Expression of genes involved in immune response and *in vitro* immunosuppressive effect of equine MSCs

Ana Rosa Remacha[a,1], Laura Barrachina[a,1], Samuel Álvarez-Arguedas[a], Beatriz Ranera[a], Antonio Romero[a,b], Francisco José Vázquez[a,b], Pilar Zaragoza[a], Rosa Yañez[c], Inmaculada Martín-Burriel[a], Clementina Rodellar[a,*]

[a] *Laboratorio de Genética Bioquímica (LAGENBIO), Facultad de Veterinaria, Universidad de Zaragoza, 50013 Zaragoza, Spain*

[b] *Hospital Veterinario, Facultad de Veterinaria, Universidad de Zaragoza, 50013 Zaragoza, Spain*

[c] *Division of Hematopoietic Innovative Therapies, CIEMAT/CIBERER/FJD, Madrid, Spain*

Abstract

The immunomodulatory capacities of mesenchymal stem cells (MSCs) have made them the subject of increased clinical interest for tissue regeneration and repair. We have studied the immunomodulatory capacity of equine MSCs derived from bone marrow (BM-MSCs) and adipose tissue (AT-MSCs) in cocultures with allogeneic peripheral blood mononuclear cells (PBMCs). Different isoforms and concentrations of phytohaemaglutinin (PHA) were tested to determine the best stimulation conditions for PBMC proliferation and a proliferation assay was performed for 7 days to determine the optimal day of stimulation of PBMCs. The effect of the dose and source of MSCs was evaluated in cocultures of 10^5 PBMCs with different ratios of AT- and BM-MSCs (1:1, 1:10, 1:20 and 1:50). Proliferation rates of the PBMCs were evaluated using BrdU ELISA colorimetric assay. PHA stimulated equine PBMCs reached their peak of growth after 3 days of culture. The immunoassay showed a decrease of the PBMCs growth at high ratio cocultures (1:1 and 1:10). Equine BM-MSCs and AT-MSCs demonstrated an ability to suppress the proliferation of stimulated PBMCs. Although MSCs derived from both sources displayed immunosuppressive effects, AT-MSCs were slightly more potent than BM-MSCs. In addition, the expression of 26 genes coding for different molecules implicated in the immune response was analyzed in cocultures of BM-MSCs and PHA stimulated PBMSCs by reverse transcriptase real time quantitative PCR (RT-qPCR). An upregulation in genes associated with the production of interleukins and cytokines such as *TNF-α* and *TGF-β1* was observed except for *IFN*-y whose expression significantly decreased. The variations of interleukins and cytokine receptors showed no clear patterns. *COX-1* and *COX-2* showed similar expression patterns while *INOs* expression significantly decreased in the two cell types present in the coculture. *Cyclin D2* and

IDO-1 showed an increased expression and *CD90, ITG-ˇ1* and *CD44* expression decreased significantly in BM-MSCs cocultured with PHA stimulated PBMCs. On the contrary, *CD6* and *VCAM1* expression increased in these cells. With regard to the expression of the five genes involved in antigen presentation, an upregulation was observed in both cocultured MSCs and stimulated PBMCs. This study contributes to the knowledge of the immunoregulatory properties of equine MSCs, which are notably important for the treatment of inflammation processes, such as tendinitis and osteoarthritis.

Keywords: Horse, Mesenchymal stem cells Bone marrow, Adipose tissue Immunomodulation

1. Introduction

Mesenchymal stem cells (MSCs) are a subpopulation of cells located within the stromal compartment of bone marrow (BM-MSCs) (Friedenstein et al., 1966) and are characterized by their phenotype for some cell surface markers, their adherence to plastic in culture and their ability to differentiate into three mesodermal lineages, chondro- cytes, osteoblasts and adipocytes (Pittenger et al., 1999). MSCs have been isolated and characterized from many other sources, including adipose tissue (AT-MSCs) (Gimble et al., 2007), peripheral blood (Huss et al., 2000; Koerner et al., 2006), umbilical cord blood (Koch et al., 2007; Reed and Johnson, 2008) and other solid mesenchymal tissues (Sakaguchi et al., 2005; Yoshimura et al., 2007). Articular tissue contains MSCs that also show the tri-lineage differentiation ability and sporadic myogenesis (de Bari et al., 2001).

MSCs have emerged as a promising therapeutic tool for tissue regeneration and repair. Their clinical interest rose after the discovery of their immunomodulatory properties in species such as humans and mice (Aggarwal and Pittenger, 2005; Siegel et al., 2009; Suva et al.,2008; Yanez et al., 2006). This characteristic suggests that MSCs might play specific roles as immunomodulators in the maintenance of peripheral tolerance, transplantation tolerance, autoimmunity, tumour evasion and foetal-maternal tolerance (Nauta and Fibbe, 2007; Patel et al., 2008), among other processes.

In humans and mice, MSCs exert a profound inhibitory effect on T cell proliferation *in vivo* and *in vitro* and display similar effects on B cells, dendritic cells and natural killer cells (Uccelli etal., 2007), and apoptosis seems not to be involved (Zappia et al., 2005). Immunosuppression seems to occur most effectively when MSCs make physical contact with immunological cells, which subsequently release soluble factors, includ-ing IL-10, IL-6 and IL-2, IFN--*y* and TNF-*a* (Aggarwal and Pittenger, 2005). However, some aspects of this process like the role of some soluble molecules and whether the increase of regulatory T cells is necessary for MSC immunomodulation remain unclear (Aggarwal and Pittenger, 2005).

During the past few years, the use of MSCs in the treatment of equine

osteoarticular injuries has led to the study of the characteristics of equine MSCs, including their pro-liferation, differentiation and surface marker pattern (Berg et al., 2009; Colleoni et al., 2009; de Mattos Carvalho et al., 2009; de Schauwer et al., 2011; Koerner et al., 2006; Ranera et al., 2011b). Although the immunomodulatory properties of equine MSCs might have profound therapeutic implications in the treatment of many diseases mediated by inflammation, injuries and autoimmune processes, very few studies have been focused in the analysis of these prop erties (Carrade et al., 2012).

Recently, some publications indicate that allogeneic MSCs can provoke an inmunoresponse resulting in rejec- tion (Ankrum et al., 2014). Although MSCs cannot be considered truly immune privileged, rejection of allo-MSCs occurs more slowly than rejection of other allogeneic cell types. The timing and severity of MSC rejection appears to be strongly dependent on context and dictated by a balance between MSC expression of immunogenic and immuno- suppressive factors. The aim of this work was to study the immunosuppressive effect of equine MSCs isolated from two different sources, bone marrow and adipose tissue, on the proliferation of stimulated peripheral blood mononu-clear cells (PBMCs). In addition, in order to clarify the mechanism by which MSCs could exert their inmunomodulatory effects, the genetic expression profile of 26 genes coding for molecules implicated in the immune response was analyzed by RT-qPCR.

Immunomodulation is a highly complex mechanism which is still unclear with many molecules involved, in order to contribute to unravel the key mechanisms we have analyzed the gene expression of 26 genes potentially involved in immunoresponse. This is the first report analysing such a large set of potential genes involved in the immunomodulatory role of equine MSCs.

2. Materials and methods

2.1. Animals

A total of 12 healthy horses (400–450 kg weight, age: 4–17 years, crossed bred) were used to characterize the immunoregulatory properties of equine MSCs. Horses were named from H1 to H12. Biological samples were obtained with owner consent and according to local animal welfare regulations. All procedures were carried out under Project Licence 31/11 approved by the in-house Ethic Committee for Animal Experiments from the University of Zaragoza. The care and use of animals were performed accordingly with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

2.2. MSC isolation, culture and expansion

137 *2.2.1. Bone marrow aspiration and isolation*

138 BM was harvested from three horses (H1, H2 and H3) to be used in the
139 immunosuppression assay and from six horses (H4–H9) for gene
140 expression analysis assay. Each BM aspirate, collected as previously
141 described (Ranera et al., 2011b), was diluted 1:3 with PBS (Gibco
142 Invitrogen Corporation California, USA) and then layered over Lymphoprep
143 (Atom, Barcelona, Spain) and centrifuged for 20 min at 1700 *g*. The MSC-
144 enriched cell population layer above the Lymphoprep was aspirated and
145 washed twice with PBS. The pellet was resuspended in 10 ml basal
146 medium, consisting of low glucose Dulbecco Modified Eagle's Medium
147 (DMEM) (Sigma-Aldrich, St. Louis, Missouri, USA) and supplemented with
148 10% foetal bovine serum, 1% glutamine (Sigma-Aldrich, St. Louis, Missouri,
149 USA) and 1% streptomycin/penicillin (Sigma-Aldrich, St. Louis, Missouri,
150 USA). Cells were counted, plated at a density of 2×10^6 nucleated
151 cells/cm^2 in 6-well plates and incubated at 37 °C, 5% CO_2.

152

153 *2.2.2. Adipose tissue harvest and isolation*

154 Adipose tissue (AT) was obtained from the same animals used for BM
155 aspiration (H1, H2 and H3). Each AT sam- ple was collected from the
156 supragluteal subcutaneous area as previously described (Ranera et al.,
157 2011b). The tissue was digested with 0.01% of activated collagenase
158 (type I) (Sigma-Aldrich, St. Louis, Missouri, USA) for 30 min at 37 °C with
159 continuous shaking. Subsequently, samples were centrifuged, the
160 supernatant removed and the cells washed with PBS by further
161 centrifugation. Finally, the pellet was resuspended in 10 ml basal medium
162 (DMEM), counted and plated at a density of 10^5 nucleated cells/cm^2 in 6
163 well plates and incubated at 37 °C, 5% CO_2.

164

165 *2.3. Cell culture, cryopreservation, thawing and characterization*

166 For culture expansion, cells were maintained in basal medium at a
167 density 5000 cells/cm^2 in T75 or T175 culture flask (Becton Dickinson,
168 Franklin Lakes, New Jersey, USA). The medium was changed every 3
169 days until cells reached approximately 80% confluence. Subsequently,
170 cells were detached by treating with 0.25% tryspin-EDTA (Sigma-
171 Aldrich, St. Louis, Missouri, USA) and counted with a haemocytometer
172 Z2 Coulter particle count and size analyser (Beckman Coulter, Inc.,
173 Brea, California, USA). Cells were subcultured until passage 3. Then, they
174 were frozen in 10^6 aliquots in cryovials (Nalgene Thermo Fisher Scientific,
175 Roskilde, Denmark) with freezing medium, consisting of 90% FBS and
176 10% DMSO. All the assays were performed with cryopreserved cells at
177 passage 3. Cells were thawed at 37 °C and set in culture for 3 days to
178 readjust the culture conditions prior to being used in the different
179 experiments. The tri-lineage characterization was performed as described
180 in previous works of our group (Ranera et al., 2011a,b).

181

2.4. PBMC isolation and proliferation

2.4.1. Blood collection and isolation

A total of 40 ml of blood was collected in heparinized tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA) from the jugular vein of nine different horses. Horses named H10, H11, H12 to be used in immunosuppressive assay and horses identified as H4–H9 for gene expression assay. PBMCs were isolated by density gradient centrifugation using Lymphoprep (Atom, Barcelona, Spain). After isolation, cells were resuspended in complete medium (CM), consisting of RPMI-1640 (Gibco Invitrogen Corpo- ration California, USA) and supplemented with 10% foetal bovine serum and 1% streptomycin/penicillin (Sigma- Aldrich, St. Louis, Missouri, USA) and adjusted to 1×10^{6} living PBMCs/ml.

2.4.2. PBMC proliferation

A proliferative assay of 7 days was performed to identify the best mitogenic conditions to be subsequently applied in the immunoregulation experiment. PBMCs (1×10^{5}) from horses H10, H11 and H12 were cultured in 96 well plates in the presence of 200 μl of CM with phytohemaglutinin isoform P (Sigma-Aldrich, St. Louis, Missouri, USA) (PHA) at a final concentration of 20 μg/ml. Unstimulated PBMCs were used as negative control and each trial was repeated in triplicate. Every day, one plate was used to measure the mitogen stimulation by the 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, Missouri, USA) assay as previous described (Mosmann, 1983). Optical density (OD) for each well was determined at 570 nm using a Biotek Synergy HT spectrophotometer. The average absorbance for triplicates of each animal was extrapolated into a standard curve. Briefly, the standard curve was elaborated determining the absorbance of a serial dilution of cells from (5×10^{5} PBMCs to 1.5×10^{4} PBMCs).

2.4.3. Allogenic PBMC and MSC cocultures

To assess the ability of MSCs to inhibit PBMC proliferation, BM- and AT-MSCs from H1, H2 and H3 horses were cocultured with PBMCs from horses H10, H11 and H12, obtaining a total of 18 coculture combinations, 9 for each type of MSCs (AT- and BM-MSCs). Proliferation assays were developed after 3 days of coculture. Initially, equine AT-MSCs or BM-MSCs were seeded into 96-well plates in diminishing concentrations (10^{5}, 10^{4}, 5×10^{3} and 2×10^{3}) and were incubated for 24 h at 37 °C with 5% CO_2. Then 200 μl of CM + PHA (20 μg/ml) containing 10^{5} PBMCs were added to the MSCs resulting in four MSC: PBMC ratios of coculture (1:1, 1:10, 1:20 and 1:50). All cocultures were set up in triplicate. Proliferation of the stimulated PBMCs was monitored by using Cell Proliferation ELISA BrdU (colorimetric) kit (Roche Applied Science, Barcelona, Spain). The procedure was carried out according to the manufacturer's protocol. The inhibitory effect of MSCs

on PBMCs proliferation was quantified subtracting the OD displayed by MSC alone cultures to the one obtained in MSC: PBMC cocultures. Proliferation rate was calculated referring to 100% the OD value of a pool of PHA stimulated PBMCs obtained from H10, H11 and H12 horses.

2.5. Gene expression profiles of 26 genes involved in equine inmunoresponse

In order to investigate the ability of MSCs to modulate an immunoresponse, the gene expression levels of 26 genes (see below) involved in equine immunoresponse were analyzed in cocultured BM-MSCs and PHA stimulated PBMCs. Genes analyzed were classified on five groups depending on their specific functions: (a) interleukins and cytokines (*TGF-ˇ1, IL-6, IL-10, TNF-˛* and *IFN*-y); (b) interleukins and cytokines receptors (*CXCR-3, CXCR-4, CXCL-1, IFN-* y*R1* and *TGF-ˇR1*); (c) enzymes related with the MSC immunosuppressive mechanism (*COX-1, COX-2, CyclinD2, iNOS* and *IDO*); (d) adhesion molecules (*CD90, ALCAM, CD44, ITGˇ1, CD6* and *VCAM1*); (e) proteins involved in antigenic presentation (*MHC-I, MHC-II, CD80, CD40* and *CD40L*).

Gene expression was analyzed in six different mixtures of cells obtained as follows: BM-MSCs from six horses (H4, H5, H6, H7, H8 and H9) were cocultured separately with six different PHA-stimulated PBMC pools. Each PBMC pool was constituted by the cells from the remaining five horses (all except one). The PBMC mix at a density of 10^5 cells/ml was cocultured in triplicate with BM-MSCs (ratio 1:1) into 25 cm^2 flasks. BM-MSCs, stimulated PBMCs and unstimulated PBMCs of the horses mentioned above were also cultivated separately. Both cell types are separated by their adherent capacity to the plastic before to extract the mRNA. Firstly we collect the culture supernatant which contents the PBMCs in suspension and secondly we detached the MSCs by treating with 0.25% tryspin-EDTA (Sigma-Aldrich, St. Louis, Missouri, USA). These two cell population were analyzed separately.

The expression levels of these genes were evaluated separately in cocultured BM-MSCs:PBMCs stimulated with PHA on day three of coculture (ratio 1:1) using RT-qPCR. mRNA levels of BM-MSCs and stimulated PBMCs separately cultured were used as controls. In order to evaluate the effect of each type of cell in the other, results were expressed referring the mRNA expression of each cocultured cellular type (BM-MSCs or PHA stimulated PBMCs) to its respective control and shown as relative quantity values.

Primers were designed using Primer Express 2.0 soft- ware (Applied Biosystems, Foster City, California, USA) based on known equine sequences. Primer details, accession numbers for equine mRNA sequences and amplicon sizes are shown in Table S1 (supplementary material). RNA obtained from PBMCs and MSCs was used as positive control to validate the primers. The specificity of the PCR products was validated by sequencing using an ABI PRISM 310 (Applied Biosystems, Foster City, California, USA).

274 Real-Time RT-PCR (RT-qPCR) was performed and monitored using the
275 StepOne Real Time PCR System (Applied Biosystems, Foster City,
276 California, USA). All reactions were carried out in a total volume of 10 μl with
277 2 μl of cDNA as template and Fast SYBR Green Master Mix (Applied
278 Biosystems, Foster City, California, USA). cDNA was amplified following
279 the manufacturer's conditions: 20 s at 95 °C for initial activation and
280 denaturation, 45 cycles consisting of 3 s at 95 °C and 30 s at 60 °C and a
281 final dissociation curve protocol. Each cDNA sample was tested in
282 triplicate and the gene expression levels were determined by the
283 comparative Ct method.

284 To improve the normalization accuracy, a normalization factor (NF) was
285 used to determine the expression level of each gene in each sample. The
286 NF was calculated as the geometric mean of the expression of 2
287 housekeeping genes (*GAPDH* and *B2M*). Primers used for
288 housekeeping gene amplification were previously described (Koerner et
289 al., 2006).

290

291 *2.5. Statistical analysis*

292 Statistical analysis was performed using the SPSS 19 software (SPSS Inc.,
293 Chicago, http://www.spss.com). Differences in proliferation were evaluated
294 with the Student's *t* test, where $p < 0.05$ was considered statistically
295 significant. Gene expression data were analyzed using Mann–Whitney test.
296 In all cases, $p < 0.05$ was considered statistically significant.

297

298 **3. Results**

299 *3.1. PBMC proliferation assay*

300 Freshly isolated PBMC cultures stimulated with PHA displayed a
301 decrease in proliferation after the first day of culture as a result of the
302 adaptation to the culture medium (Fig. 1). An increase in the number of
303 PHA stimulated cells, with respect to the initial quantity, was observed at
304 days 2 and 3 peaking on day 3. The non-stimulated cells displayed a
305 progressive decline during the same period (days 2–4). Differences
306 between the proliferation of the two culture conditions were significant on
307 days 3 and 4, and a border- line significance was detected on day 2 ($p =$
308 0.053). After day 4, the stimulated cells displayed a steady reduction in
309 proliferation until the end of the experiment. In contrast, the PBMCs
310 without PHA presented a slight augmentation after day 4, although the
311 initial number of cells was never recovered.

312

313 *3.2. Immunosupression assay*

314 Coculture of PBMCs (from horses H10, H11 and H12) with the MSCs
315 obtained from each particular animal (H1, H2 and H3) and source (AT-
316 MSCs and BM-MSCs) showed a decrease in the proliferation at high
317 MSCs:PBMCs ratios in comparison to controls (PBMCs + PHA; Fig. 2).
318 Specifically, the 1:1 ratio always showed significant differences with the

exception of the BM-MSCs of animal H3 (Fig. 2f).

With respect to AT-MSCs, the cocultures of PBMCs with AT-MSCs displayed significant inhibition of PBMCs proliferation at the 1:1 ratio in all cases. Proliferation at this ratio was also significantly lower than the one observed at the 1:50 ratio in the combinations with H1 and H2 animals (Fig. 2a and b) and at the 1:10 ratio with horse H3 AT-MSCs combination (Fig. 2c). Moreover, horse H1 AT-MSCs cocultures showed significant differences in PBMCs growth between the 1:10 ratio and the stimulated mononuclear cells (Fig. 2a). All ratios of horse H3 AT-MSC displayed statistically significant reductions in the proliferation of PBMCs with respect to PHA stimulated control (Fig. 2c).

Concerning BM-MSCs, the highest ratio (1:1) for the coculture of horse H1 BM-MSC and stimulated PBMCs dis- played a significant inhibition of PBMCs proliferation. The 1:1 ratio of PBMCs with the H2 BM-MSC showed a statistically significant reduction in PBMC proliferation compared to all the other studied ratios (Fig. 2e). Lastly, significant differences were only found between the 1:1 and 1:50 ratios for the H3 animal (Fig. 2f).

When the effect of AT-MSCs and BM-MSCs on PBMC proliferation was studied separately in the PBMCs obtained from animals H10, H11 and H12 (Fig. 3), in spite of the high variability observed, a tendency towards a more potent suppression capacity was observed for AT-MSCs. In addition, a high variability in the proliferation of the PBMCs was also found for the three different blood donors, especially at the low-ratio cocultures.

3.3. Gene expression analysis

In order to study the mechanism of the immunomod- ulatory effect of the equine BM-MSCs, we studied the expression levels of 26 genes involved in this process. Genes studied were grouped as described in Section 2.

Gene expression has been studied in both cocultured cell types. BM-MSCs and stimulated PBMCs cultured sep- arately were used as the respective controls of each cell type. Changes in the gene expression in PBMCs by effect of PHA stimulation were also calculated as relative quantity values referring to gene expression levels in unstimulated PBMCs (data not shown). Figs. 4 and 5 show relative quan- tity in gene expression obtained for cocultured MSCs and stimulated-PBMCs with respect to their controls.

3.3.1. Genes associated with the production of interleukins and cytokines

Fig. 4a shows a variable upregulation of all genes in both cocultured cell types except for *IFN*-y in cocultured PBMCs. Increases were statistically significant in the cases of *TNF-¸* and *TGF-ˇ1* for PHA-stimulated cocultured PBMCs and in *IFN*-y in cocultured BM-MSCs. The expression levels of the other genes and conditions were not found significant due to the great inter-individual variability.

3.3.2. Genes coding for interleukins and cytokines receptors

Most of the expression levels of interleukin and cytokine receptors increased in both types of cocultured cells. A significant overexpression was observed in *CXCR-3* and *IFN- γR1* in both types of cells and *CXCR-4* was significantly overexpressed in cocultured BM-MSCs. For *TGF-ˇR1* and *CXCL-1* variations were not statistically significant (Fig. 4b).

3.3.3. Genes coding for enzymes related with the immunosuppressive mechanism of MSCs

Fig. 5a shows that *COX-1* and *COX-2* were upregulated for both cell types, however the observed increasing was only significant in the case of PBMCs. INOS expression significantly decreased in the two cell types present in the coculture. The expression of *Cyclin D2* increased in both cell types but in a non-significant way. Finally, the expression for *IDO-1* significantly increased in cocultured BM-MSCs.

3.3.4. Expression of adhesion molecules

Fig. 5b shows that the gene expression of *CD90* decreased significantly in the co-cultured BM-MSC. Its expression level increased in co-cultured stimulated PBMCs. *ITG-ˇ1* and *CD44* showed an expression pattern similar to *CD90*, but only statistically significant differences were detected for cocultured BM-MSCs. In addition to these molecules, *ALCAM* displayed a similar pattern of variation for cocultured BM-MSCs and PBMCs, although none of the variations detected was significant. On the other hand, *CD6* was significantly upregulated in co-cultured BM-MSCs and PBMCs. Finally, although the expression of *VCAM1* increased in co-cultured BM-MSCs, its change was not statistically significant. The level of this gene was not modified in cocultured PBMCs.

3.3.5. Expression of genes coding for proteins involved in antigen presentation mechanism

An upregulation of the expression of the five genes involved in antigen presentation was detected in both types of cells studied. *MHC-I* and *CD40L* were significantly overexpressed in BM-MSCs and *CD80* expression levels significantly increased in both BM-MSCs and PBMCs (Fig. 5c).

4. Discussion

Recently, the immunoproperties of human and mouse MSCs have been widely studied for their applications in autoimmune diseases (Dazzi and Krampera, 2011; Uccelli et al., 2007) such as graft-*versus*-host disease (GVHD) (Patel et al., 2008; Sotiropoulou and Papamichail, 2007) and in inflammatory processes (Sotiropoulou and Papamichail, 2007). In horses, cell therapy with MSCs has been mainly used in the treatment of tendon and ligament injuries (Godwin et al., 2011). Moreover, the horse

409 is also considered by the FDA to be an animal model for the study of
410 human osteoarthritis. The proliferation and the tri- lineage capacities of
411 equine MSCs have been described (de Schauwer et al., 2011; Vidal et
412 al., 2006), but little is known about the immunoregulatory potential of
413 equine MSCs (Carrade et al., 2012).

414 Mitogens are molecules capable of stimulating the proliferation of PBMCs;
415 however, PBMCs from different species might respond differently to the
416 stimulation of different mitogens. Whereas, canine and murine cells are
417 usually stimulated by Concanavalin A (Kang et al., 2008; Yanez et al.,
418 2006), PHA has been used for human cells (Yanez et al., 2006; Zhou et
419 al., 2011) and also for equine PBMCs (Carrade et al., 2012). Before
420 determining the immunoregulatory ability of equine MSCs, Concanavalin A
421 and PHA were tested to assess their stimulatory potential on equine
422 PBMCs. In our previous tests Concanavalin A did not stimulate equine
423 PBMCs (data not shown). Consequently, different concentrations and
424 isoforms of the mitogen PHA, were tested to optimize the stimulation of
425 equine PBMCs. According to our results, PHA isoform P at 20 μg/ml was
426 chosen for using in the subsequent immunomodulation study.

427 In agreement with human and canine species (Lee et al., 2010; Yanez
428 et al., 2006), our proliferation assay of PBMCs with PHA showed the
429 maximum of stimulation on day 3. Consequently, we chose day 3 to
430 perform the immunosuppression assays of equine MSCs and PBMCs.
431 After day 3 proliferation of stimulated PBMCs decreased which it might
432 be due the consumption of the nutrients in the medium by the cells or to
433 the activation-induced cell death mechanism (AICD) because the decline
434 was abrupt (Maher et al., 2002). The number of non-stimulated PBMCs
435 remained unaltered during the entire experiment; therefore, these cells
436 do not seem to be capable of dividing in complete RPMI 1640 medium
437 without stimulation.

438 The *in vitro* immunomodulatory properties of MSCs have been described in
439 mice (Sotiropoulou and Papamichail, 2007; Yanez et al., 2006), humans
440 (Aggarwal and Pittenger, 2005; Patel et al., 2008; Yanez et al., 2006), dogs
441 (Lee et al., 2010) and horses (Carrade et al., 2012). Canine (Kang et al.,
442 2008), human and murine (Yanez et al., 2006) AT-MSCs display better
443 immunomodulatory effects at the highest ratios of coculture. In accordance
444 with these reports, the current study of equine AT-MSCs also displayed
445 greater immunosuppressive potential at the highest ratios, specifically,
446 1:1 and 1:10. Although with lower intensity than in AT-MSCs, the
447 same ratios of BM-MSC cocultures showed the greatest decrease in
448 the proliferation of PBMCs, which agrees with the findings in human
449 (Aggarwal and Pittenger, 2005). Differences between ratios of 1:1, 1:5
450 and 1:10 were not found in horses for any source of MSCs (Carrade et
451 al., 2012), in our study we did observe a higher inhibition of PBMC
452 proliferation using the highest ratio (1:1), although due to the existent
453 variability, the difference was only statistically significant in two out of the
454 three cases.

455 Similar to the previous research using human MSCs (Aggarwal and
456 Pittenger, 2005) that described different proliferation patterns depending
457 on the PBMCs donor, a high variability of reduction of the PBMCs

proliferation was observed at all the ratios of coculture in our study.

4.1. Genes associated with the production of interleukins and cytokines

MSCs secrete constitutively IL-6 in *in vitro* cultures (Park et al., 2009) however the upregulation of this cytokine by MSCs would display immunoregulatory activity on inflammatory process. According to these reports, an upregulation of the gene expression of *IL-6* was detected on MSCs, which might indicate that *IL-6* is one of the factors involved in the regulation of the proliferation of the equine lymphocytes.

Likewise, an immunosuppressive role has been assigned to IL-10 (Maher et al., 2002) in the immune system. On the contrary to IL-6, IL-10 is not constitutively secreted by MSCs, but Levy et al. reported that *IL-10* transfected MSCs were able to suppress more effectively the T-cell proliferation (Levy et al., 2013). Although significant differences were not detected in co-culture system, a twenty increase relative quantity was observed in BM-MSCs and also a slightly increase in the gene expression of PBMCs, which would contribute to the immunosuppression of the proliferation of the stimulated PBMCs. In the case of *TGF-ˇ1* no variation was observed in cocultured BM-MSCs which is consistent with that described in other studies for human MSCs (Ryan et al., 2005) while in PBMCs is significantly up- regulated as it has already been reported in canine species (Kang et al., 2008), indicating that TGF-*¡3*1 is also a suppression factor in equine immunomodulation.

IFN-*-y* is a pro-inflammatory cytokine normally secreted by natural killer cells and Th1 lymphocytes involved in the triggering of the immune response. Similarly to IL-10, MSCs do not secrete IFN-*-y* in regular *in vitro* systems, however IFN-*-y* modified MSCs have shown to be able to switch T cells from a pro-inflammatory to anti-inflammatory phenotype (Sivanathan et al., 2014). In our work, a strong effect of PBMCs in BM-MSCs was observed by a significant increase of *IFN*-y expression that was accompanied by the unalteration of gene expression of *IFN*-y of the PBMCs in the co-culture. Previous results showed a decrease of the production of this molecule by activated T cells by the inter- action with MSCs. Also, a transitory increase was observed in PBMCs when they are cocultured with MSCs (Cuerquis et al., 2014). Taken together these facts it seems to support that *IFN*-y expression by BM-MSCs contributes to the immunosuppression of equine PBMCs. TNF-*a* is the other main pro-inflammatory cytokine secreted by activated T- cells. The detection of this cytokine is normally reduced when PBMCs are cocultured with MSCs (Carrade et al., 2012), however in contrast with this, under our culture conditions the gene expression of *TNF-˛* was up-regulated. Nevertheless, this fact does not mean that the role of this molecule has to be necessarily as pro-inflammatory since it has been shown that *TNF-˛* mediated the triggering of the regulatory function of MSCs by NF-kB cascade (Dorronsoro et al., 2014).

4.2. Genes coding for interleukins and cytokines receptors

505 Chemokines regulate trafficking of leukocytes to nor- mal and inflamed
506 sites (Sallusto et al., 1998). It has also been reported that several
507 chemokine axes may operate in BM-MSCs biology and be involved in
508 the chemoattraction of BM-MSCs to inflammatory sites (Honczarenko et
509 al., 2006). The significant overexpression of the genes coding for the
510 chemokine receptors CXCR-3 and CXCR-4 found in both cocultured
511 PBMCs and BM-MSCs might be involved in the chemoattraction of both cell
512 populations to inflammatory sites. The attraction of BM-MSCs to
513 inflammatory places to exert its regulatory mechanism could indicate that
514 chemotaxis is important for the immunosuppression of equine MSCs.

515 The important role of IFN-*-y* in inflammation and as inductor of
516 immunoregulatory functions of MSCs (Cuerquis et al., 2014) leads to think
517 that the role of its receptor in the stimulation of BM-MSCs has to be
518 consider as relevant, which agrees with the significant up-regulation of the
519 gene coding for *IFN*-y*R1* in cocultured BM-MSCs. Otherwise, PBMCs might
520 up-regulate the expression of the *IFN*-y*R1* in order to capture the IFN-*-y*
521 possibly secreted by BM- MSCs, inducing a change in T-cells from pro-
522 inflammatory towards anti-inflammatory phenotype (Sivanathan et al.,
523 2014).

524

525 *4.3. Enzymes associated with the immunosuppressive mechanism of*
526 *MSCs*

527 COX-1 and COX-2 are involved in the production of PGE-2 which plays
528 a role in the MSC-mediated immunomodulatory effect (Yanez et al., 2010).
529 BM-MSCs up-regulated the expression of both *COX* although with- out
530 statistical significance. However, both were on PBMCs, which might indicate
531 that PBMCs were involved in the pro- duction of PGE-2 to regulate their own
532 proliferation.

533 Inducible nitric-oxide synthase (iNOS) is an isoform of NOS produced under
534 inflammatory stimuli. Nitric oxide (NO) is a bioactive compound which acts
535 as a vasodilator and plays a role in the immune system. NO affects
536 macrophage and T-cell functions and its production is suggested to play a
537 major role in T-cell proliferation inhibition (Sato et al., 2007). The role of NO
538 in MSC mediated immunomodulation is not completely clear since some
539 studies describe an increase in production by MSCs in co- culture with
540 PBMCs (Chabannes et al., 2007; Zhao et al., 2010) while others do not
541 consider it necessary (Ren et al., 2010) or unchanged in the process using
542 AT-MSCs (Crop et al., 2010) and others describe a synergistic role
543 between it and the PGE-2 (Siegel et al., 2009). In mouse models, NO
544 produced by MSCs have demonstrated to be able to suppress the
545 proliferation and cytokine production by lymphocytes (Ren et al., 2008),
546 whereas NO does not seem to be involved in MSC-mediated-
547 immunosuppression in humans (Ren et al., 2009). Variability in implication
548 of NO and other immunomodulatory molecules in veterinary species has
549 also been described, considering PGE2 as the primary mediator responsible
550 for inhibition of lymphocyte proliferation by horse AT-, BM-, CT-, and CB-
551 MSC (Carrade et al., 2012). According with our results, it seems that iNOS
552 does not have a central role in the equine BM-MSCs immunoregulatory

553 mechanism.

554 The role of IDO-1 enzyme is to regulate cell proliferation by the
555 catabolism of an essential amino acid like tryptophan, which impedes the
556 T cell proliferation. The up- regulation found in our results are consistent
557 with those described in the literature for other species and would point
558 out IDO-1 also as a key mediator of immunomodulation of equine MSCs.
559 This result and the up-regulation observed in the gene expression of
560 *IFN*-y would support authors results that directly implicate the presence
561 of IFN--*y* as mediator for *IDO-1* up-regulation for lymphocyte
562 immunomodulation (DelaRosa et al., 2009; Ren et al., 2009). It has been
563 reported that INF--*y* induces expression of IDO-1 and other
564 immunoregulatory molecules (Meisel et al., 2004; Ryan et al., 2007).
565 The significant upregulation of *INF*-y found in cocultured BM-MSCs could
566 be related with an autocrine stimulation mechanism, similar to that one
567 described for MHC-II (Chan et al., 2006).

568 This Cyclin D2 is a protein actively involved in the cell cycle progression
569 control from G1 to S. An increase of expression of this molecule would
570 result in increased cell proliferation. Our results showed an increase in
571 both cell types, although differences are not significant. These results do
572 not agree with the literature where the inhibition of Cyclin D2 is
573 described in the presence of human and mouse MSCs (Corcione et al.,
574 2006; Glennie et al., 2005).

575

576 *4.4. Adhesion molecules*

577 CD44, (Zhu et al., 2006) CD90 and ITG-*¡3*1 are sur- face markers used
578 to define BM-MSCs in standard culture conditions. However, the down-
579 regulation of the gene expression of these molecules might indicate a
580 change in the phenotype of BM-MSCs when they are in presence of
581 stimulated T-cells.

582 CD6 is a molecule expressed by mature T-cells and is involved in the T-cell
583 activation (Wee et al., 1993) and CD166 is a ligand of CD6 which binding is
584 essential in T- cell proliferation (Zimmerman et al., 2006). A significant
585 increase in *CD6* in BM-MSCs and PBMCs co-cultured were observed, this
586 might indicate that the two cell types inter- act by means of the CD6 and
587 ALCAM molecules. Further experiments would be necessary to elucidate
588 which type of immunoregulatory process derived from this interaction.

589 It has been described that VCAM1 is required for the interaction between
590 lymphocytes and that this adhesion is key in the MSC-mediated
591 immunoregulation process, as the immunosuppression is reversed when
592 this molecule is absent. Our results showed an increase of expression by
593 BM-MSCs, which would confirm that equine MSC- mediated
594 immunosuppression would need cell to cell contact through VCAM1.

595

596 *4.5. Proteins involved in the antigen presentation mechanisms*

597 Significant *MHC-I* upregulation in cocultured BM-MSCs could be
598 necessary to the also increased *IFN*-y expression (Chan et al., 2008),

599 as result of previously commented allogeneic PBMCs coculture. Although
600 not significant, the increase of *MHC-II* expression observed in the equine
601 MSCs when the cells were in contact with PBMCs agreed with human
602 AT-MSCs (Crop et al., 2010) and BM-MSCs reports when IFN-*-y* levels
603 are low (Chan et al., 2006). This might be the scenario on 3 day of
604 coculture as no up-regulation of *IFN*-y was found and the effect of the
605 MSCs on the PBMC proliferation is the maximal. Recent findings have
606 evidenced a conditional ability of human MSCs to present exogenous
607 antigens in presence of IFN-*y* (Chan et al., 2006). However, other authors
608 have described that MSCs show a lack of human leucocyte antigen MHC- II
609 and costimulatory molecules (CD80 and CD86) that are essential in antigen
610 presentation (Ryan et al., 2005; Schnabel et al., 2014). It has been
611 demonstrated that MSC varied widely in *MHC-II* expression despite being
612 homogeneous in terms of "stemness" marker expression and some recent
613 publications indicated that MSCs cannot be considered truly immune
614 privileged and rejection of allo-MSCs can occur, although more slowly than
615 rejection of other allogeneic cell types (Ankrum et al., 2014). It is critical to
616 consider that infused MSCs may not express *MHC-II*, but it likely will be
617 activated and/or expressed *in vivo* at sites of inflammation. The timing
618 and severity of MSC rejection appears to be strongly dependent on con-
619 text and dictated by a balance between MSC expression of immunogenic
620 and immunosuppressive factors. According to this, our study shows the
621 induction of immunogenic molecules *MHC-I* and *MHC-II* expression in
622 equine BM- MSCs cocultured with stimulated PBMCs, which could be
623 considered as an inflammatory environment, and this up-regulation could be
624 compensated by the increase on gene expression of immunoregulatory
625 molecules described above.

626 Similarly to the equine BM-MSCs studied, an increased expression of
627 *CD80* in human AT-MSCs (Crop et al., 2010) was described, although
628 other authors failed to demonstrate it (Ryan et al., 2005). Similarly to
629 *MHC-II* expression increase, the up-regulation *CD80* did not seem to
630 have a negative effect on the immunosuppression. Further experiments
631 would be necessary to determine the role of *CD80* in the
632 immunomoregulation as the mechanisms are still unclear.

633 An increase of *CD40* expression was observed in all cell conditions. The
634 role of *CD40* is associated with a wide variety of immune and
635 inflammatory responses and it is expected its increase in both cell
636 types in inflammatory conditions *in vitro* (Crop et al., 2010). Furthermore,
637 it has been reported an increased expression of this gene in human AT-
638 MSCs in coculture (Crop et al., 2010), the up-regulation under our
639 coculture conditions would agree with these reports.

640 Although most works describe an absence of CD40L on the surface of
641 MSCs (Najar et al., 2009; Ryan et al., 2005), *CD40L* was found to be
642 highly up-regulated in equine BM-MSCs. Further analyses would be needed
643 to deter- mine which might be the role of both *CD40* and *CD40L* in the
644 immunomodulatory mechanism exerted by the equine BM-MSCs.

645 In conclusion, we have demonstrated that equine BM- MSCs and AT-MSCs
646 show an immunomodulatory effect on the proliferation of PBMCs. We have
647 also showed significant variations in molecules implicated in equine immune

648 response, showing the highly complex system of the MSC- mediated
649 immuregulation. This work extends the basic knowledge of the
650 immunoregulatory properties of equine MSCs, which may be used in future
651 clinical applications to treat inflammation and autoimmune disease and
652 also allo-MSC transplants.

653

654 **Conflict of interest**

655 None of the authors has any financial or personal rela- tionships that
656 could inappropriately influence or bias the content of paper.

657

658 **Acknowledgements**

659 This work was performed as part of the AGL2011- 28609 (MICINN) and was
660 partially financed by the Gobierno de Aragón (Grupo Consolidado
661 LAGENBIO) and Instituto Aragonés de Ciencias de la Salud (ICS).

662

663 **Appendix A. Supplementary data**

664 Supplementary data associated with this article can be found, in the
665 online version, at http://dx.doi.org/10.1016/ j.vetimm.2015.04.004.

666

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916 **Figures:**

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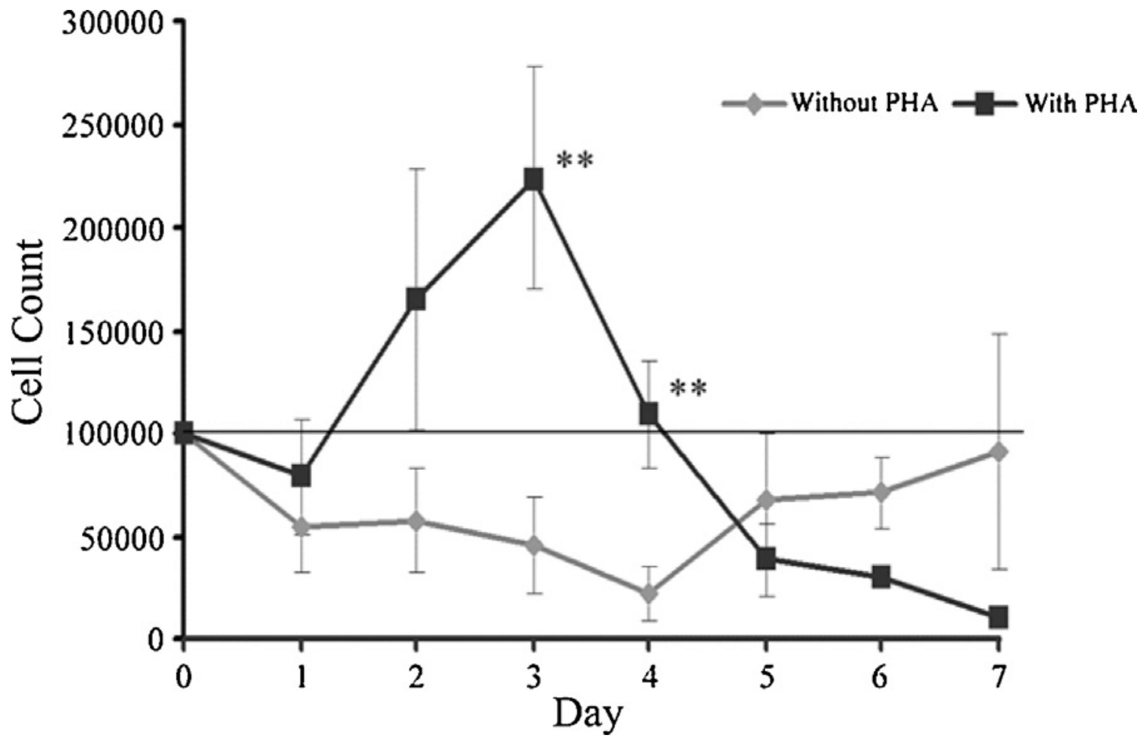
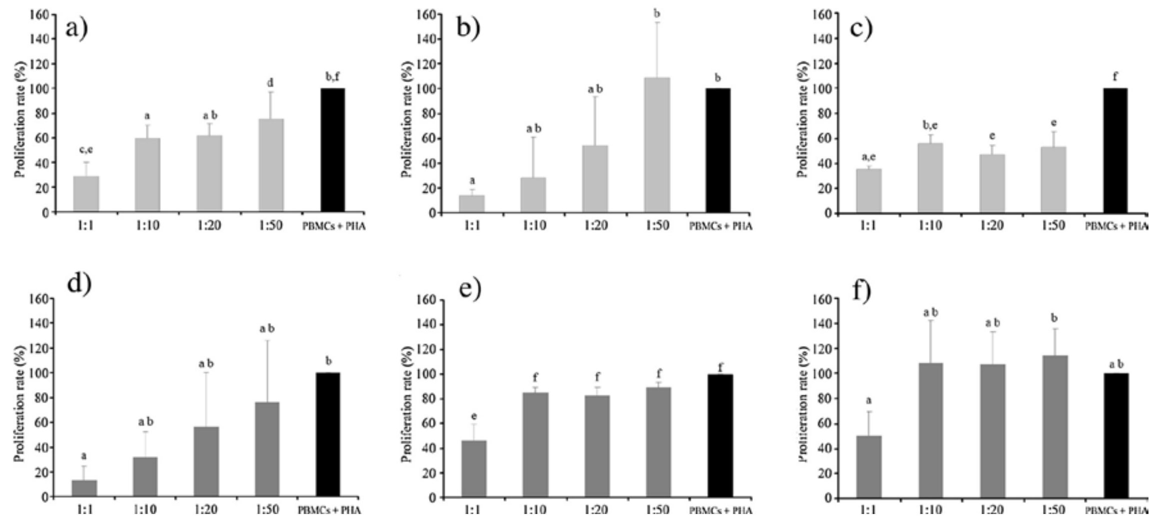


918

919 **Figure 2:** PBMCs proliferation assay (n = 3). Y axis represents the cellular
920 quantity and X axis the days in culture. Data are represented as means ±
921 standard deviations. Grey line corresponds to PBMCs cultured without PHA and
922 black lines to PBMCs with P isoform of PHA from Sigma. The horizontal line at
923 10^5 represents the initial quantity of PBMCs seeded.

924 **p < 0.01.

925

926 

927 **Figure 2:** Coculture of equine MSCs (n = 3) with allogenic PBMCs stimulated
928 with PHA (n = 3). Y axis represents the proliferation rate (%) and X axis the ratio
929 of MSCs:PBMCs tested and the control (PBMCs + PHA). Light grey bars
930 correspond to AT-MSCs, dark grey bars to BM-MSCs and black bars to control
931 (PBMCs + PHA). (a) and (d) Correspond to H1 MSCs; (b) and (e) to H2 MSCs;
932 (c) and (f) to H3 MSCs. Data are represented as means ± standard deviation.
933 Different letters differ at a, b: $p < 0.05$; c, d: $p < 0.01$; e, f: $p < 0.001$.

934

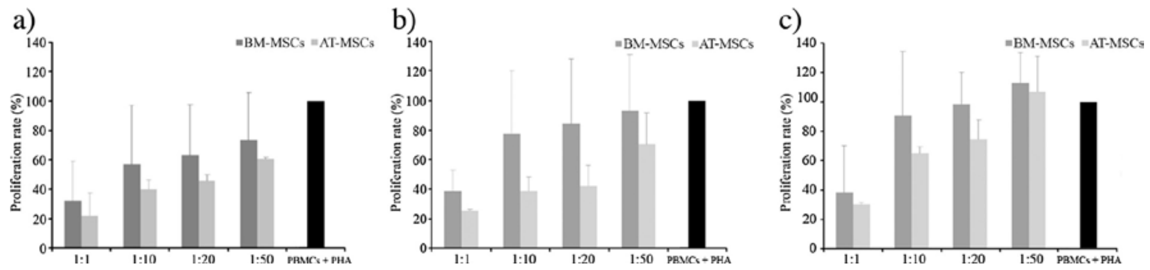


Figure 3: Coculture of BM-MSCs (n = 3) and AT-MSCs (n = 3) with allogenic PBMCs stimulated with PHA (n = 3). Y axis represents the proliferation rate (%) and X axis the ratio of MSCs:PBMCs tested and the control (PBMCs + PHA). Dark grey bars correspond to BM-MSCs, light grey bars to AT-MSCs, and black bars to control (PBMCs + PHA). (a) PBMCs from animal H10; (b) PBMCs from animal H11; (c) PBMCs from animal H12. Data are represented as means ± standard deviation. $p < 0.05$.

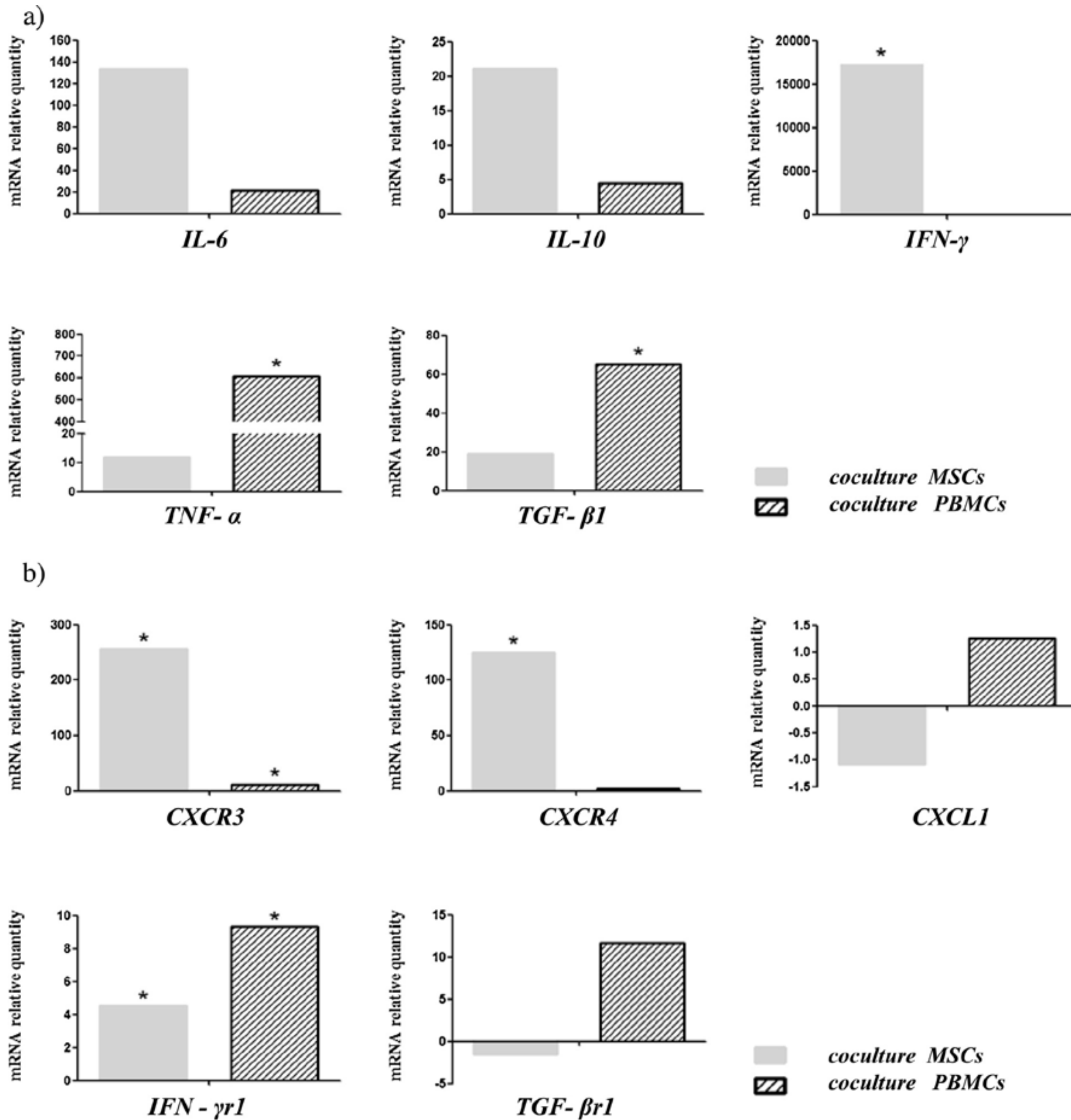
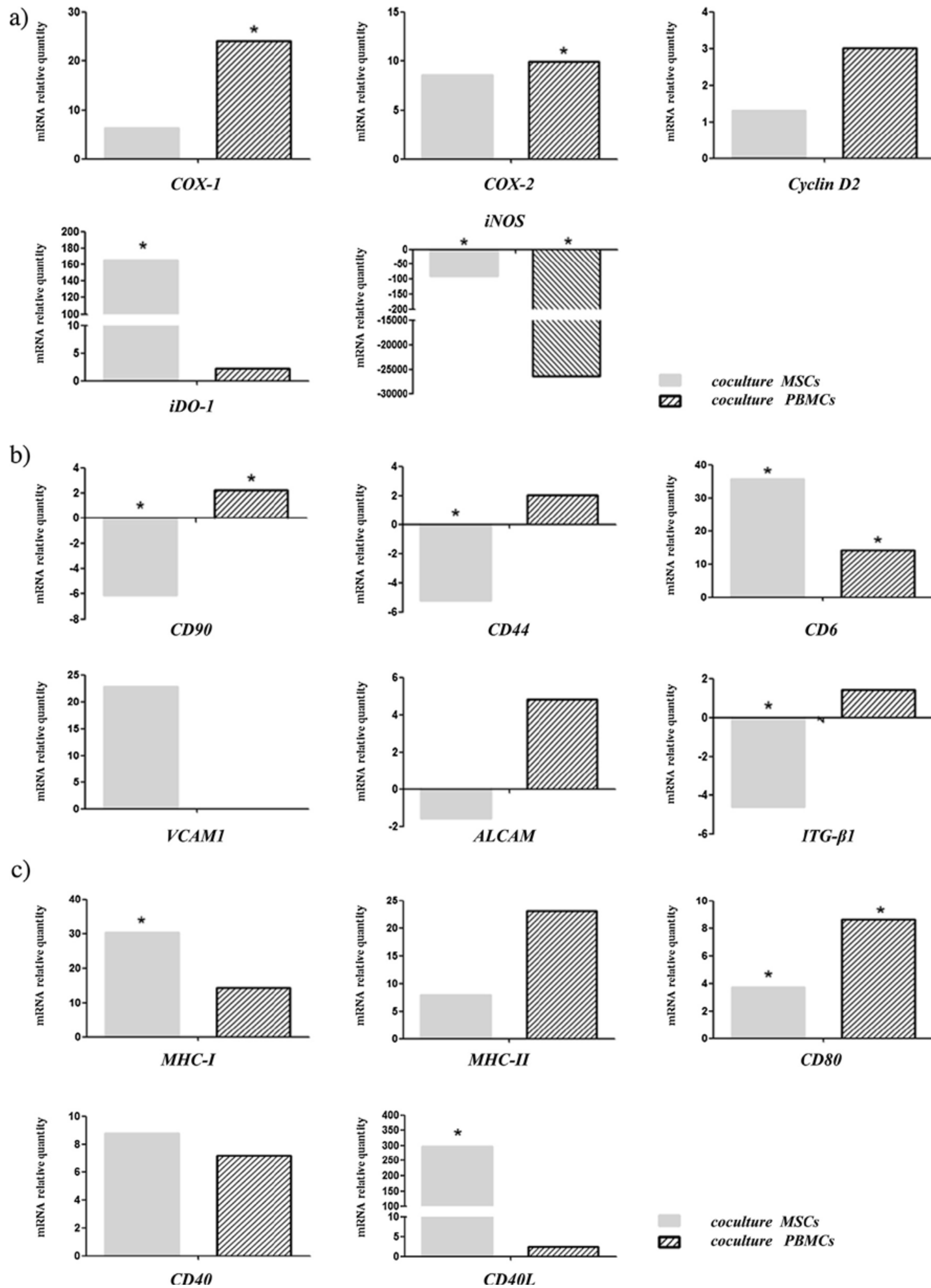


Figure 4: Coculture of BM-MSCs (n = 6) with stimulated PBMCs pools (n = 6). Y axis represents the mRNA relative quantity expression and X axis the specific gene. Light grey bars correspond to BM-MSCs and striped bars to PBMCs. (a) Genes associated with the production of interleukins and cytokines. (b) Genes coding for interleukins and cytokines receptors. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.



953

954 **Figure 5:** Coculture of BM-MSCs (n = 6) with stimulated PBMCs pools (n = 6).
955 Y axis represents the mRNA relative quantity expression and X axis represents
956 the specific gene. Light grey bars correspond to BM-MSCs and striped bars to
957 PBMCs. (a) Genes coding for enzymes related with the immunosuppressive
958 mechanism of MSCs. (b) Expression of adhesion molecules. (c) Expression of
959 genes coding for proteins involved in antigen presentation mechanism.

960 $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$