneic antigens (Pezzanite et al., 2015). Right before the intervention, scaffolds were washed with PBS three times.

Prior to any *in vivo* assay, preliminary tests were performed to check the viability of MSCs after encapsulation (Supplementary material 1.**1**), as well as to confirm their ability to differentiate into chondrocytes into the scaffolds (Supplementary material 1.**2**). In addition, a pilot study was conducted in one horse (under the same project license), where three alginate scaffolds containing green fluorescent protein (GFP)-labelled MSCs were implanted and recovered at each time-point to confirm that scaffolds were durable and would not migrate, and that MSCs would be retained inside along the duration of the study. This pilot study also allowed to optimize scaffolds placement, recovery procedures, and histological evaluation (Supplementary material 1.**3**).

2.4. Placement and retrieval of the scaffolds

Three alginate scaffolds containing 5×10^6 MSCs (either MSC-chondro, MSC-primed or MSC-naïve) each one (15×10^6 MSCs per animal in total) were placed along the neck, from cranial to caudal, using a subcutaneous pocket technique previously described (Bellas et al., 2015). The surgical intervention was carried out under appropriate sedation (detomidine 0.01 mg/kg, Sedaquick, Fatro; and butorphanol 0.02 mg/kg, Torbugesic, Pfizer) and local analgesia (lidocaine 5 %, Braun). For the placement of each scaffold, a longitudinal incision of approximately 2 cm in length was performed, leaving about 10 cm between incisions. Subcutaneous tissue was dissected distally to the incision to create a subcutaneous pocket of approximately 2.5 × 2.5 cm, where each scaffold was introduced. The incisions were closed in two layers (subcutaneous and skin) with 2/0 USP polyglyconate suture and surgical staples, respectively. All horses were clinically monitored and received a single dose of flunixin meglumine IV (1.1 mg/kg, Niglumine, Calier) right before surgery and procaine penicillin IM (15 mg/kg once daily for three days, Procapen, Livisto) postoperatively. All implant sites were ultrasonographically evaluated before and immediately after implant placement, and prior to the removal of each implant (Supplementary material 2).

Each implant was retrieved at different time-points, from cranial to caudal: at 1 (T1), 3 (T2) and 6 (T3) weeks after placement. One month after retrieving the last implant, the same procedure was repeated in the contralateral neck side and each animal received the same MSCs (MSC-chondro, MSC-primed or MSC-naïve; MHC-matched or MHC-mismatched). The three scaffolds from the second administration were also retrieved after 1 (T4), 3 (T5) and 6 (T6) weeks. The surgical intervention to recover each implant was carried out as for their placement (on station, under appropriate sedation and local analgesia). A three cm incision was made at the rostral edge of each pocket and the subcutaneous tissue was carefully dissected until retrieving the implant. Along with the implant, its surrounding tissue (*i.e.* fibrous capsule formed around the implant) was also collected for histological analysis. In a few cases, this capsule was not apparent and only the implant could be recovered. The surgical incision was closed as aforementioned, and the horses were clinically monitored during the following days.

2.5. Assessment of the local immune response and changes in MSC gene expression profile after their administration in vivo

After retrieval, each scaffold was divided into two pieces. One piece of each implant, and the corresponding surrounding tissue, were immediately fixed for histological assessment, and the other implant portion was snap frozen and kept at −80 °C to study the gene expression of molecules related to immunomodulatory and immunogenic properties of MSCs. This approach allowed for the evaluation of both the effect of MSCs on the local immune cell response under different conditions (MSC-chondro, MSC-primed, MSC-naïve; MHC-matched/mismatched), as well as the effect of such response on the immune profile of each type of MSCs.

2.5.1. Histological analysis

Scaffold pieces submitted for histology and their corresponding surrounding tissue were fixed in 4 % paraformaldehyde (PFA) in PBS (Olderøy et al., 2014; Reppel et al., 2015) overnight at 4 °C. All the preparations were embedded in paraffin, cut into 5 μm sections and the sections were hydrated with increasing gradients of alcohol and stained with haematoxylin-eosin (H&E). All sections were rinsed with distilled water, dehydrated with decreasing amounts of alcohol, and mounted (Barrachina et al., 2018b). Three preparations per sample were blindly analysed by an experienced pathologist (third author, MTS-M). Entire H&E sections were scanned using a 3D Scan Pannoramic Histech scanner (3D Histech P-1000 scanner), at 40× resolution, obtaining whole slide images. Images were analysed using SlideViewer 2.6 software (3D Histech kft.), which provides detailed morphometric analysis with precise measurements of different histological parameters at high resolution (Supplementary material 3).

The local immune response was assessed in the surrounding tissue since immune cells were not able to infiltrate intact scaffolds. Scaffold sections were used to verify the presence of the embedded MSCs inside. Each sample of surrounding tissue was evaluated using a scoring system ranging from 0 (minimal response) to 3 (maximum response), based on the presence of lymphocytes, eosinophils, histiocytes, polymorphonuclear cells. These parameters were assessed in the tissue immediately adjacent to the scaffold (*i.e.* inner part of the capsule). Additionally, the more external part of the capsule was assessed for signs of fibrosis, haemorrhage, synovial metaplasia, fibroblast proliferation, vascular proliferation, residual scaffold fragments, hemosiderin, fat, fat necrosis, and muscular fascia involvement (Supplementary material 3; **Table 3.1**). This scoring system was based on that used in previous studies (Aguiar et al., 2015) with the addition of the parameters found in our preparations. The score of each sample was normalized over the score assigned to sham-operated acellular controls (thus assigned with value 1), in order to account for the intrinsic effect of placing a subcutaneous implant.

2.5.2. Gene expression analysis

Gene expression of molecules related to the immune regulatory (*COX2*, *IL6*, *IDO1*, *iNOS2* and *VCAM1*) and immunogenic (*CD40*, *CD80*, *MHC-I* and *MHC-II*) properties of equine MSCs were analysed by real time quantitative polymerase chain reaction (RT-qPCR) to assess the profile of these cells after their administration under different conditions. After retrieving and snap-freezing the scaffolds halves, the frozen samples were disrupted and homogenized in TRI Reagent® (Zymo Research, R2050) using the TissueLyser LT instrument (Qiagen), and the Direct-Zol RNA Miniprep Kit (Zymo Research, R2052) was used to isolate mRNA from each scaffold. Prior to retrotranscription, RNA 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 was performed from 100 ng of total RNA using qScript™ cDNA SuperMix (Quanta Biosciences), according to the manufacturer's instructions. The primers used were previously designed by our group and their full information is openly accessible (Barrachina et al., 2017). All RT-qPCR reactions were performed and monitored with a QuantStudio3 Real Time PCR System device (Applied Biosystems, USA) as previously described (Cequier et al., 2022b) and gene expression was analysed using the comparative ΔΔCt method. As reference sample to calculate the relative gene expression in each scaffold, the basal scaffold containing MSC-naïve was used as reflection of MSC baseline expression prior to their administration in regular conditions. The basal (pre-implantation) expression of MSC-chondro and MSC-primed in scaffolds is also normalized over MSC-naïve, the latter being always presented with value 1 ($2^0 = 1$).

2.6. Statistical analysis

Statistical analysis was performed with the IBM SPSS Statistics version 26 statistical package. Analytical statistics were used to test for differences between types of MSC administration along the time, both for each histological parameter and for the expression of each gene.

The variables were "group" (two categories: MHC-matched and MHC-mismatched), "cell type" (three categories: MSC-chondro, MSC-primed and MSC-naïve) 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 histological score of each parameter and the expression of each gene was analysed individually, establishing them as the dependent variables. Each one of the other variables were established as factors to study the following differences: (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 10.4 was used for graphical representation.

3. Results

The results presented address the most relevant findings that may have implications for future MSCs therapies, focusing on the histological parameters and genes that showed consistent trends and significant differences between conditions. No substantial changes were noted for other histological parameters and genes, suggesting that these were not influenced by the conditions examined in this study. For comprehensive reference and potential interest in future research, graphical representations of all the analysed parameters can be found in Supplementary material 4.

Prior to presenting results, it should also be noted that 9 implants, from a total of 126 implants placed in the horses (6 implants in 21 horses, including the sham operated control), could not be recovered. This occurred due to the natural behaviour of the horses, which scratching and grooming interactions led to suture dehiscence in a few cases. In these cases, gene expression data is missing. In addition, in 19 cases, no capsule was found around the implant and thus surrounding tissue could not be recovered. Therefore, no histological values were assigned in these cases. Of note, 15 out of the 19 missing values corresponded to the first time-point (T1, one week after the first administration), which might suggest that there was not enough time for capsule formation. The detailed information about missing implants and missing capsules is included in Supplementary material 4.1.

3.1. Changes induced by MSCs on the recipients' immune response

The local immune response induced by MSC-chondro, MSC-primed or MSC-naïve conditions was evaluated in the tissue surrounding the MSC-containing scaffolds that were placed subcutaneously in autologous, MHC-matched or mismatched horses, and subsequently recovered at different time-points. As aforementioned, there were some cases in which the tissue surrounding the implant could not be recovered, mostly at the first time-point. Interestingly, autologous and MHC-matched horses did not develop such capsule after the first administration (T1). However, also at T1, a capsule around the implant was formed in some horses from the MHC-mismatched groups, regardless of the type of MSCs administered (Supplementary Material 4.1) and in the sham-operated (acellular scaffolds) horse. All the retrieved implants presented MSCs embedded within. Even in the few cases where complete removal of the implants was not possible, the surrounding tissue consistently presented inclusion of alginate fragments containing cells. These findings confirm the persistent local presence of MSCs throughout the study period.

3.1.1. Equine MSCs induced a local immune response that was influenced by the type of MSCs

The response of lymphocytes and eosinophils differed upon the type of MSC received (MSC-chondro, MSC-primed or MSC-naïve) (Fig. 2), while a similar immune response was observed in terms of presence of histiocytes, polymorphonuclear cells, multinucleated cells, fibrosis, haemorrhage, synovial metaplasia, fibroblast proliferation, vascular proliferation, residual scaffold fragments regardless of the MSC condition (Supplementary material 4.**2**).

Both MHC-matched and mismatched recipients of MSCs overall showed a higher degree of lymphocyte recruitment than the sham-operated control (value = 1), revealing a significant effect of MSC administration on the local immune response. 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, after the second administration, MSC-primed promoted an earlier and higher recruitment of lymphocytes, both in MHC-matched and mismatched recipients. Indeed, the highest degree of lymphocyte infiltration was observed at one week after the second administration of MSC-primed in MHC-mismatched animals (T4, $p < 0.05$ over both MSC-chondro and MSC-naïve) (Fig. 2A).

Regarding the recruitment of eosinophils, the scores were overall lower than for lymphocyte recruitment, and MSCs promoted a local increase in eosinophils compared to the sham-operated control (value = 1) only under certain conditions. After the first administration, MSC-naïve induced higher eosinophil scores than the other MSC types, both in the MHC-matched and in the MHC-mismatched group (T2, over MSC-chondro MHC-mismatched $p < 0.05$; T3, over MSC-chondro MHC-matched, $p < 0.05$; T3, over MSC-primed MHC-mismatched, $p < 0.05$). Nevertheless, the eosinophil score only increased over the control (value >1) in the MHC-mismatched group: after one week (T1) for all the three types of MSCs, and after three weeks (T2) only for MSC-naïve.

After the second administration, the eosinophil scores in the MHC-matched group remained similar for MSC-naïve while they increased for MSC-chondro and MSC-primed. Specifically, at one week after the second administration, MSC-chondro and MSC-primed promoted a higher eosinophil recruitment than MSC-naïve (T4, $p < 0.01$ for MSC-chondro; $p < 0.05$ for MSC-primed). In contrast, at the same time-point but in the MHC-mismatched group, MSC-primed recruited significantly less eosinophils than MSC-chondro and MSC-naïve ($p < 0.01$ in both cases) (Fig. 2B).

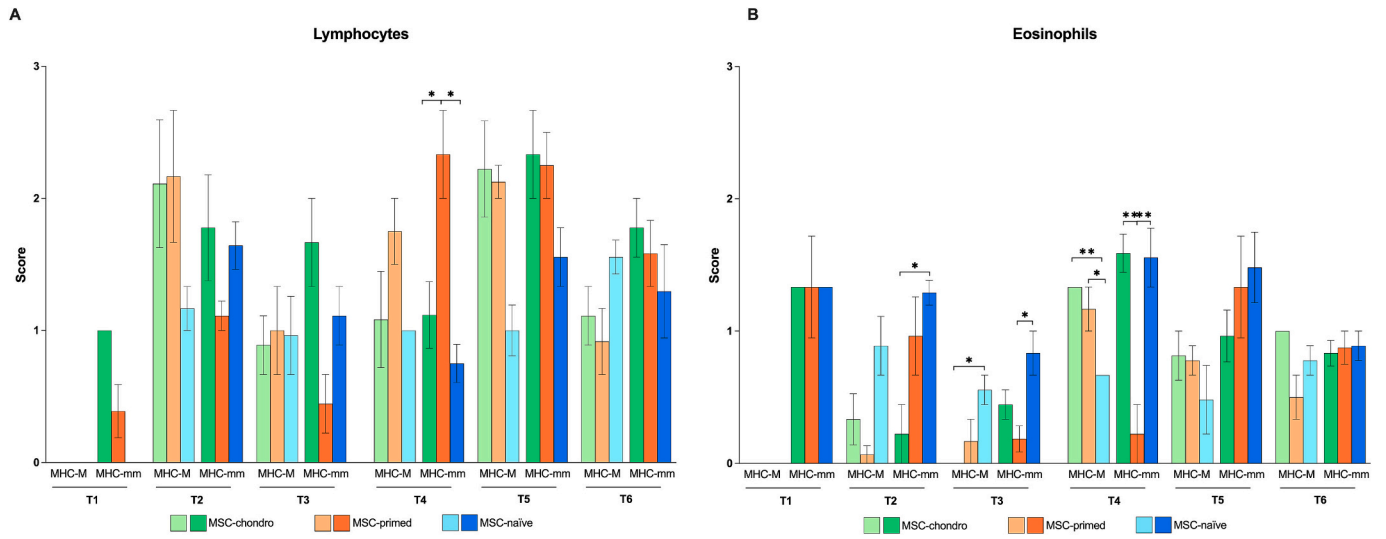

Fig. 2. Evolution of scores for lymphocytes (A) and eosinophils (B) along the time in MHC-matched (MHC-M) and MHC-mismatched (MHC-mm) horses following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Scores are normalized over the sham-operated acellular control (value 1) and represented as mean ± S.E.M. Comparison between MSC types is presented within each recipient group and for specific time-points: Significant differences between MSC-chondro, MSC-primed and MSC-naïve within each recipient group (MHC-matched or mismatched) are represented at each time-point by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.1.2. The local immune response elicited by equine MSCs is dynamic along the time and is influenced by MHC compatibility

Regarding lymphocyte recruitment, no significant differences were observed between MHC-matched and MHC-mismatched groups. However, this parameter presented evolution patterns along the time that differed between MHC-matched and mismatched recipients, and which were also dependent on the type of MSCs. For MSC-chondro, both in the MHC-matched and mismatched groups, the highest lymphocyte scores were found at three weeks after each administration (T2 and T5) compared to all the other time-points, but the increase was statistically significant only over T4 in the MHC-matched group (T2 and T5 over T4, $p < 0.05$ in both cases) (Fig. 3A). For MSC-primed, the first administration promoted more lymphocyte recruitment in the MHC-matched group than in the MHC-mismatched group, while the opposite was observed after the second administration. Thus, both MHC-matched and mismatched recipients presented higher lymphocyte scores after the second administration compared to the first one, but the magnitude of this increase was higher for MHC-mismatched recipients (T4 and T5 over T1, $p < 0.05$ in both cases; T6 over T3, $p < 0.05$) (Fig. 3B). Finally, recipients of MSC-naïve presented less variation in lymphocyte recruitment over time. The highest values were observed in the MHC-mismatched group at three weeks after each administration, similarly to MSC-chondro (T2 and T5 over T4, $p < 0.05$ in both cases) (Fig. 3C).

Regarding eosinophils, their recruitment was generally higher after the second MSC administration. Recipients of MSC-chondro developed slightly higher scores at one week after each administration (Fig. 3D). MSC-primed induced more recruitment of eosinophils in MHC-mismatched recipients after the first administration, but this parameter significantly decreased over the MHC-matched group after the second administration (T4, $p < 0.05$) (Fig. 3E) ($p < 0.05$). In contrast, MSC-naïve led to higher eosinophil scores in the MHC-mismatched group over the MHC-matched group after the second administration (T4 and T5, $p < 0.05$ in both cases) (Fig. 3F).

Histiocyte scores were generally low and with minor changes along the time. At some time-points, significantly higher scores were noted in MHC-mismatched recipients of both MSC-chondro (T2 and T6, $p < 0.05$) and MSC-primed (T2 and T5, $p < 0.05$) compared to the MHC-matched group (Fig. 3G, H).

Regarding the infiltration of polymorphonuclear cells, MSC-chondro promoted a significant increase in MHC-mismatched recipients compared to the MHC-matched group after the first administration (T2 and T3, $p < 0.05$ in both cases) (Fig. 4A), but this parameter decreased in the MHC-mismatched group after the second administration (T6 over T4, $p < 0.05$). In MHC-recipients at one week after the second administration of MSC-chondro, polymorphonuclear infiltration was also detected but it subsequently decreased (T5 over T4, $p < 0.05$). MSC-primed induced a quick increase in polymorphonuclear cells after each administration into MHC-mismatched recipients, which subsequently decreased (T5 over T4, $p < 0.05$) (Fig. 4B). Polymorphonuclear infiltration also increased shortly after the second administration of MSC-primed into MHC-matched recipients, but significantly decreased at the next time-point (T5 over T4, $p < 0.05$) (Fig. 4B). Similarly, each administration of MSC-naïve promoted an increase in polymorphonuclear scores in MHC-mismatched recipients, which was diminished over time (T5 over T4, $p < 0.01$; T6 over T4, $p < 0.05$) (Fig. 4C).

With regard to the presence of multinucleated cells, MSC-chondro promoted a marked increase from three weeks onwards after each administration, which was similar in both recipient groups (Fig. 4D). A similar pattern was observed for MSC-primed, but these cells had a minimal effect in MHC-mismatched recipients while comparatively induced a significantly higher reaction in MHC-matched animals (T3, $p < 0.01$; T5, $p < 0.05$; T6, $p < 0.05$) (Fig. 4e). In contrast, MSC-naïve induced a higher presence of multinucleated cells in MHC-mismatched recipients (T2 and T3, $p < 0.05$) (Fig. 4F).

The placement of scaffolds into subcutaneous pockets led to the peripheral formation of fibrosis, with a similar degree for MSC-containing scaffolds than for the sham-operated acellular scaffolds (normalized value 1). However, higher fibrosis scores were detected at three weeks after each MSC administration (T2 and T5) for all the three types of MSCs and in both recipient groups, which subsequently decreased (Fig. 5A, B, C) (First administration: MSC-chondro MHC-matched, T3 over T2, $p < 0.05$; MSC-naïve MHC-matched, T3 over T2, $p < 0.05$; Second administration: MSC-chondro MHC-matched and mismatched, T4 and T6 over T5, $p < 0.05$; MSC-primed MHC-mismatched, T6 over T5, $p < 0.05$; MSC-naïve MHC-matched, T5 over T2, $p < 0.05$; MSC-naïve MHC-mismatched, T6 over T5, $p < 0.05$). The degree of fibrosis formation was similar between the MHC-matched and the MHC-

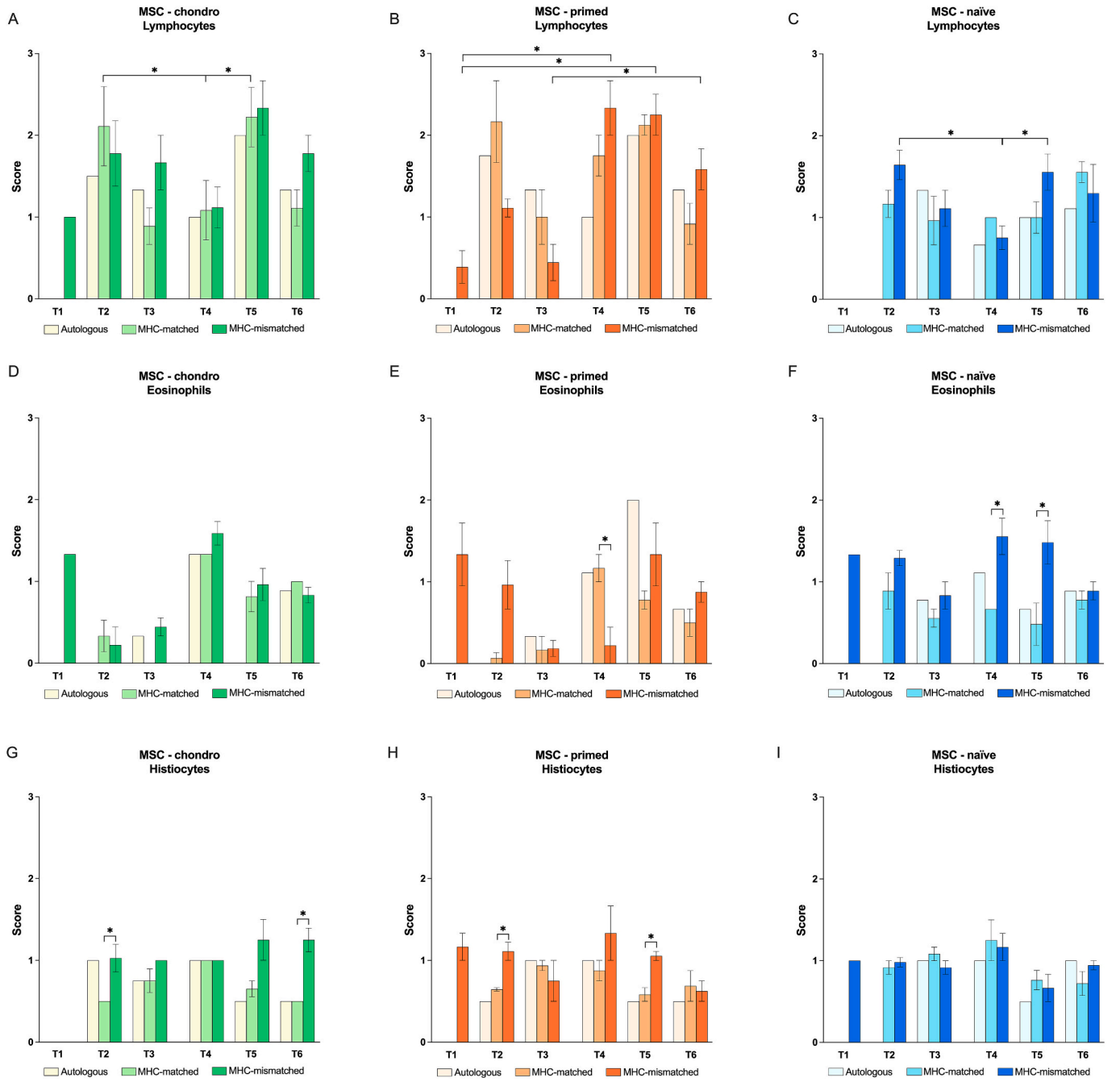

Fig. 3. Evolution of lymphocytes (A, B, C), eosinophils (D, E, F) and histiocytes (G, H, I) scores along the time in autologous, MHC-matched and MHC-mismatched recipient horses following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Scores are normalized over acellular control (value 1) and represented as mean ± S.E.M. Comparison between recipient groups and along the time-points within each MSC type: significant differences between autologous, MHC-matched and MHC-mismatched recipients, and between time-points within the same recipient group, are represented separately for each MSC type (MSC-chondro, MSC-primed, MSC-naïve) by a squared line with an asterisk (*, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mismatched recipients, but at some points it was higher for the latter (MSC-chondro, T2, $p < 0.05$; MSC-naïve, T5, $p < 0.05$) (Fig. 5A, C).

The degree of haemorrhagic reaction observed was overall low in all cases. For MSC-chondro, no conditions exceeded the values of the sham-operated control. However, after the first administration, MHC-mismatched recipients generally presented higher haemorrhage scores than the MHC-matched group (T2, $p < 0.05$) (Fig. 5D). On the contrary, MSC-primed tended to produce more haemorrhage in the MHC-matched than in the MHC-mismatched recipients (T6, $p < 0.05$), but all the values were closer to or lower than the sham-operated control (Fig. 5E). Similarly to MSC-chondro, MSC-naïve also produced more haemorrhagic reaction in the MHC-mismatched recipients. Indeed, the MHC-mismatched group showed significantly higher values after both the first and the second administration (T2, $p < 0.05$; T4, $p < 0.05$) (Fig. 5F).

3.2. *Changes in the gene expression of markers related to equine MSCs immunomodulation and immunogenicity*

The changes in the expression of relevant genes involved in the immunomodulatory properties of equine MSCs were analysed (*COX2*,

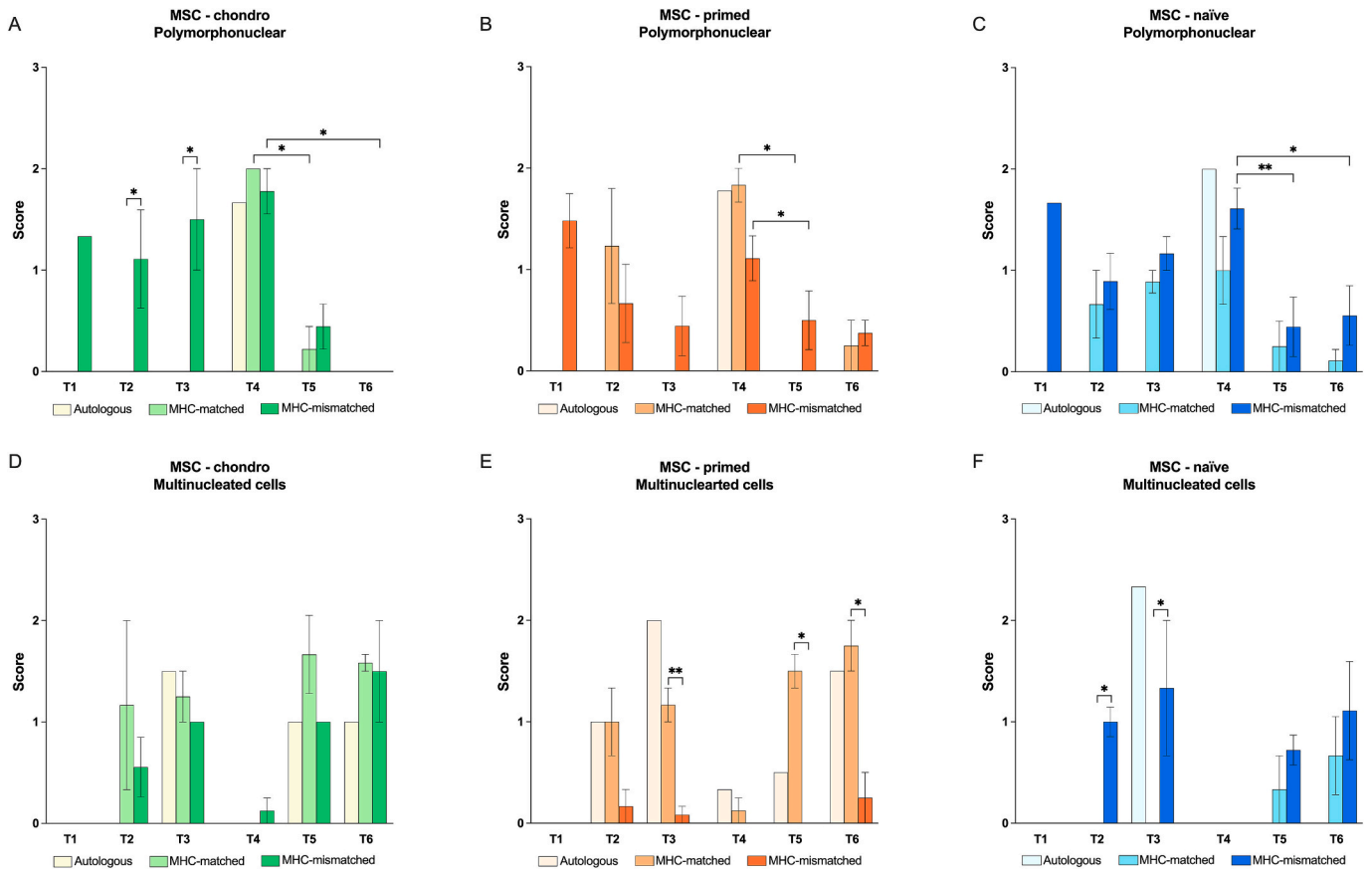

Fig. 4. Evolution of polymorphonuclear (A, B, C) and multinucleated cells (D, E, F) scores along the time in autologous, MHC-matched and MHC-mismatched recipient horses following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Scores are normalized over acellular control (value 1) and represented as mean ± S.E.M. Comparison between recipient groups and along the time-points within each MSC type: significant differences between autologous, MHC-matched and MHC-mismatched recipients, and between time-points within the same recipient group, are represented separately for each MSC type (MSC-chondro, MSC-primed, MSC-naïve) by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

IL6, *IDO1*, *iNOS2* and *VCAM1*). Additionally, the expression of genes related to antigen presentation, including *CD40*, *CD80*, *MHC-I* and *MHC-II*, was assessed as reflection of changes in MSC immunogenicity. No relevant changes in the expression of these genes were observed between the types of MSCs (MSC-chondro, MSC-primed or MSC-naïve) and thus are not presented in the main manuscript, but these data can be found in the Supplementary material 4. On the contrary, there were significant differences between groups of recipients (MHC-matched and MHC-mismatched) and along the time-points (T1-T6), which are presented below.

3.2.1. *Gene expression of markers related to equine MSC immunomodulation*

The exposure of equine MSCs to the *in vivo* environment (subcutaneous placement within scaffolds) resulted in different changes in the immune profile of such cells, depending on the conditions in which they were administered (MSC-chondro, MSC-primed or MSC-naïve; MHC-matched or mismatched). Overall, immunomodulatory genes were upregulated in all the MSC types shortly after each administration. In addition, the influence of MHC matching was mostly detected for MSC-chondro and MSC-naïve, but it was not so marked for MSC-primed: when MSC-chondro or MSC-naïve were administered into MHC-matched recipients, these cells showed a higher immunomodulatory upregulation than when administered into MHC-mismatched recipients, while the difference between recipient groups was less consistent for MSC-primed. Interestingly, none of the three MSC conditions showed a significant upregulation of immunomodulatory genes in the MHC-mismatched group compared to the MHC-matched group.

MSC-chondro administered into MHC-matched animals significantly increased their expression of immunomodulatory genes. Specifically, *COX2*, *IL6* and *IDO1* exhibited higher expression in MSC-chondro recovered from the MHC-matched group compared to the MHC-mismatched group at the first time-point (T1, $p < 0.05$ in all cases) (Fig. 6A, D, H). Similarly, shortly after the second administration (T4), MSC-chondro administered to the MHC-matched group showed a higher expression of *COX2*, *IDO1*, and *VCAM1* compared to the MHC-mismatched group ($p < 0.05$) (Fig. 6A, H, N). Such overexpression found after each administration progressively decreased along the time: In the case of *COX2*, MSC-chondro significantly downregulated its expression to close-to-basal levels at T2 and T3 over T1, and at T6 over T4 (Fig. 6A); and for *IL6* at T3 over T1 (Fig. 6B) ($p < 0.05$ in all cases).

MSC-primed overall tended to reduce the expression of immunomodulatory genes after their *in vivo* administration, as compared to basal (pre-administration) MSC-primed. These genes were progressively further downregulated along the time post-administration. Such downregulation was statistically significant for *VCAM1* after the second administration compared to the first one in the MHC-matched group (T4 and T5 over T1 $p < 0.05$) (Fig. 6O).

The expression of immunomodulatory genes in MSC-naïve followed a similar pattern than in MSC-chondro. Overall, MSC-naïve showed higher expression of immunomodulatory genes when administered into MHC-matched recipients, although significant differences over the MHC-mismatched group were only punctually observed (*COX2*, T6; *iNOS2*, T1; $p < 0.05$ in both cases) (Fig. 6C, M). After their

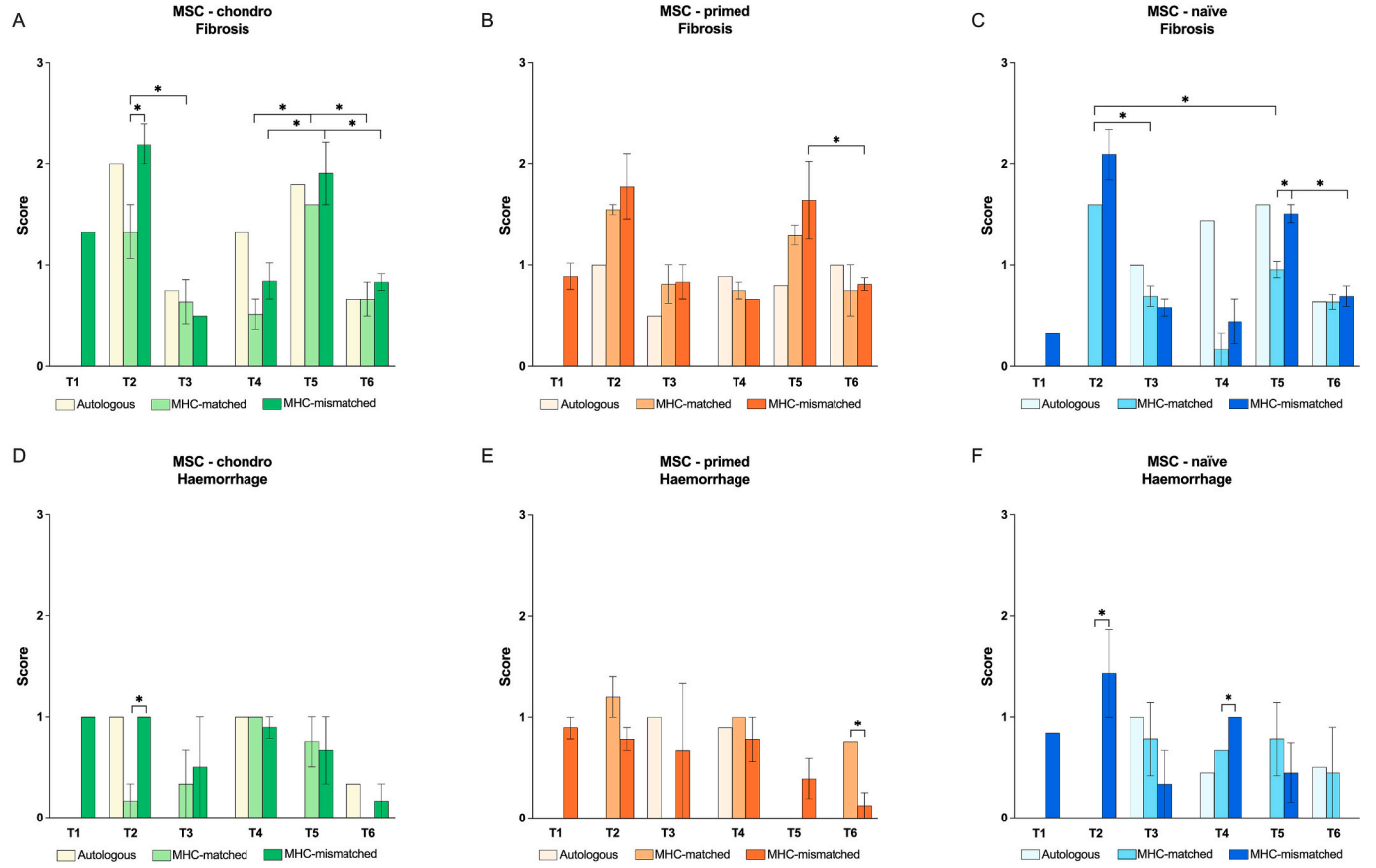

Fig. 5. Evolution of fibrosis (A, B, C) and haemorrhage (D, E, F) scores along the time in autologous, MHC-matched and MHC-mismatched horses following the administration of MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars). Scores are normalized over acellular control (value 1) and represented as mean ± S.E.M. Comparison between recipient groups and along the time-points within each MSC type: significant differences between autologous, MHC-matched and MHC-mismatched recipients, and between time-points within the same recipient group, are represented separately for each MSC type (MSC-chondro, MSC-primed, MSC-naïve) by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

administration, MSC-naïve tended to immediately increase (T1) the expression of *COX2, IL6, IDO1* and *VCAM1,* followed by a progressive downregulation. Such downregulation was significant at T2 and T3 over T1 for *COX2* in the MHC-mismatched group (Fig. 6C), and for *VCAM1* in the MHC-matched group ($p < 0.05$) (Fig. 6P). On the other hand, the expression of *iNOS2* presented a progressive upregulation and peaked at longer time-points (Fig. 6M). *VCAM1* also showed the highest values upon re-exposure (T5 over T2, $p < 0.05$) (Fig. 6P).

3.2.2. *Gene expression of markers related to equine MSC immunogenicity*

When compared to the basal reference (pre-administration MSC-naïve embedded into a scaffold, value 1), most of the equine MSCs exposed to the *in vivo* environment showed a downregulation (mean < 1) of the immunogenic genes *CD40*, *CD80* and *MHC-I*. This was seen in almost all cases, with a few exemptions, at one week after each administration (T1, T4), regardless of the type of MSCs (MSC-chondro, MSC-primed, MSC-naïve) and the type of recipient (MHC-matched or mismatched). Therefore, there was not an immediate raise in the immunogenic profile of equine MSCs after their *in vivo* administration, in spite of the inflammation produced by the surgery to place the scaffolds, and even when the administration was repeated (Fig. 7A-7I). Nevertheless, there were some differences at later time-points in the expression of immunogenicity-related genes among the MSC types. Moreover, in general there was a higher expression of immunogenic genes in equine MSCs administered into MHC-mismatched recipients, contrary to what was observed for immunomodulatory genes, which expression tended to be higher in MHC-matched recipients.

The expression of *CD40* only exceeded the basal values in MSCs administered to MHC-mismatched recipients. Specifically, MSC-chondro upregulated *CD40* at the latest time-point after the first administration (T3 over T6, $p < 0.05$) (Fig. 7A), and MSC-primed at the second time-point after each administration (T2 over T3, $p < 0.05$; T5 over T4, $p < 0.01$) (Fig. 7B). Furthermore, the expression of *CD40* was significantly higher in MSC-naïve administered into MHC-mismatched recipients compared to the MHC-matched group at T2 ($p < 0.05$) (Fig. 7C).

Regarding *CD80*, MSC-chondro maintained an expression below the reference sample (MSC-naïve pre-administration, value 1) along the time, but this expression slightly increased over MSC-chondro pre-administration. Significant differences were punctually found when MSC-chondro were administered to the MHC-mismatched group (T1 over T4, $p < 0.05$) (Fig. 7D). Similarly, MSC-primed also maintained *CD80* expression below the reference (< 1), but it slightly increased over basal (pre-administration) MSC-primed in the MHC-mismatched group, even though significant differences were not detected (Fig. 7E). MSC-naïve neither increased their *CD80* expression above the baseline, except when administered into MHC-mismatched recipients at T2 (over MHC-matched and over T3, $p < 0.05$ in both cases) (Fig. 7F).

The expression of *MHC-I* in MSC-chondro did not exceed the reference level (pre-administration MSC-naïve, value 1) in any case, but it was markedly higher in MSC-chondro administered into MHC-mismatched compared to MHC-matched horses, and also over MSC-chondro basal value, at all the time-points. However, likely due to the large deviation observed in the MHC-mismatched group, the difference between recipient groups was not statistically significant (Fig. 7G).

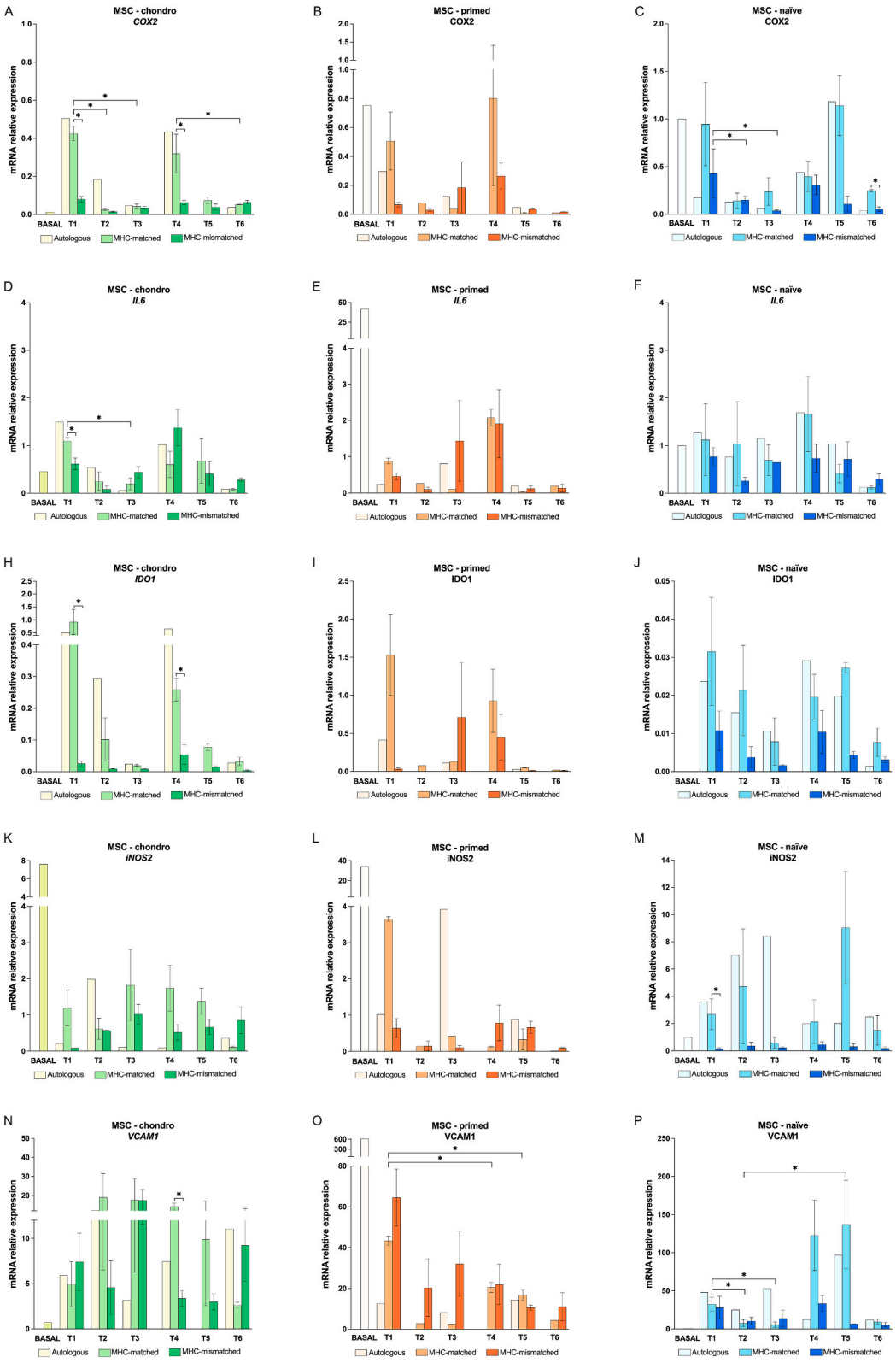

Fig. 6. Changes in the gene expression of the immunomodulatory markers cyclooxygenase 2 (*COX2*), interleukin 6 (*IL6*), indoleamine 2,3-dioxygenase 1 (*IDO1*), inducible nitric oxide synthase 2 (*iNOS2*) and vascular cell adhesion molecule 1 (*VCAM1*) in MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars) after their administration into MHC-matched and MHC-mismatched horses. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using basal MSC-naïve scaffold as reference sample (value 1). Basal values (pre-administration) of each MSC-chondro and MSC-naïve are also provided, and have been normalized over basal MSC-naïve too. Comparison between recipient groups and along the time-points within each MSC type: significant differences between autologous, MHC-matched and MHC-mismatched recipients, and between time-points within the same recipient group, are represented separately for each MSC type (MSC-chondro, MSC-primed, MSC-naïve) by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

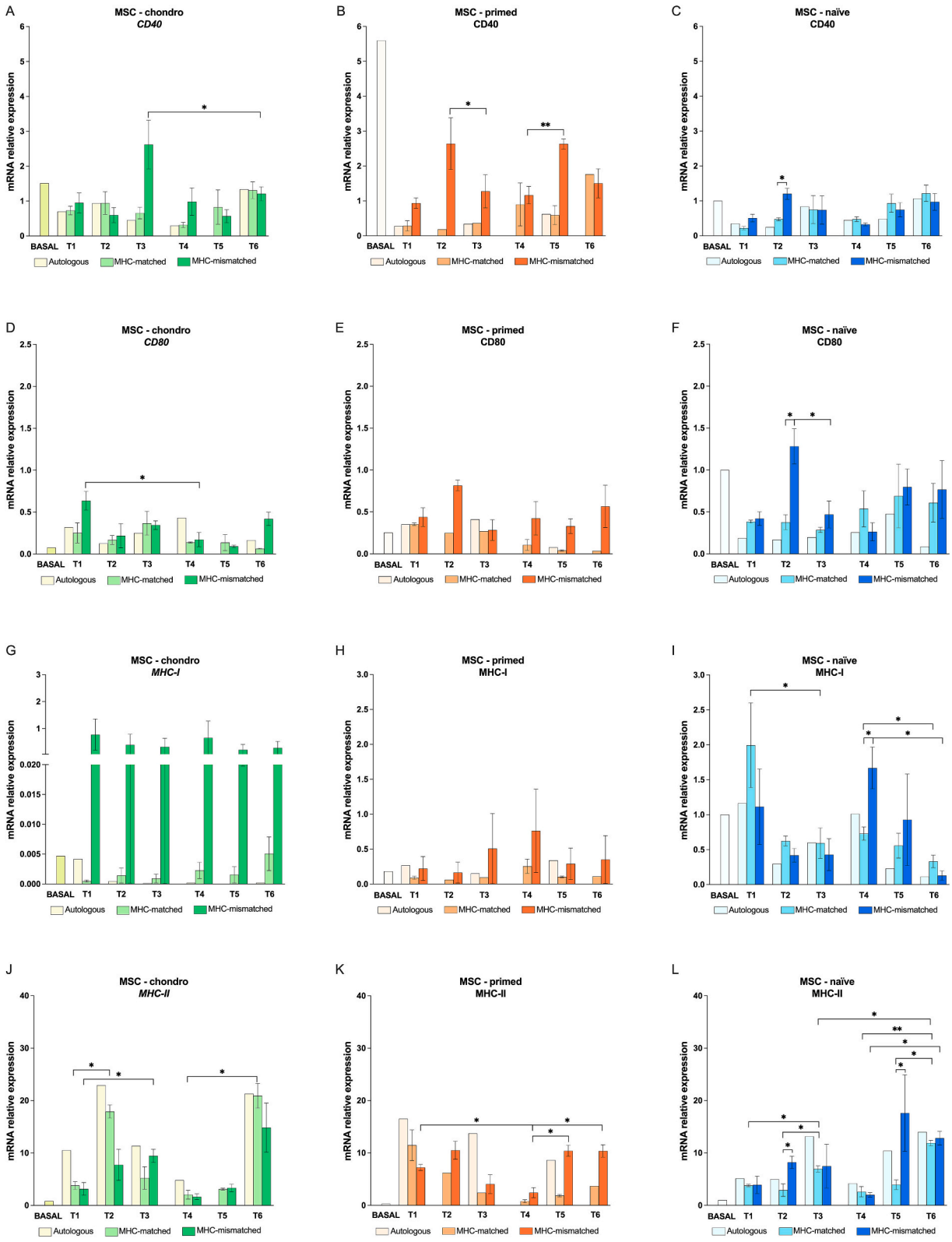

Fig. 7. Changes in the gene expression of the immunogenic markers *CD40*, CD80, *MHC-I* and *MHC-II* in MSC-chondro (green bars), MSC-primed (orange bars) and MSC-naïve (blue bars) after their administration into MHC-matched and MHC-mismatched horses. Changes in gene expression are represented as mean ± S.E.M of the relative mRNA expression, using basal MSC-naïve scaffold as reference sample (value 1). Basal values (pre-administration) of MSC-chondro and MSC-naïve are also provided, and have been normalized over basal MSC-naïve too. Comparison between recipient groups and along the time-points within each MSC type: significant differences between autologous, MHC-matched and MHC-mismatched recipients, and between time-points within the same recipient group, are represented separately for each MSC type (MSC-chondro, MSC-primed, MSC-naïve) by a squared line with an asterisk (*, $p < 0.05$; **, $p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Similarly, the expression of *MHC-I* in MSC-primed tended to be higher when the cells were administered into MHC-mismatched animals, particularly after the second administration, but the expression levels did not exceed the reference value, neither significant differences were detected (Fig. 7K). On the other hand, MSC-naïve presented a higher expression of *MHC-I* after their first administration into MHC-matched animals, which subsequently decreased (T1 over T3, $p < 0.05$). After the second administration, *MHC-I* continued decreasing in MSC-naïve administered to the MHC-matched group (T4 over T6, $p < 0.05$), while showed an increase in the MHC-mismatched group (T4, $p < 0.05$), but it also decreased subsequently (T4 over T6, $p < 0.05$) (Fig. 7I).

In terms of *MHC-II* expression, and contrary to that observed for the other immunogenic genes, there was an upregulation compared to basal values (pre-administration scaffolds) in almost all cases (Fig. 7J, K, L). After the first administration, MSC-chondro increased *MHC-II* expression (T2 over T1 in the MHC-matched group, $p < 0.05$; T3 over T1 in the MHC-mismatched group, $p < 0.05$). The *MHC-II* expression returned to close-to-basal values after the second administration, but it increased again at the latest time-point (T6 over T4, MHC-matched group, $p < 0.05$) (Fig. 7J). In MSC-primed, the initial increase in *MHC-II* expression was progressively reduced in the MHC-matched group. MSC-primed administered into MHC-mismatched recipients also decreased their *MHC-II* expression (T4 over T1, $p < 0.05$), but a significant increase was observed again after their second administration (T5 and T6 over T4, $p < 0.05$ in both cases) (Fig. 7K). Expression of *MHC-II* in MSC-naïve administered to the MHC-matched group progressively increased after each administration (T3 over T1, $p < 0.05$; T3 over T2, $p < 0.05$; T6 over T3, $p < 0.05$, T6 over T4, $p < 0.01$; T6 over T5, $p < 0.05$) (Fig. 7L). Similarly, *MHC-II* expression also tended to increase along the time in MSC-naïve administered to the MHC-mismatched group, particularly after the second administration (T6 over T4, $p < 0.05$). Expression of *MHC-II* tended to be higher when MSC-naïve were administered into MHC-mismatched animals compared to MHC-matched animals (T2, $p < 0.05$; T5, $p < 0.05$) (Fig. 7L).

4. Discussion

The immunomodulatory and immunogenic properties of equine MSCs play a crucial role in their therapeutic effectiveness, including their ability to avoid detection by the immune system after allogeneic administration (Voga et al., 2020). The immune properties of MSCs are heavily influenced by their surrounding environment, and their response to changes in such microenvironment 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). Although many studies have explored the effects of equine MSCs on different immune cell populations *in vitro* (Caffi et al., 2020; Cassano et al., 2018), little is known about how immune cells *in vivo* can lead to changes in equine MSCs that may have therapeutic implications.

To the best of the authors' knowledge, this is the first study conducted in the equine species that simultaneously evaluated both the local immune response elicited by equine MSCs, and how the immune profile of MSCs is modified *in vivo*. Moreover, this study takes into account several key factors for the administration of MSCs, including their pre-treatment (proinflammatory priming, chondrogeneic differentiation), the compatibility of the MHC between donor and recipient, and the use of repeated administrations of MSCs.

4.1. Main findings

We initially hypothesized that equine MSCs primed with pro-inflammatory cytokines would be more efficient in evading immune recognition while chondrogeneically differentiated MSCs would be more easily recognized, and that MHC compatibility would facilitate immune evasion in all cases. We observed that MSC-primed, in contrast, triggered an early local immune response accompanied by a decrease in their immunomodulatory profile. Nevertheless, MSC-primed did not upregulate, or even downregulate, their immunogenic profile *in vivo*. MSC-primed tended to produce higher responses in MHC-mismatched recipients, but interestingly, the effect of MHC compatibility was less marked for these cells. Also contrary to that expected, MSC-chondro were not more immunogenic, but their administration to MHC-mismatched horses induced a stronger local immune response, accompanied by a downregulation of immunomodulatory genes, compared to MHC-matched horses. Similarly, and according to our initial hypothesis, MSC-naïve administered into MHC-mismatched horses also produced a stronger local immune response after both administrations. Moreover, MHC-mismatched administration promoted a higher immunogenic profile and a lower immunomodulatory profile of MSC-naïve.

Therefore, a number of interesting findings can be extracted from our results. First, the local immune response to equine MSCs was lower when donors were MHC-matched with recipients, regardless of the type of MSC (MSC-chondro, MSC-primed or MSC-naïve). Second, the expression of immunomodulatory genes was similar across the different types of MSCs administered when the recipients were MHC-matched. Moreover, when administered into MHC-matched recipients, the three types of MSCs tended to increase their expression of immunomodulatory genes, suggesting a potential for a better therapeutic effect. Third, and in contrast, when the donor and recipient were MHC-mismatched, the type of MSC differently influenced both the immune response and the changes in the expression of immunogenic genes.

4.2. Limitations of the study and particular considerations

Prior to engaging into further discussion of our results, it is important to mention the limitations of this study and to take into account several considerations. First, a minor subcutaneous lesion needed to be created in order to place the MSC-containing scaffolds in a way that later allowed their recovery for assessment. Therefore, there was an intrinsic response of the recipients similar to that in a cutaneous wound and its subsequent healing. Consequently, several of the histological patterns initially observed were likely due to the natural tissue repair process, including haemostasis, inflammation, proliferation, and remodelling. To isolate the "MSCs effect" from the "scaffold effect" and from the "natural wound healing effect", acellular scaffolds were used in a sham-operated control animal. Moreover, while this study did not intend to evaluate the therapeutic effects of equine MSCs, their potential effect in the healing of these surgical wounds should not be overlooked.

Second, the limited sample size could have potentially impacted the observation of more statistically relevant trends and differences. Owing to the high variability of MHC-haplotypes (Sadeghi et al., 2018), it was complex to find more MHC-matched animals. In addition, working with a large species like horses, and considering their particular requirements, it is challenging to work with a high number of them. To this consideration it must be added that the social behaviour of horses prevented the recovery of 9 of the implants. In spite of the efforts in monitoring and managing the herd, while allowing horses to express their natural behaviour, some of the implants were lost along the study due to grooming and scratching interactions.

Third, the subcutaneous placement of MSC-containing scaffolds is not an accurate representation of the most common routes for MSC local administration in equine clinics (*i.e.* intra-lesion injection). Nevertheless, the use of encapsulated MSCs has been proposed as a treatment strategy for focal joint defects in equine and in other species (Maihöfer et al., 2021; Ribitsch et al., 2021). In line with previous research, we found that embedding MSCs into alginate hydrogels did not impact their viability (de Souza et al., 2021; de Vries-van Melle et al., 2014; Santos et al., 2018). Additionally, encapsulating MSCs within an alginate scaffold is critical to maintain cell retention at the site of injury (Ryan et al., 2014) and we verified that, in our conditions, MSCs were retained into the scaffolds along the whole study. Therefore, the administration system used in this study was chosen for several reasons: first, to make

MSCs easily reached by the immune system; second, to retain the MSCs in a definite anatomic location, which third, and importantly, allowed their recovery for analysis. Nonetheless, it should be acknowledged that the potential effect of hydrogel as a physical barrier for the interaction of MSCs and immune cells (Bellas et al., 2015; Ryan et al., 2014).

Fourth, and also related to the route for MSC administration, the placement of scaffolds within subcutaneous pockets led in most cases to the formation of a surrounding capsule, which has also been reported in other studies (Doloff et al., 2017). This capsule might 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). In the conditions of this study, the formation of a surrounding capsule at the first time-point appeared to be associated with MHC compatibility, as they formed only in some animals from the MHC-mismatched group. However, implants were also encapsulated in the sham-operated animal. A potential explanation might be related to slight differences in the surgical technique. Even though the same surgeon (ninth author, AR) placed all the scaffolds, horses were intervened on two different batches: 1) autologous and MHC-matched recipients, 2) MHC-mismatched recipients and sham-operated animal. Thus, the influence of small technical variations between batches cannot be completely disregarded. Nevertheless, the presence or not of a surrounding capsule did not seem to influence the variables assessed in this study.

4.3. Interplay between equine mesenchymal stem/stromal cells and the immune response

We initially hypothesized that the type of equine MSC (MSC-chondro, MSC-primed and MSC-naïve) and the type of administration (MHC-matched and mismatched) would result in different local immune responses, and thus in different changes in the immune profile of MSCs *in vivo*. Contrary to our hypothesis, the local immune response was similar for all three types of equine MSCs. Equine MSCs induced a minimal local immune response in all cases, taking the sham-operated acellular control as reference. On the other hand, according to our hypothesis, compatibility for the MHC had a more marked effect on the immune response and influenced the expression of immunomodulatory and immunogenic genes in MSCs. Our findings suggest that MHC compatibility may play a role in attenuating the local immune response induced by equine MSCs, particularly when MSC-naïve or MSC-chondro are administered. However, MSC-primed produced a similar response regardless of the MHC compatibility. Furthermore, the interplay between MSCs and the immune system was dynamic along the time. Our results indicate that MSCs were able to maintain their immunomodulatory profile in the short term; however, the recipient's immune response may have eventually induced the expression of immunogenic genes in the longer term.

After an injury, immune cells arrive at the site of the lesion. Typically neutrophils and macrophages arrive first, and lymphocytes arrive later (Short et al., 2022). Neutrophils are polymorphonuclear cells that act as the first line of defense in the innate immune system and are known to play a crucial role in the immune response (Short et al., 2022). Previous studies reported the presence of neutrophils surrounding alginate scaffolds, and suggested that macrophages and neutrophils played a significant part in the formation of a thick fibrotic layer around alginate scaffolds in immunocompetent mice (Rezaa Mohammadi et al., 2018). In our study, neutrophils were attracted in higher numbers by MHC-mismatched MSC-containing scaffolds compared to MHC-matched implants across various time points. Nevertheless, the degree of polymorphonuclear infiltration was similar or even lower to that in the sham-operated acellular control, except at one week after the second administration (T4): of note, all the MSC-containing scaffolds (including those autologous) induced higher polymorphonuclear scores at T4 compared to the acellular control, indicating the potential chemoattractive properties of MSCs at the site of injury.

Soon after the arrival of neutrophils, macrophages (histiocytes) are also attracted to the site of injury. Similarly to the response of polymorphonuclear cells, a lower histiocyte reaction was observed in MHC-matched recipients. In this case, the MHC-matched recipients of MSC-chondro and MSC-primed showed less histiocyte infiltration compared to the MHC-mismatched group and even to the acellular control. These findings suggest that when equine MSCs are MHC-mismatched, their ability to regulate this response of histiocytes may be more limited.

Eosinophils do not usually play a central role in the typical inflammatory response following an injury. Their presence is limited to specific situations where their function is required, such as in allergies or parasitic infections. Eosinophils tend to arrive later, usually after macrophages, and in some cases depending on the immunological context, either before or simultaneously to lymphocytes (Berek, 2016; Quirce et al., 2023). In this study, eosinophils were similarly attracted by MHC-matched and MHC-mismatched equine MSCs, contrary to that observed for neutrophils and macrophages that were more strongly attracted by MHC-mismatched MSCs. Of note, this similar degree of eosinophil infiltration was noticed in MSC-chondro and MSC-primed, but not in MSC-naïve. The administration of MSC-naïve resulted in a more pronounced increase in the eosinophilic score within the MHC-mismatched group, and particularly after the second administration. While eosinophils can play a beneficial role in tissue engineering by promoting wound healing, they can also contribute to the expansion of B cells and promote immunoglobulin secretion (Weller and Spencer, 2018). In a previous study from our group, the repeated administration of MSC-naïve and MSC-chondro into MHC-mismatched horses induced more proliferation of circulating B cells compared to MSC-primed, and also compared to MHC-matched MSC-naïve (Cequier et al., 2024). Moreover, the intra-articular administration of equine MSC-naïve into both MHC-matched and MHC-mismatched horses resulted in the latter in an increased humoral response (Rowland et al., 2021). This finding further emphasizes the significance of MHC compatibility when implementing therapy using non-manipulated (non-primed, non-differentiated) MSCs.

Lymphocytes usually are the latest immune cells arriving to the injury site. According to our results, MHC compatibility did not have a marked effect on attracting lymphocytes, similarly to that observed for eosinophils. On the other hand, different types of equine MSCs may have varying effects on lymphocyte recruitment over time. Interestingly, lymphocytes arrived earlier in horses that received MSC-primed after the second administration. This observation may be attributed to a combination of factors, including the post-administration down-regulation of immunomodulatory genes such as *IL6* and *COX2*, which are responsible for inhibiting T cell proliferation by equine MSCs (Carrade Holt et al., 2014), and the upregulation of *MHC-II* expression, which will be discussed below.

The evaluation of gene expression in equine MSCs exposed to the *in vivo* environment revealed that, in general terms, all the three MSC conditions in both MHC combinations tended to upregulate immunomodulatory genes after each administration (T1 and T4), but this upregulation tended to decrease over time. These observations may be explained by an inconsistent exposure to an inflammatory environment, as all the genes assessed are typically induced by inflammation (Barrachina et al., 2017), which would be higher at one week after the surgery to place the scaffolds.

We initially hypothesized that MSC-chondro would present low immune regulatory capacity. In contrast, our results indicated that MSC-chondro can display an immunomodulatory profile *in vivo*, also contrary to previous studies in horses (Van Hecke et al., 2021) and rodents (Ryan et al., 2014) that pointed out at a loose in MSC regulatory activity after differentiation. However, MSC-chondro showed a decrease in *COX2*, *IL6* and *IDO1* expression along the time, suggesting that the immunomodulatory properties of differentiated MSCs may not be sustained over time.

We also hypothesized that MSC-primed would show a higher

immune regulatory profile. On the contrary, and even though previous *in vitro* studies have shown an increase in *COX2*, *IL6* and *iNOS2* expression in equine MSCs after cytokine priming (Caffi et al., 2020; Cassano et al., 2018; Connard et al., 2021; Lee et al., 2021), our *in vivo* study found that MSC-primed downregulated the expression of most of the immunomodulatory genes over time in both MHC-matched and mismatched groups. These results suggest that the effect of pro-inflammatory cytokine treatment is short-termed and is lost over time after *in vivo* administration. Another possible explanation could be that MSC-primed require a sustained inflammatory stimuli to keep activated their immunomodulatory profile, which would be better facilitated in injury situations (Barrachina et al., 2018a) but not by the mild and transient inflammation associated with scaffold placement in our study.

Finally, we hypothesized that MHC-mismatching would increase the local immune reaction elicited by equine MSCs, and thus their immunogenic profile. Accordingly, all the three types of MSCs upregulated their immunogenic profile when administered into MHC-mismatched horses. Specifically, MSC-naïve showed a higher expression of immunogenic markers and produced a stronger local immune response after both administrations into MHC-mismatched recipients, suggesting that these conditions would result in a less immune evasive scenario. On the other hand, MHC-matched administration promoted the immunomodulatory profile of equine MSCs, particularly in MSC-naïve and MSC-chondro. However, there were no significant differences in the gene expression of MSC-primed depending on the type of receptor, suggesting that MHC compatibility might not be so critical for licensed MSCs.

In spite of the differential expression of immunogenic genes between MHC-matched/mismatched recipients and along the time, their expression levels remained similar to the baseline (pre-administration) in most cases, except for *MHC-II*. Our study revealed an upregulation of *MHC-II* in equine MSCs *in vivo*, as compared over basal (pre-administration) scaffolds. Such upregulation took place after the first administration of all the three types of MSCs and in both MHC-matched or mismatched recipients. Interestingly, the highest expression of *MHC-II* was not observed immediately after each administration but at later time-points. This observation suggests that the immunogenicity of equine MSCs can keep changing after administration, likely in response to the immune response developed in the first place. In addition, this progressive increase in *MHC-II* expression over time suggests a potential role in further triggering immune responses against equine MSCs, 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 equine MSCs donors with low *MHC-II* expression to minimize immune response (Schnabel et al., 2014). However, according to our *in vivo* results, it should also be considered that MHC expression in MSCs may undergo changes after administration. Such changes in the MHC expression in equine MSCs *in vivo* could be influenced by the local inflammatory environment and may not be predicted by the baseline characteristics of the donor cells. It should be noted, however, that in this study we assessed the gene expression but not the surface expression or the secretion of bioactive molecules, which does not always correlate (Cassano et al., 2018).

5. Conclusions

We have confirmed that the immune profile of equine MSCs is dynamic *in vivo,* changing in response to their surrounding microenvironment. Furthermore, the changes in MSCs might simultaneously modify the immune response against them. This study underscores the relevance of the MHC compatibility for the administration of MSCs in horses. While the conditions in which MSCs are administered (proinflammatory priming, chondrogeneic differentiation) appear to have little impact on the long-term local immune response, the use of MHC-matched donors would be highly recommended, particularly when planning for multiple MSC treatments.

Our findings indicate that MSCs from MHC-matched donors could increase the expression of immunomodulatory genes, while MSCs from MHC-mismatched donors tend to upregulate the gene expression of immunogenic markers. Moreover, the immunomodulatory potential of equine MSCs may diminish over time, thus immune evasion may be favoured by using MHC-matched MSCs.

The findings of this study can have important implications for the therapeutic use of equine MSCs, and highlight the importance of strategies like selecting MSC donors by their MHC. Further research is crucial to explore additional factors and mechanisms that could contribute to the immune evasiveness of MSCs, and thus, to optimize their therapeutic potential resulting in more effective and safer cell therapies for veterinary and human patients.

CRediT authorship contribution statement

Alina Cequier: Writing – original draft, Methodology, Formal analysis, Data curation. **Mª. Belén Serrano:** Writing – original draft, Methodology, Data curation. **Mª. Teresa Soler-Monsó:** Writing – review & editing, Methodology, Formal analysis. **Elvira Bernad:** Writing – review & editing, Methodology, Data curation. **Francisco José Vázquez:** Writing – review & editing, Resources, Methodology. **Arantza Vitoria:** Writing – review & editing, Resources, Methodology. **Sara Fuente:** Writing – review & editing, Resources, Methodology. **Pilar Zaragoza:** Writing – review & editing, Project administration, Funding acquisition. **Antonio Romero:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Clementina Rodellar:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Laura Barrachina:** Writing – review & editing, Supervision, Data curation, Conceptualization.

Ethical statement

The animal study was approved by Advisory Ethics Committee for Animal Research from the University of Zaragoza (Project License PI 15/16). The study was conducted in accordance with the local legislation and institutional requirements.

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Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2025.105889.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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