Manuscript
Click here to view linked References

1 **Effect of inflammatory environment on equine bone marrow derived mesenchymal**
2 **stem cells immunogenicity and immunomodulatory properties**
3
4

5 L. Barrachina[a, b], A.R. Remacha[a], A. Romero[a, b], F.J. Vázquez[a, b], J. Albareda[a, c], M.
6 Prades[a, d], B. Ranera[1], P. Zaragoza[a], I. Martín-Burriel[a], C. Rodellar[a*]
7
8 [a] *Laboratorio de Genética Bioquímica LAGENBIO, Universidad de Zaragoza. C/Miguel*
9 *Servet, 177. 50013 Zaragoza (Spain)*
10 [b] *Servicio de Cirugía y Medicina Equina, Hospital Veterinario, Universidad de*
11 *Zaragoza. C/Miguel Servet, 177. 50013 Zaragoza (Spain)*
12 [c] *Servicio de Cirugía Ortopédica y Traumatología. Hospital Clínico Universitario*
13 *Lozano Blesa, Zaragoza. Avda. San Juan Bosco, 15. 50009 Zaragoza (Spain)*
14 [d] *Departament de Medicina i Cirugia Animal, Universidad Autónoma de Barcelona.*
15 *Edifici H, UAB, 08193 Bellaterra, Barcelona (Spain)*
16
17 *Corresponding author. Telephone: +34 976 761620; Fax: +34 976 762949
18 E-mail address: rodellar@unizar.es (C. Rodellar)
19
20 E-mail address of co-authors:
21
22 Laura Barrachina: lbarrach@unizar.es
23 Ana Rosa Remacha: aremacha@unizar.es
24 Antonio Romero: aromerol@unizar.es
25 Francisco José Vázquez: pvazquez@unizar.es

26 Jorge Albareda: albaredajorge@gmail.com

27 Marta Prades: marta.prades@uab.cat

28 Beatriz Ranera: beatrizranera@gmail.com

29 Pilar Zaragoza: pilarzar@unizar.es

30 Inmaculada Martín-Burriel: minma@unizar.es

31 Clementina Rodellar: rodellar@unizar.es

32

33 **Word count: 4.868**

34

35 **Abstract**

36

37 Mesenchymal stem cells (MSCs) are being investigated for the treatment of equine joint
38 diseases because of their regenerative potential and their ability to differentiate into
39 specialized articular tissue cell types, but recently, the focus mainly has addressed to
40 their immunomodulatory capacities. Inflammation plays a central role in joint
41 pathologies, since the release of proinflammatory mediators to the synovial fluid (SF)
42 leads to the activation of enzymatic degradation of the cartilage. MSCs can modulate
43 the local immune environment through direct or paracrine interaction with immune
44 cells, suppressing their proliferation and re-addressing their functions. Proinflammatory
45 molecules can induce MSC immunoregulatory potential, but they could also increase
46 the expression of immunogenic molecules. Studying the effect of inflammatory
47 environment on MSC immunomodulation and immunogenicity profiles is a key step to
48 improve cellular therapies. The aim of this study was to analyse the response of equine
49 bone marrow MSCs (eBM-MSCs) to three inflammatory conditions. Equine BM-MSCs
50 from three animals were exposed to: a) 20% allogeneic inflammatory SF (SF); b) 50
51 ng/ml of TNFα and IFNγ (CK50) and c) 20 ng/ml of TNFα and IFNγ (CK20). After 72
52 hours of exposure, expression of immunogenic and immunomodulation-related
53 molecules, including cell-to-cell contact and paracrine signalling molecules, were
54 analysed by RT-qPCR and flow cytometry. The gene expression of *ALCAM* and *VCAM-*
55 *1* was significantly upregulated by inflammatory conditions. CK culture conditions
56 significantly upregulated the expression of *COX-2*, *iNOS*, *IDO* and *IL-6*. *MHC-I* gene
57 expression was upregulated by all the conditions, whereas *MHC-II* was upregulated
58 only after CK priming. The expression of *CD40* did not significantly change, whereas
59 the ligand, *CD40L*, was significantly downregulated in CK20 conditions. Flow

cytometry showed an increase of IL-6, MHC-I and MHC-II positive cells at CK50 conditions, supporting the gene expression results. These outcomes reinforce the change of the immunophenotype of the eBM-MSCs according to the surrounding conditions. Inflammatory synovial environment did not lead to significant changes, so the environment found by eBM-MSCs when they are intraarticular administered may not be enough to activate their immunomodulatory potential. CK priming at tested doses enhances the immunoregulatory profile of eBM-MSCs, which may promote a therapeutic benefit. Even if CK priming induced an upregulation of MHC expression, costimulatory molecule expression however was not upregulated, suggesting that immunogenicity could not be increased. This study provides a better understanding about the behaviour of eBM-MSCs inside the inflamed joint and constitutes a previous step to improve MSC-based therapies for equine joint diseases.

Keywords: Horse, Mesenchymal stem cells, synovial fluid, proinflammatory cytokines, immunomodulation, immunogenicity

Introduction

Degenerative joint diseases (DJD), such osteoarthritis (OA), are prevalent articular pathologies in both equine and human species. In addition, horses have a double role in joint pathologies because, firstly, they commonly suffer from OA and osteochondrosis (OC), important diseases for equine clinicians (Schlueter and Orth, 2004). Moreover, horses are considered the most suitable animal model for testing cell based therapies for humans joint injuries (Cellular, Tissue and Genic Therapies Advisory Committe2005). Current therapeutic strategies for DJD are focused on reducing pain, decreasing disability, and limiting deterioration of articular structures (Walker-Bone et al., 2000; Goodrich and Nixon, 2006). Inflammation plays a central role in the pathophysiology of joint diseases like OA, due to the release of proinflammatory molecules like Tumor necrosis factor α (TNFα) or Interleukin 1β (IL-1β). These cytokines promote inflammation and pain, and drive to cartilage breakdown (Goodrich and Nixon, 2006; Sellam and Berenbaum, 2010). Therefore, an optimal therapeutic approach to OA should try to limit this inflammation, the subsequent cartilage degradation and try to stimulate the regeneration of articular structures. Several studies have focused on MSC intra-articular (IA) based therapies for both horses and human, with promising results (Noth et al., 2008; Broeckx et al., 2014; Ferris et al., 2014;) demonstrating their therapeutic potential.

Traditionally, the regenerative role of MSCs was mainly attributed to their differentiation ability into target tissue cells. MSCs seem to engraft in articular tissues like synovium or menisci, but not in the cartilage. However, a delaying effect on progressive degeneration of OA articular cartilage has been observed associated with

101 MSCs administration. This effect has been attributed to the anti-inflammatory and
102 immunoregulatory properties of MSCs (Whitworth and Banks, 2014), suggesting that
103 the MSC therapeutic mechanism may be due to an immunoregulation exerted by cells
104 promoted by the environment (Kode et al., 2009; Meirelles Lda et al., 2009). This
105 phenomenon seems to be a multifactorial process that requires both direct cell-to-cell
106 contact and contact-independent paracrine signalling governed through different
107 molecules such interleukin 6 (IL-6), interleukin 10 (IL-10), indoleamine 2,3-
108 dioxygenase (IDO), inducible nitric oxide synthase (iNOS), prostaglandin E2 (PGE2) or
109 tumor necrosis factor-inducible gene 6 protein (TSG-6), among others. These molecules
110 are involved in proliferation, differentiation, migration or apoptosis in different immune
111 cells (Ma et al., 2014), varying between species (Ren et al., 2009). The expression of
112 several molecules related to paracrine and cell-contact mechanisms, like chemokines
113 and adhesion molecules, is regulated in MSCs by pro-inflammatory molecules such
114 interferon γ (IFNγ), especially in combination with TNFα, interleukin 1α (IL-1α) or IL-
115 1β (Ren et al., 2008). This fact suggests that for a full regulatory function of MSCs,
116 MSC activation or licensing through exposure to an inflammatory environment might
117 be needed (Renner et al., 2009; Cuerquis et al., 2014).
118
119 Inflammatory synovial fluid (SF) contains variable amounts of several inflammatory
120 molecules that may alter MSC function and characteristics. Some studies in equine and
121 human species have reported that changes in the composition of inflammatory
122 molecules of the SF might affect the expression and production of paracrine signalling
123 molecules by MSCs, acting as inductors of their immunomodulatory potential (Leijs et
124 al., 2012; Vézina Audette et al., 2013). However, the stimulus exerted by SF could be
125 heterogeneous because it depends on the variable amounts of the different mediators,

126 which is defined by the SF inflammatory level (Leijs et al., 2012). *In vitro*, the
127 combination of definite doses of INFγ with another proinflammatory cytokine, such
128 TNFα, has demonstrated a change in the immunoregulatory abilities of MSCs, inducing
129 the expression or the secretion of anti-inflammatory and regulatory factors and
130 suppressing T cell proliferation in co-culture experiments (Cuerquis et al., 2014;
131 Paterson et al., 2014). However, an increase of the immunogenicity has been also
132 observed due to this priming (Chan et al., 2006; Chan et al., 2008), indicating a possible
133 limitation to use allogeneic primed MSCs. The enhancement of MSC immunoregulatory
134 properties, without detriment of their immune-evasive status, could improve the MSC
135 therapeutic efficacy and allow their allogeneic use (Ankrum et al., 2014).
136
137 Despite the recent findings, it is little known about the effects and behaviour of MSCs
138 when they are administered IA in an injured joint. Pre-existing joint inflammation may
139 impact the secretome of MSCs altering their therapeutic efficacy (Roberts et al., 2011),
140 therefore, a deep understanding of the changes induced in MSC immunogenicity and
141 immunoregulatory abilities by an inflammatory synovial environment is needed to
142 study. Since immunoregulatory potential of MSCs seems to be crucial for the
143 therapeutic potential, stimulating this ability could be of major interest in order to
144 improve the therapeutic benefit for the regenerative medicine. In consequence, knowing
145 how the synovial environment affects administered MSCs, and the way in which we can
146 stimulate them to enhance their immunoregulatory potential, will be the key for
147 developing effective joint regenerative treatments.
148
149 The aim of the present study was to assess the effect of different inflammatory stimuli
150 on eBM-MSCs immunoregulatory ability and immunogenicity, studying the expression

151 of immunogenic and immunomodulation-related molecules. Firstly, the influence of
152 allogeneic inflammatory SF on eBM-MSCs was investigated, and subsequently, the
153 effect of priming eBM-MSCs with a combination of the two pro-inflammatory
154 molecules IFNγ and TNFα, was tested at two different doses. This work contributes to
155 understand the effects of inflammatory exposure on eBM-MSCs, as a previous step to
156 enhance the clinical use *in vivo* in equine joint diseases.
157

158 Materials and Methods

159

160 Experimental design

161

162 Three experiments were designed to investigate the *in vitro* response of eBM-MSCs to
163 different inflammatory stimuli:
164 Experiment 1 (SF). The culture medium was supplemented with 20% of equine
165 allogeneic inflammatory SF.
166 Experiment 2 (CK50). The culture medium was supplemented with pro-inflammatory
167 cytokines: IFNγ (50ng/ml) and TNFα (50ng/ml).
168 Experiment 3 (CK20). The culture medium was supplemented with pro-inflammatory
169 cytokines: IFNγ (20ng/ml) and TNFα (20ng/ml).
170

171 Equine BM-MSCs were cultured in all inflammatory conditions for 72 hours.
172 Subsequently, the expression of molecules related to immunoregulation and
173 immunogenicity was analysed by flow cytometry and RT-qPCR.
174

175 Animals

176

177 Four geldings (age: 6–12 years; weight: 450–500 kg,) were used in this study, named as
178 057, 059, 060 and 063. Animals 057, 059 and 060 were patients from the Veterinary
179 Hospital of the University of Zaragoza determined to be healthy based on clinical and
180 hematologic examination. Biological samples (BM or SF) were obtained with owner
181 consent and according to local animal welfare regulations. All procedures were carried
182 out under Project License 31/11 approved by the in-house Ethic Committee for Animal
183 Experiments from the University of Zaragoza. The care and use of animals were
184 performed accordingly with the Spanish Policy for Animal Protection RD53/2013,
185 which meets the European Union Directive 2010/63 on the protection of animals used
186 for experimental and other scientific purposes.

187

188 **Isolation, culture and characterization of eBM-MSCs**

189

190 Forty ml of BM from sternum were aspirated in heparinized syringes using a 4″ 11G
191 Jamshidi needle from horses 057, 059 and 060. Mononuclear cells were isolated by
192 gradient centrifugation on Lymphoprep (Atom) for 20 min at 1700 rpm. The cells were
193 plated at 2×10^6 nucleated cells/cm^2 in 6-well plates (Becton Dickinson) in growth
194 medium consisting of low glucose Dulbecco’s Modified Eagle’s Medium (DMEM)
195 supplemented with 10% Fetal Bovine Serum (FBS), 1% Glutamine and 1%
196 Streptomycin/ Penicillin (all from Sigma-Aldrich) (Ranera et al., 2011). The cells were
197 expanded until third passage and were characterized by tri-lineage differentiation and
198 phenotype determination as previously described (Ranera et al., 2011). The MSC
199 phenotype was examined by analysing the mRNA levels of six genes coding for the
200 mesenchymal cell markers *CD90, CD105, CD44* and *CD73*, and the haematopoietic

201 markers *CD34* and *CD45*, using RT-qPCR. Complementary, three mesenchymal
202 surface markers were also analysed by flow cytometry (CD90, CD105 and CD44).
203 Osteogenic, adipogenic and chondrogenic differentiation potentials were induced using
204 specific media as previously described (Ranera et al., 2011). Subsequently, cells were
205 cryopreserved in frozen medium consisting of 10% DMSO and 90% FBS (both from
206 Sigma-Aldrich) until experiments started.

207

208 **Collection of synovial fluid**

209

210 Inflammatory SF for the Experiment 1 was obtained from one tarso-crural joint of
211 animal 063, which presented aseptic synovitis. Arthrocentesis was performed to reduce
212 the joint effusion and SF aspirated was collected in heparin-treated tubes (Beckton
213 Dickinson) and used for the Experiment 1. SF inflammatory status was verified
214 measuring total protein (g/dl) with a portable optical refractometer (RHB-32 Hand-held
215 brix refractometer, Spectrum Technologies) and determining the concentration of the
216 acute phase proteins (APP) serum amyloid A (SAA) and haptoglobin (Hp). The
217 methodology for SAA and Hp determination is described in the Supplementary data 1.
218 Cytological examination of the SF was also performed. SF was centrifuged at 3000g/15
219 min and supernatant was stored at -80ºC, whereas cellular fraction was discarded. All
220 processes were carried out in sterile conditions to prevent from culture contamination.

221

222 **Inflammatory culture media preparation**

223

224 Growth medium described above was used as basal culture medium. For Experiment 1,
225 20% inflammatory allogeneic SF was added to the basal culture medium (SF medium).

226 For Experiments 2 and 3, basal medium was supplemented with recombinant equine
227 TNFα and recombinant equine IFNγ (both from R&D Systems). Fifty ng/ml of each
228 proinflammatory cytokine was added to the basal culture medium for Experiment 2
229 (CK50 medium) whereas 20 ng/ml of every cytokine were added for Experiment 3
230 (CK20 medium). Basal medium was used as culture control in the three experiments
231 (Control medium).

232

233 **Equine BM-MSC culture in inflammatory conditions**

234

235 Approximately 10^6 cells in passage 3 from animals 057, 059 and 060 were thawed at
236 37ºC and cultured for 3 days to allow re-adjust conditions prior to being used for the
237 different experiments. In all experiments, eBM-MSCs from each animal were seeded at
238 5000 cells/cm^2 with basal medium. The cells were expanded at 37ºC and 5% CO_2 with
239 twice-week culture medium change. At 90-100% confluence, SF-, CK50-, CK20- or
240 control media were added to the cells and maintained for 72 hours. All conditions in all
241 experiments were carried out in triplicate for each animal. After 72 hours, control and
242 inflammatory exposed eBM-MSCs were detached with 0.25% trypsin-EDTA (Sigma-
243 Aldrich), counted and destined for different assays.

244

245 **mRNA isolation from eBM-MSCs**

246

247 Total mRNA was isolated from approximately 10^6 of eBM-MSCs from all samples with
248 RNAspin Mini RNA Isolation Kit (GE Healthcare) according to manufacturer's
249 instructions. DNAse Turbo (Ambion) was used to remove genomic DNA according to

250 manufacturer's instructions. 1,5 µg of mRNA from each sample were retrotranscripted
251 to cDNA with the Superscript Reverse Trancriptase Kit (Life Technologies).
252
253 **Real time quantitative polymerase chain reaction (RT-qPCR)**
254
255 Primers were designed using Primer Express 2.0 software based on known equine
256 sequences. Primer details, accession numbers for equine mRNA sequences and
257 amplicon sizes are shown in Table 1.
258
259 Genes were grouped based on their functions and implications, in order to facilitate the
260 posterior analysis, as it follows:
261
262 *Adhesion molecules and chemokine receptors:* Vascular Cell Adhesion Molecule 1
263 (*VCAM-1* or *CD106*), Activated Leukocyte Cell Adhesion Molecule (*ALCAM* or
264 *CD166*), chemokine receptor 4 (*CXCR4*), *CD90,* Endoglin (*CD105*) and Hyaluronate
265 Receptor (*CD44*).
266
267 *Immunomodulation-related paracrine molecules:* cyclooxygenase 2 (*COX-2*),
268 indoleamine 2 3-dioxygenase (*IDO-1*), inducible nitric oxide synthase (*iNOS*),
269 interleukin 6 (*IL-6*), transforming growth factor beta 1 (*TGF-β1*), tumor necrosis factor-
270 inducible gene 6 protein (*TSG-6*), interleukin 10 (*IL-10*).
271
272 *Antigen presenting-related molecules*: Major Complex of Histocompatibility I (*MHC-I*),
273 Major Complex of Histocompatibility II (*MHC-II*). Co-stimulatory molecules: Cluster
274 of Differentiation 40 (*CD40*), Cluster of Differentiation 40 Ligand (*CD40L*).

275 RT-qPCR was performed and monitored using the StepOne Real Time PCR System
276 device (Applied Biosystems). All reactions were carried out in a total volume of 10 µl
277 with 2 µl of cDNA as a template and Fast SYBR Green Master Mix (Applied
278 Biosystems). cDNA was amplified following the manufacturer's protocol: 20 s at 95°C
279 of an initial activation and denaturation, followed by 40 cycles consisting of 3 s at 95°C
280 and 30 s at 60°C. Each cDNA sequence was tested in triplicate and a dissociation curve
281 protocol was run after all PCR reactions. The levels of gene expression were determined
282 by the comparative Ct method. A normalization factor (NF) was used to determine the
283 expression level of each gene in each sample. The NF was calculated as the geometric
284 mean of the quantity of 2 housekeeping genes (*GAPDH* and *B2M*). Primers used for
285 housekeeping gene amplification were previously described (Kolm et al., 2006).
286
287 **Flow cytometry of eBM-MSCs**
288
289 Flow cytometry was used at two different moments. First, for immunophenotyping cells
290 prior to be used in the experiments, and second, to study the surface expression of the
291 adhesion molecules CD90, CD105 and CD44, antigen presenting molecules MHC-I and
292 MHC-II, and the immunomodulatory related molecule IL-6, after inflammatory
293 exposure. The cells were suspended in PBS/2mM EDTA at 10^6 cells/ml. Fifty µl
294 aliquots of cells were transferred to FACS tubes and incubated for 15min at 4ºC with
295 CD90-PE (BD Pharmingen), CD105-FITC (R&D Systems), CD44-FITC (Abcam),
296 HLA-ABC-FITC (Beckman Coulter), HLA-DR-APC (Immunostep) and IL-6-PE (BD
297 Pharmigen) monoclonal antibodies. Anti-horse reactivity of antibodies was previously
298 described (Ranera et al., 2011) or tested. Subsequently, cells were washed with PBS

299 (Gibco) and diluted in 500 μL of PBS/2mM EDTA and analysed with the fluorescence-
300 activated cell sorter (FACSARIA, BD Biosciences).
301
302 **Statistical analysis**
303
304 Data obtained from RT-qPCR and flow cytometry analysis were subjected to statistical
305 analysis using the SPSS 15.0. Gene expression data is reported as mean (n=3) fold
306 change increase or decrease of stimulated eBM-MSC gene expression over unstimulated
307 control eBM-MSCs. Differences between eBM-MSCs from each inflammatory
308 condition (SF, CK50 and CK20) and the counterpart controls were analysed by a paired
309 Student's t test. Flow cytometry data were represented as mean (n=3) of percentage of
310 positive stimulated and unstimulated eBM-MSCs for each experiment. Differences in
311 the surface expression between stimulated and unstimulated eBM-MSCs were analysed
312 by paired Student's t test, separately for each experiment. 95% confidence intervals
313 were provided for the fold changes in gene expression and percentage of expression in
314 flow cytometry analysis. Significance level was set at $P<0.05$ for all analyses.
315
316 **Results**
317
318 **Isolation and characterization of eBM-MSCs**
319
320 Plastic-adherent fibroblast-like cells were observed in all samples obtained from BM
321 aspirates within the first days of culture. The cells showed capacity for attachment to
322 plastic and the ability of differentiation into osteoblast, adipocyte and chondrocyte (data
323 not shown). Isolated cells from three animals were positive for the surface markers

324 CD90 (90.80% ± 7.51), CD105 (42.53 ± 1.67) and CD44 (97.46% ± 0.35) by flow
325 cytometry. All the samples expressed transcripts for *CD90*, *CD105*, *CD44 and CD73*,
326 but not for *CD34* and *CD45*, by RT-qPCR (data not shown).
327
328 **Inflammatory synovial fluid obtainment**
329
330 Total protein measure in the SF obtained from animal 063 was 2,25g/dl. The SAA
331 concentration was 5.85 μg/ml and the Hp concentration was $2.38 \cdot 10^{-4}$ μg/ml. These data
332 confirmed the inflammatory status of the SF and along with the cytological
333 examination, a septic origin of the joint inflammation was discarded.
334
335 **Effect of inflammatory environment on immunomodulatory and immunogenic**
336 **gene expression of eBM-MSCs**
337
338 Gene expression data from RT-qPCR analysis are represented in Figure 1. The
339 expression of each gene in each inflammatory condition is expressed as relative
340 expression respect to corresponding control. The results for the grouped genes were:
341
342 *Genes coding for molecules implied in MSC immunomodulatory mechanisms*
343
344 A) Molecules related with cell-to-cell contact mechanism: adhesion molecules and
345 chemokine receptors
346
347 *ALCAM* gene expression was found significantly higher, whereas *CXCR4* gene
348 expression non-significantly decreased, under inflammatory SF conditions. For CK50

349 and CK20 conditions, *VCAM-1* expression was significantly upregulated, whereas a
350 *CXCR4* significant downregulation in CK20 was observed (Figure 1.A). *CD90*, *CD105*
351 and *CD44* gene expression did not display differences between control and
352 inflammatory exposed eBM-MSCs in any Experiment (data not shown).
353
354 B) Molecules related with paracrine signalling mechanism
355
356 In SF-Experiment (Figure 1.B), non- significant down-regulation of genes coding for
357 *COX-2* and *iNOS* was observed. In addition, no expression for *IDO* was detected. Slight
358 non-significant increase for *IL-6* was also observed. The opposite pattern was observed
359 in CK50-Experiment, where *COX-2, IDO, iNOS* and *IL-6* gene expression was
360 significantly up-regulated. For CK20 conditions, similar gene expression pattern to
361 CK50-Experiment was shown, although differences for *iNOS* and *COX-2* expression
362 were not significant. Anti-inflammatory molecules *IL-10, TGF-β1* and *TSG-6* presented
363 a similar pattern for three conditions (SF, CK50 and CK20): no-significant down-
364 regulation was described in general, but *IL-10* significantly down-regulated in CK20
365 conditions.
366
367 *Antigen presenting-related molecules*
368
369 Gene expression data from RT-qPCR analysis is represented in Figure 1.C. Non-
370 significant gene expression increase was observed for *MHC-I* in all conditions. Gene
371 expression of *MHC-II* was up-regulated in both CK50 and CK20 conditions, being
372 significant only in CK50 conditions. *CD40* expression did not significantly change in

373 any conditions, whereas *CD40L* was down-regulated, being significant for CK20
374 conditions.
375
376 **Effect of the inflammatory environment on cell immunophenotype**
377
378 Cell immunophenotype was studied after inflammatory exposure. Due to the difficulty
379 of finding antibodies with reactivity against equine species, not all the molecules
380 analysed by RT-qPCR could have been also analysed by flow cytometry (Ibrahim et al.,
381 2007). Flow cytometry from CD90, CD105 and CD44, antigen presenting molecules
382 MHC-I and MHC-II and the immunomodulatory related molecule IL-6 were performed
383 in control and inflammatory exposed cells of experiments SF and CK50 (Figure 2) but
384 not in CK20 experiment due to the low number of available cells.
385
386 In the SF-experiment, the surface expression of CD105 and CD44 molecules was
387 significantly lower in SF condition respect to its control, whereas the number of positive
388 cells for CD90 and IL-6 was maintained (Figure 2.A). Non-significant changes were
389 detected in the studied surface expression pattern between CK50 condition and its
390 control, but the increase of 26.62% in IL-6 expression under CK50 condition is
391 remarkable (Figure 2.B).
392
393 MHC-I and MHC-II surface expression were also assessed after inflammatory
394 stimulation. MHC-I expression in SF exposed cells was very similar to their control
395 (66.90% for SF-cells and 61.45% for control cells) (Figure 2.C), whereas no significant
396 increase was observed for CK50 (99.3% for CK50-cells and 71.43% for control cells).

397 A mean expression of 8.62% for MHC-II was observed at CK50 conditions, whereas
398 only 0.06% of control cells were positive for this marker (Figure 2.D).
399
400 **Discussion**
401
402 The cultured cells showed capacity for attachment to plastic and the ability of
403 differentiation into osteoblast, adipocyte and chondrocyte, as criteria established to
404 define human MSCs (Dominici et al., 2006). Equine BM-MSCs displayed a gene and
405 cell surface expression pattern similar to previous reports for this species and showed a
406 normal growth pattern, with a proliferation rate and viability similar to other studies
407 (Ranera et al., 2011). Inflammatory conditioned media were prepared based on recent
408 publications on this field. Inflammatory SF was obtained from an allogeneic donor and
409 added in concentration and time previously described (Vézina Audette et al., 2013).
410 SAA and Hp were used to determine the inflammatory status of the SF. Both APP were
411 high according to the ranges of references established by Jacobsen et al and Basile et al.
412 (Jacobsen et al., 2006; Basile et al., 2013). SAA is the major APP defined for horses and
413 experiments marked increases, whereas Hp is a moderate APP with mild modifications
414 (Jacobsen and Andersen, 2007), which is in agreement with the values of SAA and Hp
415 found in the SF used in this study. For CK-conditioned media, the synergy displayed by
416 the pro-inflammatory cytokines TNFα and IFNγ (Zimmermann and McDevitt, 2014)
417 supported the decision of using them to reproduce an inflammatory environment in the
418 already tested doses 20 ng/ml and 50 ng/ml (Ren et al., 2008; van Buul et al., 2012).
419
420 Several mechanisms have been proposed to participate in the immunoregulatory
421 function of MSCs. Both cell-to-cell contact and paracrine signalling appear to be

422 involved, including the participation of chemokine axis, adhesion molecules and soluble
423 factors (Ma et al., 2014). Adhesion molecules would play an essential role in the cell-to-
424 cell contact mechanism and in the induction of immune response in MSCs. The co-
425 culture of MSCs in the presence of T cells triggers the expression of *VCAM-1* (Ren et
426 al., 2010), according to the results obtained in our CK20 and CK50 inflammatory
427 conditions. More adhesion molecules were up-regulated in presence of inflammatory
428 environment, the mRNA transcripts of *ALCAM* increased in SF experiment. These
429 findings might suggest the relationship of cell-to-cell contact in the immunoregulatory
430 mechanism. Adhesion molecules are also related to migration of MSCs, a mechanism
431 that could be critical for recruitment of MSCs into wound sites for tissue regeneration.
432 Many factors and molecules are involved in this process and diverse authors gather the
433 complexity of MSC migration in inflammatory environments. Some studies describe the
434 enhancement of MSC migratory property under inflammatory stimulation (Ries et al.,
435 2007; Shi et al., 2007), whereas others report a decrease in this property depending on
436 the time of exposure (Waterman et al., 2010). The migration-related molecules analysed
437 in this work showed that, under our tested inflammatory conditions, MSC migration
438 might be diminished. CD44 is a hyaluronan receptor related to the migration of the cells
439 through the extracellular matrix by binding to the present hyaluronic acid (Zhu et al.,
440 2006). Nevertheless, CD44 and CD105 surface expression was found significantly
441 lower in SF conditions, whereas in CK conditions no change was observed by either
442 RT-qPCR nor by flow cytometry. Furthermore, chemokine receptor *CXCR4*, also
443 involved in chemotaxis and migration of MSCs (Honczarenko et al., 2006), was found
444 down-regulated under exposure to CK20 inflammatory environment. The loss of this
445 chemokine receptor is accompanied by a decrease in the expression of other adhesion

446 molecules, while CD90 and CD105 are not modified (Honczarenko et al., 2006),
447 similarly to the gene and surface expression results described for our CK conditions.
448

449 Paracrine mechanism also plays an important role in the MSC immunomodulation.
450 Inflammatory SF is able to modulate the transcription of paracrine signalling molecules
451 in eBM-MSCs (Vézina Audette et al., 2013) and influence the effect of human MSCs
452 on lymphocyte proliferation (Leijs et al., 2012). The slight changes observed in our
453 conditions in SF exposed MSCs may be due to the inflammatory degree of the SF used,
454 which might not have been sufficient to induce significant changes. Moreover, the
455 stimulus exerted by SF could be variable depending on the inflammatory level, which is
456 heterogeneous even in the same pathology group (Leijs et al., 2012).
457

458 The IDO, iNOS, IL-6 and COX-2, through PGE2 secretion, are consistently reported *in*
459 *vitro* as the most important mediators in the paracrine signalling MSC
460 immunoregulatory mechanism to suppress T-cell proliferation (Noel et al., 2007;
461 Nemeth et al., 2009; Bouffi et al., 2010; Ghannam et al., 2010). These soluble
462 immunosuppressive factors are demonstrated to be produced or transcribed by MSCs
463 from veterinary species (Carrade and Borjesson, 2013). IDO and iNOS are enzymes
464 stimulated under inflammatory conditions. IDO causes depletion of local tryptophan,
465 required for immune cell proliferation, and accumulation of toxic breakdown products
466 for inflammatory cells (Ryan et al., 2007). iNOS produces nitric oxide (NO), which
467 inhibits proliferation and function of macrophage and T-cells (Sato et al., 2007). PGE2
468 and IL-6 also act as powerful immune suppressants, decreasing T-cell proliferation,
469 stopping differentiation into mature cells, and inhibiting the production of
470 proinflammatory cytokines (Jiang et al., 2005; Djouad et al., 2007; Xu et al., 2007;

471 Raffaghello et al., 2008; Ghannam et al., 2010) . The anti-inflammatory effects
472 produced by the panel of molecules secreted by MSCs have been reported to act locally
473 inside the inflamed synovium, producing the phenomena described above of decreasing
474 the proliferation and function of immune cells (Bouffi et al., 2010). iNOS product, NO,
475 has vasodilatory effects, increases vascular permeability and promotes angiogenesis in
476 the inflamed joint (Sutton et al., 2009), and PGE2 inhibits the production of collagenase
477 MMP-1 and regulates the gene expression of extra-cellular matrix components of the
478 articular cartilage by chondrocytes (Sutton et al., 2009). Proinflammatory cytokines
479 such TNFα and IFNγ induce the gene expression or the secretion of these anti-
480 inflammatory and regulatory molecules by MSCs from different species and different
481 sources (Meisel et al., 2004; Aggarwal and Pittenger, 2005; English et al., 2007; Ryan et
482 al., 2007; Ren et al., 2008; Hemeda et al., 2010; Hegyi et al., 2012). According to these
483 reports, both of our CK conditions would significantly up-regulate the gene expression
484 of these molecules. Gene expression results of *IL-6* were supported by flow cytometry
485 as the percentage of positive cells expressing IL-6 was also higher under CK50. Taken
486 together, these findings indicate an enhancement of immunoregulatory role of eBM-
487 MSCs after tested cytokine priming.
488
489 IL-10, TGF-β1 and TSG-6 participation in MSC immune mechanisms is reported (Di
490 Nicola et al., 2002; Aggarwal and Pittenger, 2005; Ma et al., 2014) and some authors
491 describe an increase in the production of TGF-β1 in rabbit BM-MSCs after IFNγ
492 treatment (Liu et al., 2006) and secretion of TSG-6 by TNFα-activated human MSCs
493 (Choi et al., 2011). However, some studies have described no change in *IL-10* and *TGF-*
494 *β1* gene expression after TNFα and IFNγ stimulation (Yoo et al., 2009), and no TGF-β1
495 secretion after exposure to activated lymphocytes in equine BM-MSCs (Carrade et al.,

496 2012). In our conditions, we have observed that the gene expression of the anti-
497 inflammatory molecule *IL-10* was significantly down-regulated by CK20 treatment and
498 no significant changes for *TGF-β1* and *TSG-6* gene expression were observed in any
499 experiment. Variability in immunoregulatory mechanisms has been described associated
500 with the species or the MSCs source (Carrade and Borjesson, 2013). Since several
501 factors could participate in the mechanisms underlying the immune suppression exerted
502 by MSCs, we hypothesize that the downregulation of some molecules could be
503 compensated by the overexpression of other factors such *COX-2* (PGE2), *IDO*, *iNOS* or
504 *IL-6*, as commented above.
505
506 The differences in immune-regulation mechanisms depending on species, sources and
507 inflammatory stimuli may explain some differences between our results and previous
508 reports. Despite of that fact, our data demonstrate a change in the gene expression and
509 marker surface pattern of eBM-MSC, confirming that the increase of the MSC
510 immunoregulatory potential is induced by inflammatory environments.
511
512 Preservation of low immunogenicity in MSCs after stimulation is important to allow
513 allogeneic transplantation. Allogeneic MSC therapy would be an "off-the-shelf" product
514 due to the scarcity of MSCs, especially in cases where MSC are low and the quality
515 might be committed as it happens in aged individuals and disease, and also in situations
516 where autologous MSCs might have the same genetic defects as the patient (Chen and
517 Tuan, 2008). The three inflammatory conditions tested in the present work induced a
518 non-significant increase of *MHC-I* gene expression, but only CK50 and CK20
519 conditions induced an increase in *MHC-II* gene expression, significant for CK50
520 condition. The increase in MHC-I and MHC-II surface expression detected by flow

521 cytometry was only observed for CK50 condition. Inflammatory conditions have been
522 reported to induce the expression of MHC (Najar et al., 2012). In fact, IFNγ is
523 considered as MHC inductor in MSCs, but not TNFα, in a non-dose-dependent manner
524 (Chan et al., 2006; Chan et al., 2008; Chinnadurai et al., 2014) . IFNγ-upregulated MHC
525 expression is not accompanied by an immunogenicity increase, due to the lack of
526 expression of the co-stimulatory molecules CD40, CD80 and CD86 (Tse et al., 2003)
527 along with the immune-suppressive properties of MSCs, which attenuate the local
528 immune system response (Ankrum et al., 2014). Some reports show that IFNγ
529 stimulated-MSCs display MHC-I molecules up-regulated and induce the expression of
530 MHC-II, but are not able to modify the expression of co-stimulatory molecules (Tse et
531 al., 2003; Klyushnenkova et al., 2005). According to these authors, in CK50 conditions
532 we have observed a significant diminution in *CD40L* gene expression. CD40L acts as a
533 ligand for the co-stimulatory molecule CD40, expressed by the professional antigen
534 presenting cells. It has been described that blocking CD40L, immune-tolerance is
535 allowed (Briones et al., 2011), thus the down-regulation of the gene coding for this
536 molecule might be implicated in the attenuation of the immunogenicity of eBM-MSCs.
537 This lack of change in co-stimulatory molecule gene expression, combined with up-
538 regulation of the immunoregulatory molecules, suggest that eBM-MSC might remain
539 immune-evasive after inflammatory priming.
540
541 In summary, eBM-MSCs are able to change their gene expression profile in response to
542 inflammatory stimuli, and several of these changes are correlated with modifications in
543 the eBM-MSC surface expression profile. Inflammatory synovial environment does not
544 promote significant and homogeneous immunoregulatory changes in the expression
545 patterns, indicating that the inflammatory environment of an injured joint might not be

546 sufficient to activate eBM-MSCs to display immunosuppression potential. Controlled
547 concentrations of TNFα and IFNγ treatment would induce the changes in a significant
548 and more homogenous manner. In consequence, IA cellular therapy would improve the
549 efficiency by prior *ex vivo* priming of eBM-MSCs. The up-regulation of MHC-II
550 produced by CK priming may mean an immunogenicity increase, but it might be
551 relieved by the absence of co-stimulatory molecules to trigger an immune response and
552 the enhancement of immunomodulatory factor expression by the eBM-MSCs
553 themselves. Further studies, including *in vivo* experiments of immune response, would
554 be needed to elucidate the effect of cytokine priming on the immunogenicity of MSCs.
555

556 **Conclusion**

557

558 Several important findings on eBM-MSC behaviour and immunoregulatory responses,
559 relevant to their IA therapeutic applications, were identified in the current investigation.
560 Firstly, the eBM-MSC immunomodulatory gene expression pattern was modified when
561 exposed to inflammatory environment, and was dependent on the type of stimuli, but
562 not necessarily in a dose-dependent manner. Secondly, cytokine priming induced the
563 expression of MHC, but not of the co-stimulatory molecules, therefore, the
564 immunogenicity was not necessarily increased. The immunomodulatory profile
565 enhancement reached treating eBM-MSCs with TNFα and IFNγ could be advantageous
566 to the modulation of the immune response, but further studies to ensure that it does not
567 affect the immune-evasive status of eBM-MSCs negatively are needed.
568

569 **Acknowledgements**

570

This study was supported by the Ministerio de Economía y Competitividad, España (AGL2011-28609) and by the Gobierno de Aragón (Grupo de Investigación LAGENBIO). Laura Barrachina is funded by a doctoral grant from the Gobierno de Aragón. Ana Rosa Remacha is funded by a doctoral grant (EPIF) from the University of Zaragoza. We acknowledge the horse owners for allowing their animals to be part of this study, and the Veterinary Hospital of the University of Zaragoza for facilitating access to equine patients and allowing the use of its facilities for sample collection. We also acknowledge the Departamento de Bioquímica y Biología Molecular y Celular from the University of Zaragoza for their support in the analysis of APPs in the SF used in this study.

Authors' contribution

Laura Barrachina: acquisition of data, analysis and interpretation of the data, drafting the article.

Ana Rosa Remacha: acquisition of data, analysis and interpretation of the data.

Antonio Romero: conception and design of the study, critical revision of the article for important intellectual content, provision of study materials or patients.

Francisco José Vázquez: conception and design of the study, critical revision of the article for important intellectual content, provision of study materials or patients.

Jorge Albareda: critical revision of the article for important intellectual content.

Marta Prades: critical revision of the article for important intellectual content.

593 Beatriz Ranera: acquisition of data, analysis and interpretation of the data.

594 Pilar Zaragoza: critical revision of the article for important intellectual content,
595 obtaining of funding.

596 Inmaculada Martín-Burriel: critical revision of the article for important intellectual
597 content, obtaining of funding.

598 Clementina Rodellar: conception and design of the study, critical revision of the article
599 for important intellectual content, obtaining of funding, final approval of the version to
600 be submitted.

601

602 **Conflict of interest**

603

604 None of the authors has any financial or personal relationships that could
605 inappropriately influence or bias the content of paper.

606

607 **Role of the funding source**

608

609 The study sponsors had no involvements in the study design, collection, analysis and
610 interpretation of data; in the writing of the manuscript; and in the decision to submit the
611 manuscript for publication.

612

613 **References**

614

615 Aggarwal, S., Pittenger, M.F., 2005. Human mesenchymal stem cells modulate
616 allogeneic immune cell responses. Blood 105, 1815-1822.

617 Ankrum, J.A., Ong, J.F., Karp, J.M., 2014. Mesenchymal stem cells: immune evasive,
618 not immune privileged. Nature biotechnology 32, 252-260.

619 Basile, R.C., Ferraz, G.C., Carvalho, M.P., Albernaz, R.M., Araújo, R.A., Fagliari, J.J.,
620 Queiroz-Neto, A., 2013. Physiological Concentrations of Acute-Phase Proteins and
621 Immunoglobulins in Equine Synovial Fluid. Journal of Equine Veterinary Science 33,
622 201-204.

623 Bouffi, C., Bony, C., Courties, G., Jorgensen, C., Noel, D., 2010. IL-6-dependent PGE2
624 secretion by mesenchymal stem cells inhibits local inflammation in experimental
625 arthritis. PLoS One 5, e14247.

626 Briones, J., Novelli, S., Sierra, J., 2011. T-cell costimulatory molecules in acute-graft-
627 versus host disease: therapeutic implications. Bone marrow research 2011, 976793.

628 Broeckx, S., Zimmerman, M., Crocetti, S., Suls, M., Marien, T., Ferguson, S.J., Chiers,
629 K., Duchateau, L., Franco-Obregon, A., Wuertz, K., Spaas, J.H., 2014. Regenerative
630 therapies for equine degenerative joint disease: a preliminary study. PLoS One 9,
631 e85917.

632 Carrade, D.D., Borjesson, D.L., 2013. Immunomodulation by mesenchymal stem cells
633 in veterinary species. Comparative medicine 63, 207-217.

634 Carrade, D.D., Lame, M.W., Kent, M.S., Clark, K.C., Walker, N.J., Borjesson, D.L.,
635 2012. Comparative Analysis of the Immunomodulatory Properties of Equine Adult-
636 Derived Mesenchymal Stem Cells. Cell medicine 4, 1-11.

637 Cellular, T.a.G.T.A.C., 2005. Cellular products for joint surface repair. FDA Center for
638 Biologics Evaluation and Research March 3–4.

639 Cuerquis, J., Romieu-Mourez, R., Francois, M., Routy, J.P., Young, Y.K., Zhao, J.,
640 Eliopoulos, N., 2014. Human mesenchymal stromal cells transiently increase cytokine
641 production by activated T cells before suppressing T-cell proliferation: effect of
642 interferon-gamma and tumor necrosis factor-alpha stimulation. Cytotherapy 16, 191-
643 202.

644 Chan, J.L., Tang, K.C., Patel, A.P., Bonilla, L.M., Pierobon, N., Ponzio, N.M.,
645 Rameshwar, P., 2006. Antigen-presenting property of mesenchymal stem cells occurs
646 during a narrow window at low levels of interferon-gamma. Blood 107, 4817-4824.

647 Chan, W.K., Lau, A.S., Li, J.C., Law, H.K., Lau, Y.L., Chan, G.C., 2008. MHC
648 expression kinetics and immunogenicity of mesenchymal stromal cells after short-term
649 IFN-gamma challenge. Experimental hematology 36, 1545-1555.

650 Chen, F.H., Tuan, R.S., 2008. Mesenchymal stem cells in arthritic diseases. Arthritis
651 research & therapy 10, 223.

652 Chinnadurai, R., Copland, I.B., Patel, S.R., Galipeau, J., 2014. IDO-independent
653 suppression of T cell effector function by IFN-gamma-licensed human mesenchymal
654 stromal cells. Journal of immunology 192, 1491-1501.

655 Choi, H., Lee, R.H., Bazhanov, N., Oh, J.Y., Prockop, D.J., 2011. Anti-inflammatory
656 protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse
657 peritonitis by decreasing TLR2/NF-kappaB signaling in resident macrophages. Blood
658 118, 330-338.

659 Di Nicola, M., Carlo-Stella, C., Magni, M., Milanesi, M., Longoni, P.D., Matteucci, P.,
660 Grisanti, S., Gianni, A.M., 2002. Human bone marrow stromal cells suppress T-
661 lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood
662 99, 3838-3843.

663 Djouad, F., Charbonnier, L.M., Bouffi, C., Louis-Plence, P., Bony, C., Apparailly, F.,
664 Cantos, C., Jorgensen, C., Noel, D., 2007. Mesenchymal stem cells inhibit the
665 differentiation of dendritic cells through an interleukin-6-dependent mechanism. Stem
666 Cells 25, 2025-2032.

667 Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D.,
668 Deans, R., Keating, A., Prockop, D., Horwitz, E., 2006. Minimal criteria for defining
669 multipotent mesenchymal stromal cells. The International Society for Cellular Therapy
670 position statement. Cytotherapy 8, 315-317.

671 English, K., Barry, F.P., Field-Corbett, C.P., Mahon, B.P., 2007. IFN-gamma and TNF-
672 alpha differentially regulate immunomodulation by murine mesenchymal stem cells.
673 Immunology letters 110, 91-100.

674 Ferris, D.J., Frisbie, D.D., Kisiday, J.D., McIlwraith, C.W., Hague, B.A., Major, M.D.,
675 Schneider, R.K., Zubrod, C.J., Kawcak, C.E., Goodrich, L.R., 2014. Clinical outcome
676 after intra-articular administration of bone marrow derived mesenchymal stem cells in
677 33 horses with stifle injury. Veterinary surgery : VS 43, 255-265.

678 Ghannam, S., Bouffi, C., Djouad, F., Jorgensen, C., Noel, D., 2010. Immunosuppression
679 by mesenchymal stem cells: mechanisms and clinical applications. Stem cell research &
680 therapy 1, 2.

681 Goodrich, L.R., Nixon, A.J., 2006. Medical treatment of osteoarthritis in the horse - a
682 review. Vet J 171, 51-69.

683 Hegyi, B., Kudlik, G., Monostori, E., Uher, F., 2012. Activated T-cells and pro-
684 inflammatory cytokines differentially regulate prostaglandin E2 secretion by
685 mesenchymal stem cells. Biochemical and biophysical research communications 419,
686 215-220.

687 Hemeda, H., Jakob, M., Ludwig, A.K., Giebel, B., Lang, S., Brandau, S., 2010.
688 Interferon-gamma and tumor necrosis factor-alpha differentially affect cytokine
689 expression and migration properties of mesenchymal stem cells. Stem cells and
690 development 19, 693-706.

691 Honczarenko, M., Le, Y., Swierkowski, M., Ghiran, I., Glodek, A.M., Silberstein, L.E.,
692 2006. Human bone marrow stromal cells express a distinct set of biologically functional
693 chemokine receptors. Stem Cells 24, 1030-1041.

694 Ibrahim, S., Saunders, K., Kydd, J.H., Lunn, D.P., Steinbach, F., 2007. Screening of
695 anti-human leukocyte monoclonal antibodies for reactivity with equine leukocytes. Vet
696 Immunol Immunopathol 119, 63-80.

697 Jacobsen, S., Andersen, P.H., 2007. The acute phase protein serum amyloid A (SAA) as
698 a marker of inflammation in horses. Equine Veterinary Education 19, 38-46.

699 Jacobsen, S., Thomsen, M.H., Nanni, S., 2006. Concentrations of serum amyloid A in
700 serum and synovial fluid from healthy horses and horses with joint disease. American
701 journal of veterinary research 67, 1738-1742.

702 Jiang, X.X., Zhang, Y., Liu, B., Zhang, S.X., Wu, Y., Yu, X.D., Mao, N., 2005. Human
703 mesenchymal stem cells inhibit differentiation and function of monocyte-derived
704 dendritic cells. Blood 105, 4120-4126.

705 Klyushnenkova, E., Mosca, J.D., Zernetkina, V., Majumdar, M.K., Beggs, K.J.,
706 Simonetti, D.W., Deans, R.J., McIntosh, K.R., 2005. T cell responses to allogeneic
707 human mesenchymal stem cells: immunogenicity, tolerance, and suppression. Journal of
708 biomedical science 12, 47-57.

709 Kode, J.A., Mukherjee, S., Joglekar, M.V., Hardikar, A.A., 2009. Mesenchymal stem
710 cells: immunobiology and role in immunomodulation and tissue regeneration.
711 Cytotherapy 11, 377-391.

712 Kolm, G., Klein, D., Knapp, E., Watanabe, K., Walter, I., 2006. Lactoferrin expression
713 in the horse endometrium: relevance in persisting mating-induced endometritis.
714 Veterinary immunology and immunopathology 114, 159-167.

715 Leijs, M.J., van Buul, G.M., Lubberts, E., Bos, P.K., Verhaar, J.A., Hoogduijn, M.J.,
716 van Osch, G.J., 2012. Effect of Arthritic Synovial Fluids on the Expression of
717 Immunomodulatory Factors by Mesenchymal Stem Cells: An Explorative in vitro
718 Study. Frontiers in immunology 3, 231.

719 Liu, H., Kemeny, D.M., Heng, B.C., Ouyang, H.W., Melendez, A.J., Cao, T., 2006. The
720 immunogenicity and immunomodulatory function of osteogenic cells differentiated
721 from mesenchymal stem cells. The Journal of Immunology 176, 2864-2871.

722 Ma, S., Xie, N., Li, W., Yuan, B., Shi, Y., Wang, Y., 2014. Immunobiology of
723 mesenchymal stem cells. Cell Death Differ 21, 216-225.

724 Meirelles Lda, S., Fontes, A.M., Covas, D.T., Caplan, A.I., 2009. Mechanisms involved
725 in the therapeutic properties of mesenchymal stem cells. Cytokine & Growth Factors
726 Review 20, 419-427.

727 Meisel, R., Zibert, A., Laryea, M., Gobel, U., Daubener, W., Dilloo, D., 2004. Human
728 bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-
729 dioxygenase-mediated tryptophan degradation. Blood 103, 4619-4621.

730 Najar, M., Raicevic, G., Fayyad-Kazan, H., De Bruyn, C., Bron, D., Toungouz, M.,
731 Lagneaux, L., 2012. Immune-related antigens, surface molecules and regulatory factors
732 in human-derived mesenchymal stromal cells: the expression and impact of
733 inflammatory priming. Stem cell reviews 8, 1188-1198.

734 Nemeth, K., Leelahavanichkul, A., Yuen, P.S., Mayer, B., Parmelee, A., Doi, K.,
735 Robey, P.G., Leelahavanichkul, K., Koller, B.H., Brown, J.M., Hu, X., Jelinek, I., Star,
736 R.A., Mezey, E., 2009. Bone marrow stromal cells attenuate sepsis via prostaglandin
737 E(2)-dependent reprogramming of host macrophages to increase their interleukin-10
738 production. Nature medicine 15, 42-49.

739 Noel, D., Djouad, F., Bouffi, C., Mrugala, D., Jorgensen, C., 2007. Multipotent
740 mesenchymal stromal cells and immune tolerance. Leukemia & lymphoma 48, 1283-
741 1289.

742 Noth, U., Steinert, A.F., Tuan, R.S., 2008. Technology insight: adult mesenchymal stem
743 cells for osteoarthritis therapy. Nature clinical practice. Rheumatology 4, 371-380.

744 Paterson, Y.Z., Rash, N., Garvican, E.R., Paillot, R., Guest, D.J., 2014. Equine
745 mesenchymal stromal cells and embryo-derived stem cells are immune privileged in
746 vitro. Stem cell research & therapy 5, 90.

747 Raffaghello, L., Bianchi, G., Bertolotto, M., Montecucco, F., Busca, A., Dallegri, F.,
748 Ottonello, L., Pistoia, V., 2008. Human mesenchymal stem cells inhibit neutrophil
749 apoptosis: a model for neutrophil preservation in the bone marrow niche. Stem Cells 26,
750 151-162.

751 Ranera, B., Lyahyai, J., Romero, A., Vazquez, F.J., Remacha, A.R., Bernal, M.L.,
752 Zaragoza, P., Rodellar, C., Martin-Burriel, I., 2011. Immunophenotype and gene
753 expression profiles of cell surface markers of mesenchymal stem cells derived from
754 equine bone marrow and adipose tissue. Veterinary immunology and immunopathology
755 144, 147-154.

756 Ren, G., Su, J., Zhang, L., Zhao, X., Ling, W., L'Huillie, A., Zhang, J., Lu, Y., Roberts,
757 A.I., Ji, W., Zhang, H., Rabson, A.B., Shi, Y., 2009. Species variation in the
758 mechanisms of mesenchymal stem cell-mediated immunosuppression. Stem Cells 27,
759 1954-1962.

760 Ren, G., Zhang, L., Zhao, X., Xu, G., Zhang, Y., Roberts, A.I., Zhao, R.C., Shi, Y.,
761 2008. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action
762 of chemokines and nitric oxide. Cell Stem Cell 2, 141-150.

763 Ren, G., Zhao, X., Zhang, L., Zhang, J., L'Huillier, A., Ling, W., Roberts, A.I., Le,
764 A.D., Shi, S., Shao, C., Shi, Y., 2010. Inflammatory cytokine-induced intercellular
765 adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells
766 are critical for immunosuppression. The Journal of Immunology 184, 2321-2328.

767 Renner, P., Eggenhofer, E., Rosenauer, A., Popp, F.C., Steinmann, J.F., Slowik, P.,
768 Geissler, E.K., Piso, P., Schlitt, H.J., Dahlke, M.H., 2009. Mesenchymal stem cells
769 require a sufficient, ongoing immune response to exert their immunosuppressive
770 function. Transplantation proceedings 41, 2607-2611.

771 Ries, C., Egea, V., Karow, M., Kolb, H., Jochum, M., Neth, P., 2007. MMP-2, MT1-
772 MMP, and TIMP-2 are essential for the invasive capacity of human mesenchymal stem
773 cells: differential regulation by inflammatory cytokines. Blood 109, 4055-4063.

774 Roberts, S., Genever, P., McCaskie, A., De Bari, C., 2011. Prospects of stem cell
775 therapy in osteoarthritis. Regenerative medicine 6, 351-366.

776 Ryan, J.M., Barry, F., Murphy, J.M., Mahon, B.P., 2007. Interferon-gamma does not
777 break, but promotes the immunosuppressive capacity of adult human mesenchymal
778 stem cells. Clinical and experimental immunology 149, 353-363.

779 Sato, K., Ozaki, K., Oh, I., Meguro, A., Hatanaka, K., Nagai, T., Muroi, K., Ozawa, K.,
780 2007. Nitric oxide plays a critical role in suppression of T-cell proliferation by
781 mesenchymal stem cells. Blood 109, 228-234.

782 Schlueter, A.E., Orth, M.W., 2004. Equine osteoarthritis: a brief review of the disease
783 and its causes. Equine and Comparative Exercise Physiology 1, 221-231.

784 Sellam, J., Berenbaum, F., 2010. The role of synovitis in pathophysiology and clinical
785 symptoms of osteoarthritis. Nature reviews. Rheumatology 6, 625-635.

786 Shi, M., Li, J., Liao, L., Chen, B., Li, B., Chen, L., Jia, H., Zhao, R.C., 2007. Regulation
787 of CXCR4 expression in human mesenchymal stem cells by cytokine treatment: role in
788 homing efficiency in NOD/SCID mice. Haematologica 92, 897-904.

789 Sutton, S., Clutterbuck, A., Harris, P., Gent, T., Freeman, S., Foster, N., Barrett-Jolley,
790 R., Mobasheri, A., 2009. The contribution of the synovium, synovial derived
791 inflammatory cytokines and neuropeptides to the pathogenesis of osteoarthritis. Vet J
792 179, 10-24.

793 Tse, W.T., Pendleton, J.D., Beyer, W.M., Egalka, M.C., Guinan, E.C., 2003.
794 Suppression of allogeneic T-cell proliferation by human marrow stromal cells:
795 implications in transplantation. Transplantation 75, 389-397.

796 van Buul, G.M., Villafuertes, E., Bos, P.K., Waarsing, J.H., Kops, N., Narcisi, R.,
797 Weinans, H., Verhaar, J.A., Bernsen, M.R., van Osch, G.J., 2012. Mesenchymal stem
798 cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic
799 synovium and cartilage explant culture. Osteoarthritis and cartilage / OARS,
800 Osteoarthritis Research Society 20, 1186-1196.

801 Vézina Audette, R., Lavoie-Lamoureux, A., Lavoie, J.P., Laverty, S., 2013.
802 Inflammatory stimuli differentially modulate the transcription of paracrine signaling
803 molecules of equine bone marrow multipotent mesenchymal stromal cells.
804 Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society 21, 1116-1124.

805 Walker-Bone, K., Javaid, K., Arden, N., Cooper, C., 2000. Regular review: medical
806 management of osteoarthritis. Bmj 321, 936-940.

807 Waterman, R.S., Tomchuck, S.L., Henkle, S.L., Betancourt, A.M., 2010. A new
808 mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1
809 or an Immunosuppressive MSC2 phenotype. PLoS One 5, e10088.

810 Whitworth, D.J., Banks, T.A., 2014. Stem cell therapies for treating osteoarthritis:
811 prescient or premature? Vet J 202, 416-424.

812 Xu, G., Zhang, Y., Zhang, L., Ren, G., Shi, Y., 2007. The role of IL-6 in inhibition of
813 lymphocyte apoptosis by mesenchymal stem cells. Biochemical and biophysical
814 research communications 361, 745-750.

815 Yoo, K.H., Jang, I.K., Lee, M.W., Kim, H.E., Yang, M.S., Eom, Y., Lee, J.E., Kim,
816 Y.J., Yang, S.K., Jung, H.L., Sung, K.W., Kim, C.W., Koo, H.H., 2009. Comparison of
817 immunomodulatory properties of mesenchymal stem cells derived from adult human
818 tissues. Cellular immunology 259, 150-156.

819 Zhu, H., Mitsuhashi, N., Klein, A., Barsky, L.W., Weinberg, K., Barr, M.L., Demetriou,
820 A., Wu, G.D., 2006. The role of the hyaluronan receptor CD44 in mesenchymal stem
821 cell migration in the extracellular matrix. Stem Cells 24, 928-935.

822 Zimmermann, J.A., McDevitt, T.C., 2014. Pre-conditioning mesenchymal stromal cell
823 spheroids for immunomodulatory paracrine factor secretion. Cytotherapy 16, 331-345.

824

825

826 **Tables**

827

828 **Table 1.** Genes analysed by RT-qPCR. GenBank accession numbers of the sequences
829 used for primers design. Primers (F: Forward and R: Reverse) and length of the
830 amplicon in base pair (bp). Genes were grouped in agreement with the functions and
831 implications of encoded molecules, in order to facilitate the posterior analysis: House-
832 keeping, characterization cell surface markers, adhesion molecules and chemokine
833 receptors, immunomodulation-related paracrine molecules and antigen presenting-
834 related molecules

835

GENE	Accession number	Primer sequence (5'–3')	Amplicon size
HOUSE-KEEPING			
GAPDH	NM_001163856	F:GGCAAGTTCCATGGCACAGT R:CACAACATATTCAGCACCAGCAT	128
B2M	NM_001082502.2	F: TCGTCCTGCTCGGGCTACT R: ATTCTCTGCTGGGTGACGTGA	102
CHARACTERIZATION CELL SURFACE MARKERS, ADHESION MOLECULES AND CHEMOKINES RECEPTORS			
CD90	EU881920	F:TGCGAACTCCGCCTCTCT R:GCTTATGCCCTCGCACTTG	93
CD105	XM_001500078	F:GACGGAAAATGTGGTCAGTAATGA R:GCGAGAGGCTCTCCGTGTT	100
CD73	XM_001500115	F:GGGATTGTTGGATACACTTCAAAAG R:GCTGCAACGCAGTGATTTCA	90
CD44	NM_001085435	F: CCCACGGATCTGAAACAAGTG R: TTCTGGAATTTGAGGTCTCCGTAT	95
CD45	AY_114350	F:TGATTCCCAGAAATGACCATGTA R:ACATTTTGGGCTTGTCCTGTAAC	100
CD34	XM_001491596	F:CACTAAACCCTCTACATCATTTTCTCCTA R:GGCAGATACCTTGAGTCAATTTCA	150
VCAM-1 (CD106)	DQ246452	F: TCTATGCTACGCTCTGGCTACG R: TTGATGGTCTCCCCGATGA	127
ALCAM (CD166)	XM_001494152	F: TCACGACTTCATCGAGCACATC R: GGCGAACTTAAAGTCAGTGGCA	118
CXCR4	XM_001490165	F: TGCAGCAGCAGGTAGCAAAGT R: ATATACGGAACCCGTCCATGG	97
IMMUNOMODULATION-RELATED PARACRINE MOLECULES			
COX-2	AB041771	F: GTTTGCATTTTTTGCCCAGC R: ACTTAAATCCACCCCGTGACC	103

IDO-1	XM_001490681	F: TCATGACTACGTGGACCCAAAA R: CGCCTTCATAGAGCAGACCTTC	104
iNOS	AY027883	F: CCAACAATGGCAACATCAGGT R: TGAGCATTCCAGATCCGGA	85
IL-6	EU438770	F: AACAGCAAGGAGGTACTGGCA R: CAGGTCTCCTGATTGAACCCA	95
TGFβ-1	AF175709	F: GTCCTTTGATGTCACCGGAGT R: TGGAACTGAACCCGTT	137
TSG-6	AY919871.1	F: GGAAGAGGCTCACGGATGG R: TTCCAGACCGTGCTTCTCTGT	101
IL-10	EU438771	F: GACATCAAGGAGCACGTGAACT R: TGGAGCTTACTGAAGGCACTCT	140
ANTIGEN PRESENTING-RELATED MOLECULES			
MHC-I	AB525081	F: CGTGAGCATCATTGTTGGC R: TCCCTCTTTTTTCACCTGAGG	92
MHC-II	NM_001142816	F: AGCGGCGAGTTGAACCTACAGT R: CGGATCAGACCTGTGGAGATGA	172
CD40	AY514017	F: ACAAATACTGCGACCCCAACC R: TTTCACAGGCATCGCTGGA	114
CD40L	XM_001490011	F: AGTTCGAAGGCTTCGTCAAGG R: CGCAATTTGAGGCTCCTGAT	101

836

837

Figure legends

Figure 1. Equine BM-MSCs expression of genes coding for immunomodulatory related molecules analysed by RT-qPCR. Gene expression of each gene in every experiment (SF, CK50 and CK20) is represented as Mean ± S.E.M (n=3) of the relative expression regarding correspondent control. **A)** Expression of genes coding for molecules participating in direct cell-cell contact mechanism of MSCs *VCAM-1, ALCAM*, *CXCR4*; **B)** Expression of genes coding for immunomodulatory molecules implied in the MSCs paracrine signalling mechanism *COX-2, IDO-1, iNOS, IL-6*, *IL-10, TGFβ-1, TSG-6.* **C)** Expression of genes coding for immunogenic molecules participating in the antigenic presentation *MHC-1, MHC-II, CD40* and *CD40L* (* = $p<0.05$).

Figure 2. Equine BM-MSCs surface expression of molecules related with immunomodulatory functions and immunogenic molecules related with antigenic presentation, analysed by flow cytometry. Expression of each molecule in every experiment (SF and CK50) is represented as Mean ± S.E.M (n=3) of percentage of positive cells. **A)** Control and SF-stimulated eBM-MSCs (Experiment 1) positive for CD90, CD105, CD44 and IL-6. **B)** Control and CK50-stimulated eBM-MSCs (Experiment 2) positive for CD90, CD105, CD44 and IL-6. **C)** Control and SF-stimulated eBM-MSCs (Experiment 1) positive for MHC-I and MHC-II. **D)** Control and CK50-stimulated eBM-MSCs (Experiment 2) positive for MHC-I and MHC-II (* = $p<0.05$).

Figure 1

Click here to download high resolution image

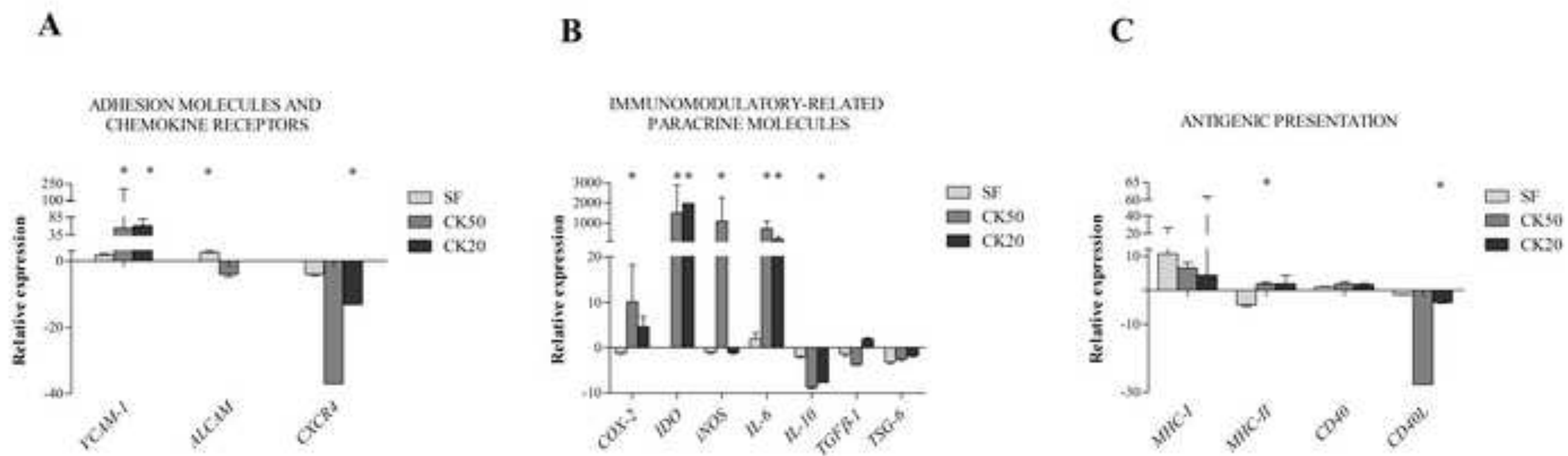
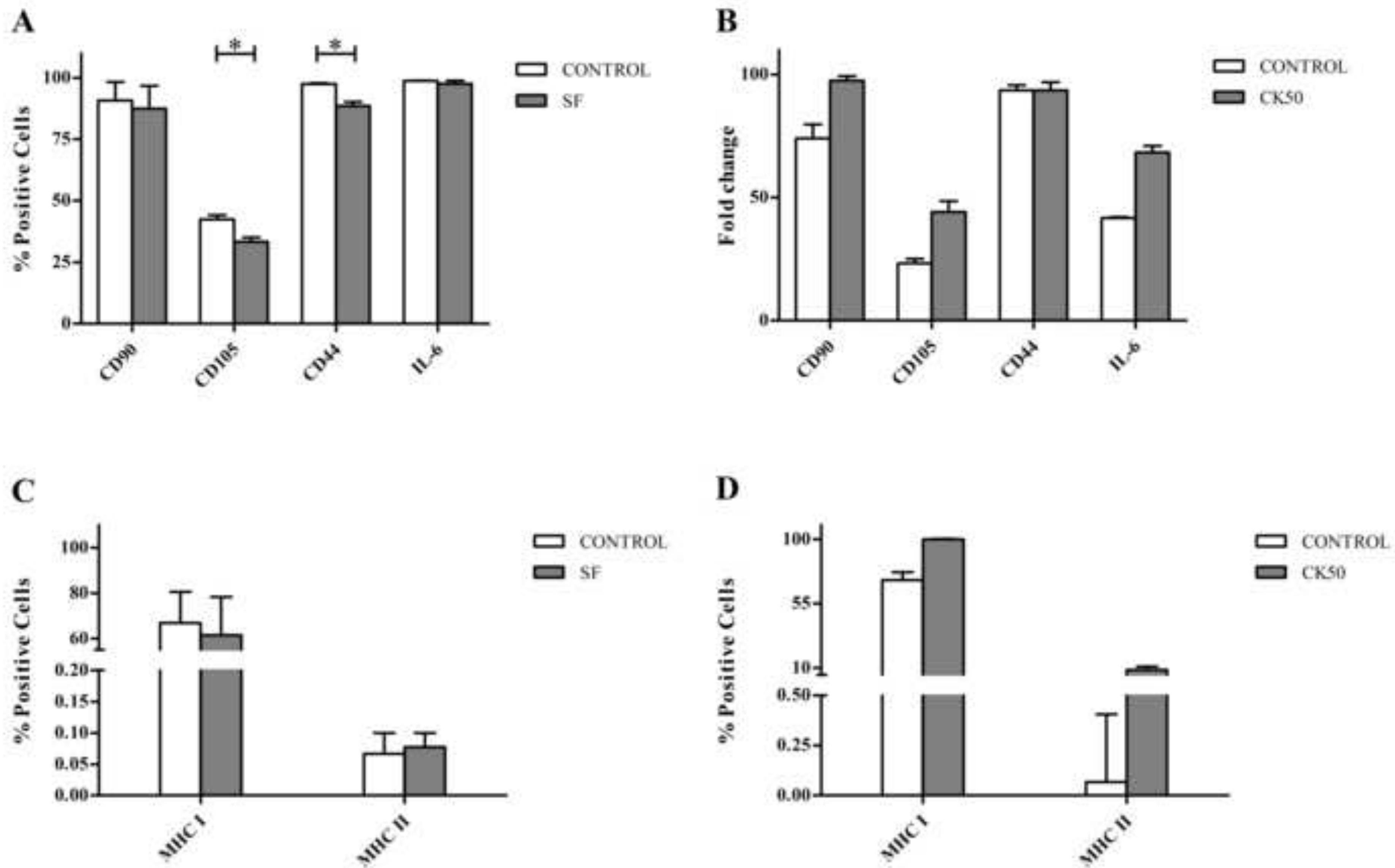


Figure 2

Click here to download high resolution image



Highlights

Joint inflammation did not activate the eBM-MSCs immunomodulatory potential.

Cytokine priming upregulates the immunoregulatory profile of eBM-MSCs

Cytokines lead to an increase in MHC, but not in costimulatory molecules expression.

Equine BM-MSCs change their immune-phenotype according to their environment.