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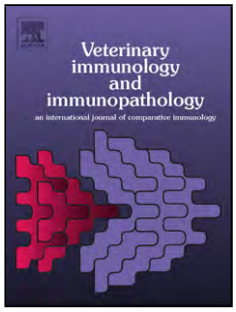

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Title

Differentiation of equine bone marrow derived mesenchymal stem cells increases the expression of immunogenic genes

Authors

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Highlights

- Immunogenicity of differentiated equine mesenchymal stem cells (MSCs) was unknown
- Adipogenesis and osteogenesis, but not chondrogenesis, upregulated MHC-I in equine MSCs
- MHC-II expression increased after equine MSCs differentiation into the three lineages
- CD40 and CD80 expression was not induced in equine MSCs after differentiation
- MHC upregulation after differentiation might increase equine MSC immunogenicity

Abstract

Mesenchymal stem cells (MSCs) are a promising treatment for equine musculoskeletal injuries because of their ability to regulate the inflammation and to differentiate into other cell types. Since interest in allogeneic therapy is rising, concerns about MSC immunogenicity need to be addressed. Differentiated MSCs from several species increase their expression of immunogenic molecules and induce alloresponses, but equine MSC immunogenic profile after differentiation has not been reported. Therefore, the aim of this study was to assess the gene expression of immunogenic markers in tri-lineage differentiated equine bone marrow derived MSCs (eBM-MSCs). For this purpose, eBM-MSCs (n=4) were differentiated into osteoblasts, adipocytes and chondrocytes. Differentiation was confirmed by specific staining and gene expression of lineage-related markers. Subsequently, gene expression of *MHC-I*, *MHC-II*, *CD40* and *CD80* was analyzed in undifferentiated (control) and tri-lineage differentiated eBM-MSCs. Osteogenesis and adipogenesis, but not chondrogenesis, significantly upregulated *MHC-I*; *MHC-II* expression significantly increased in the three lineages, while *CD40* and *CD80* expression did not change. Despite this, *MHC-I* and *MHC-II* upregulation after differentiation might lead to increased immunogenicity and risk of allorecognition, either eBM-MSCs differentiate in vivo after administration or they are differentiated prior to administration, with potential negative consequences for effectiveness and safety of allogeneic therapy.

Keywords.- Horse; Mesenchymal stem cells; Allogeneic; Immunogenicity; Differentiation

Word count.- 2,787 words

Introduction

Mesenchymal stem cells (MSCs) are raising great interest for the treatment of equine musculoskeletal injuries since these have a huge impact in this species (Thorpe et al., 2010), and because of the suitability of the horse as animal model (Colbath et al., 2017). Mesenchymal stem cells exert their therapeutic effects through different mechanisms, including anti-inflammatory and immunomodulatory properties, and their ability to differentiate into cells such as chondrocytes, osteoblasts or tenocytes (da Silva Meirelles et al., 2009). The regulatory properties of MSCs are currently focusing most of the interest, but differentiation should also be taken into account as it can occur in vivo after administration even if at low rates (Murphy et al., 2003; Mokbel et al., 2011). Furthermore, some therapeutic strategies are based on the implantation of differentiated MSCs, such as chondrocytes for treating joint pathologies (Broeckx et al., 2014a; Ham et al., 2015). The implantation of chondrocytes derived from MSCs may overcome the limitations of other techniques using autologous chondrocytes or chondroprogenitor cells, which are technically demanding and may involve donor site morbidity related to tissue harvesting (Jayasuriya and Chen, 2015; Cokelaere et al., 2016). Moreover, bioreactors are being developed to produce tissue-engineered constructs derived from MSCs for the repair of tissues such as cartilage, bone or tendon (Xie et al., 2013; Youngstrom et al., 2016).

The optimal moment for MSC administration has yet to be determined, but several studies suggested enhanced effects with early administration (Koch et al., 2009; Mokbel et al., 2011), which might be limited by the use of autologous MSCs. Autologous therapy may also be limited in elderly patients or patients with genetic disorders. Hence, allogeneic therapy has raised great interest (Zhang et al., 2015). However, MSCs are not truly immune-privileged as they may induce both cellular and humoral immune

responses, thus their immune allorecognition would lead to negative effects both in terms of effectiveness, because of the elimination of the cells, and safety, because of potential adverse effects for the patient (Berglund et al., 2017b).

In spite of the aforementioned, clinical implications of allogeneic equine MSCs administration are not entirely clear. While single and repeat intra-articular administration of allogeneic MSCs have been reported as clinically safe in both healthy (Carrade et al., 2011; Pigott et al., 2013a, b; Ardanaz et al., 2016) and pathologic equine joints (Broeckx et al., 2014a, b), other authors reported altered synovial parameters compared to autologous MSCs after second administration (Joswig et al., 2017).

These contradictory results might be due to different levels of major histocompatibility complex (MHC) expression and MHC compatibility between donor and recipient, since positive and heterogeneous MHC-II expression level (Schnabel et al., 2014) and in vivo generation of antibodies against MHC-mismatched equine MSCs (Berglund and Schnabel, 2016) have been reported. MSC differentiation might affect their MHC expression and, consequently, their immunogenicity. Hence, differentiation may imply negative consequences for allogeneic MSC survival, potentially hampering their effectiveness and compromising patient safety (Lohan et al., 2014).

Upregulation or induction of MHC-I and MHC-II and costimulatory molecules such as CD40 or CD80 has been shown after MSC differentiation into several lineages in human and small laboratory animals (Le Blanc et al., 2003; Liu et al., 2006a, b; Huang et al., 2010; Ryan et al., 2014; Yang et al., 2017). However, to the best of our knowledge, immunogenic profile of differentiated MSCs has not been assessed in the horse. Since allogeneic MSCs may differentiate in vivo albeit at low rates (Mokbel et al., 2011) or they can be differentiated to create tissue constructs prior to implantation

(Youngstrom et al., 2016), questions about immunogenicity of equine differentiated MSCs need to be addressed.

The aim of this study was to assess the gene expression of immunogenic markers after tri-lineage differentiation of equine bone marrow derived MSCs (eBM-MSCs) in order to provide preliminary results about the potential consequences of eBM-MSC differentiation for their allogeneic administration.

Material and methods

Isolation of equine BM-MSCs

Twenty ml of BM from sternum were collected in heparinized syringes using a 4″11G Jamshidi needle from four healthy horses (Shetland ponies geldings, 4-7 years, 138-162 kg) under approval of the Ethic Committee for Animal Experiments from the University of Zaragoza (Project License 31/11). Gradient density separation technique (Lymphoprep, Atom) was used to isolate mononuclear cells, which were expanded in basal culture medium consisting of low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1%Glutamine, 1%Streptomycin/Penicillin and 10%Fetal Bovine Serum (FBS) (Sigma-Aldrich) until passage three and then characterized as eBM-MSCs by their phenotype (Ranera et al., 2011) and by their tri-lineage differentiation potential as it will be explained in the next section.

Tri-lineage differentiation of equine BM-MSCs

Equine BM-MSCs (n=4) were exposed to induction media (differentiation) or basal medium (undifferentiated control) in triplicates according to each differentiation assay. Differentiation potential was assessed by specific staining and gene expression of

markers for each lineage. Methodology used was previously described by Ranera et al. (2011).

For inducing osteogenic differentiation, 20,000 cells/cm^2 were seeded in 24-well plates. Osteogenic medium consisted of basal culture medium described above supplemented with 10nmol/l dexamethasone, 10mmol/l β-glycerophosphate and 100µmol/l ascorbate-2-phosphate (Sigma-Aldrich). After 7 days, cells were fixed with 70%ethanol for 1 hour at room temperature (RT), stained with 2%Alizarin Red stain (pH 4.6) (Sigma-Aldrich) for 10' RT and washed with PBS (Gibco).

Equine BM-MSCs were seeded at 5,000 cells/cm^2 in 12-well plates for the adipogenic differentiation with induction medium consisting of 1µmol/l dexamethasone, 500µmol/l 3-isobutyl-1-methylxanthine, 200µmol/l indomethacin and 15% rabbit serum (Sigma-Aldrich) supplemented basal medium. After 15 days, cells were fixed with 10%formalin (Sigma-Aldrich) for 15' RT, stained with 0,3%Oil Red O stain (Sigma-Aldrich) (dissolved in 60:40; isopropanol:distilled water) for 30' at 37ºC and washed with distilled water.

To achieve chondrogenic differentiation, approximately 300,000 eBM-MSCs were transferred to conic bottom 15ml tube, 400µl of differentiation medium were added and then centrifuged at 1,750rpm 5' to pellet the cells. Chondrogenic medium consisted of 10%FBS, 10ng/ml TGFβ-3 (R&D Systems), ITS+premix (Beckton Dickinson), 40µg/ml proline, 50µg/ml ascorbate-2-phosphate and 0.1µmol/l dexamethasone supplemented high glucose DMEM (Sigma-Aldrich). After 21 days, pellets were fixed in 10%formalin, embedded in paraffin and cut into 5µm sections. The sections were hydrated with increasing gradients of alcohols, stained with Mayer's haematoxylin and

3%Alcian Blue dyes, rinsed with distilled water, dehydrated with decreasing amounts of alcohol and mounted.

Differentiation was also assessed by analyzing the gene expression of the osteogenic markers *Alkaline phosphatase (ALP)* and *Runt-related transcription factor 2 (RUNX2)*, the adipogenic markers *Lipoprotein lipase (LPL)* and *Peroxisome proliferator-activated receptor γ (PPARγ)*, and the chondrogenic markers *Collagen type II alpha I (COL2A1)* and *Aggrecan (ACAN).* Methodology used for gene expression analysis will be further explained in the next section.

Real time quantitative polymerase chain reaction (RT-qPCR)

Expression of genes coding for the lineage-associated markers aforementioned and immunogenic markers *MHC-I*, *MHC-II*, *CD40* and *CD80* was analyzed by RT-qPCR. Isolation of mRNA and complementary DNA (cDNA) retrotranscription from all samples were performed with the kit Cells-to-cDNA II (Ambion) according to manufacturer's instructions.

RT-qPCR reactions were performed and monitored with a StepOne RT-PCR System device (Applied Biosystems), using Fast SYBR Green Master Mix (Applied Biosystems) and 2 µl of cDNA as template. Amplification was performed in triplicate as follows: 20'' at 95°C, followed by 40 cycles consisting of 3''/95°C and 30''/ 60°C. A dissociation curve protocol was run after every reaction. Gene expression levels were obtained using the comparative Ct method. Normalization factor was calculated as the geometric mean of the quantity of two housekeeping genes, *GAPDH* and *B2M* (Ranera et al., 2011). Primers were designed with the Primer Express 2.0 software based on known equine sequences and cDNA obtained from equine peripheral blood

mononuclear cells (PBMCs) was used as positive control to validate the primers (Remacha et al., 2015). Information about primers is shown in Table 1.

Statistical analyses

Statistical analyses were performed using the SPSS 15.0 (SPSS Inc.). Normality of each data group was tested with the Shapiro-Wilk test. Differences in the expression level of each gene were assessed between undifferentiated (control) and differentiated cells within each lineage by the non-parametric paired Wilcoxon test. Significance level was set at $P<0.05$ for all analyses.

Results and Discussion

Equine MSCs were successfully isolated from BM from all animals and displayed similar phenotype (data not shown) and differentiation potential to that previously described (Ranera et al., 2011), as shown by specific staining (Figure 1.A) and by upregulated gene expression of lineage markers ($p<0.05$) (Figure 1.B). Control cells (not exposed to induction media) did not spontaneously differentiate in any case.

Based on previous studies in other species (Le Blanc et al., 2003; Liu et al., 2006a, b; Huang et al., 2010; Ryan et al., 2014; Yang et al., 2017), controls with separate components of the differentiation media were not performed. This approach was based on the assumption that possible MHC upregulation would be related to phenotypic changes experienced by differentiated MSCs, rather than to the direct effect of media composition. Actually, dexamethasone may decrease MHC-II expression in constitutively expressing human cells (Schwiebert et al., 1995). Indomethacin is a non-steroidal anti-inflammatory agent which inhibits prostaglandin(PG)-E2 production. Since PGE2 may participate in MHC regulation, their inhibitors might influence MHC

expression (Otsuka et al., 1991; Kim et al., 2010). However, to the best of our knowledge it has not been reported modification of MHC expression in MSCs by indomethacin. Members of the TGFβ family are usually found in immune-privileged tissues (Siglienti et al., 2007) and TGFβ-2 diminished MHC expression in equine MSCs (Berglund et al., 2017a), so it would not be expected that the isoform TGF-β3 had upregulated MHC. Finally, as far as we are concerned, the other components used in the differentiation media have not been described to affect MHC expression.

The results presented in this study are refered to gene expression, which has been widely used to study differentiated MSCs as it reflects their response to their environment in terms of control of protein production. However, mRNA traduction into proteins is a complex process so gene expression may not exactly correlate with protein expression. Nevertheless, increased MHC expression has been observed in both terms of gene and surface (protein) expression in undifferentiated equine MSCs exposed to inflammatory stimuli (Barrachina et al., 2016). Moreover, previous studies in other species assessing MHC expression in differentiated MSCs reported its increase in terms of both gene and protein expression (Huang et al., 2010; Xia and Cao, 2013). Therefore, gene expression changes found in this study may be related to surface protein changes, albeit at different extent. Further analyses, including protein expression and cytotoxicity assays, are warranted; but current gene expression results provide a preliminary insight about changes in the immunogenicity of differentiated equine MSCs.

Gene expression of *MHC-I* was significantly upregulated, compared to the undifferentiated control, in both osteogenic ($p<0.01$) and adipogenic ($p<0.01$) differentiated eBM-MSCs, but not in the cells undergoing chondrogenesis (Figure 2.A). On the contrary, MHC-I expression did not increase in human MSCs differentiated into osteogenic and adipogenic lineages but was upregulated after chondrogenesis (Le Blanc

et al., 2003). Interestingly, in the study of Le Blanc, MHC-I expression after adipogenesis was slightly reduced, while in our study this lineage showed the greatest *MHC-I* upregulation.

MHC-II gene expression significantly increased in eBM-MSCs differentiated into the three lineages ($p< 0.05$ for osteogenesis and adipogenesis, $p<0.01$ for chondrogenesis) (Figure 2.B). However, differentiated human MSCs did not show increased MHC-II expression in any lineage (Le Blanc et al., 2003), as well as no MHC-II expression was found in insulin-producing cells derived from human MSCs (Yang et al., 2017). On the contrary, human MSCs undergoing neuronal differentiation (Liu et al., 2006a) and murine MSCs differentiated into osteoblast (Zhang et al., 2009) and myocardiocytes (Huang et al., 2010) did upregulate MHC-II expression.

On the other hand, neither *CD40* nor *CD80* gene expression significantly changed in differentiated eBM-MSCs compared to undifferentiated cells (Figure 2.C and 2.D). In basal conditions, MSCs do not express costimulatory molecules such as CD40, CD80 or CD86 (Wang et al., 2014) but inflammatory exposure may lead to CD40 upregulation (Najar et al., 2012; Barrachina et al., 2017). Similarly to our results, insulin-producing cells derived from human MSCs showed no expression of CD40 or CD80 (Yang et al., 2017). Osteogenic differentiation neither induced CD80 nor CD86 expression in murine MSCs (Zhang et al., 2009), but osteogenesis (Shi et al., 2010) and neurogenesis (Liu et al., 2006a) of human MSCs led to CD80 expression.

Thus, a great variability has been observed in the immunogenic profile of differentiated MSCs among species and lineages. Therefore, it is needed to assess these changes in each case to clarify the potential implication of MSC differentiation in their immunogenicity and, consequently, in their effective and safe administration. To the

best of our knowledge, this is the first study reporting the influence of the osteogenic, adipogenic and chondrogenic differentiation of equine MSCs on their immunogenic gene expression profile.

Hence, upregulation of *MHC-I* and *MHC-II* might be related to an increased risk of immune allorecognition of the differentiated eBM-MSCs. Allorecognition of MHC molecules can be triggered by both direct and indirect pathways, that is, through the direct recognition of MHC-I and MHC-II molecules expresed by donor MSCs by recipient CD8+ or CD4+ T-cells (Chan et al., 2006; Afzali et al., 2008), or through the internalization of allogeneic MHC-I and MHC-II fragments by the recipient antigen presenting cells and their subsequent presentation to B and T-cells, potentially leading to cellular and humoral responses involving the generation of immune memory, which may limit repeat administration (Consentius et al., 2015). Furthermore, natural killer (NK)-cells could also target allogeneic MSCs that are missing self-MHC since they would be unable to activate the NK-cell inhibitory receptors (Spaggiari et al., 2006).

Equine MSCs expressing MHC-II directly induced proliferation of allogeneic MHC-mismatched T-cells in vitro (Schnabel et al., 2014), so MHC upregulation after differentiation might play a similar role. Activation of naïve T-cells requires costimulatory signals provided by costimulatory molecules such as CD40 and CD80 (Tse et al., 2003), which were not upregulated after differentiation of eBM-MSCs in this study. Even though the lack of costimulatory molecules expression in MSCs has been proposed to partially explain the absence of alloresponse in some situations (Consentius et al., 2015), the solely recognition of allogeneic MHC molecules may activate memory and effector T-cells, leading to an immune rejection (Benichou et al., 2017).

Consequently, MHC upregulated gene expression might be hypothesized to potentially increase the risk of immune allorecognition of eBM-MSCs after differentiation, even though costimulatory molecules were not upregulated. However, previous studies in other species did not always correlate the changes in the immunogenic profile of differentiated MSCs with an increased immune response in vitro (Le Blanc et al., 2003; Liu et al., 2006a; Zhang et al., 2009).On the contrary, other studies observed that differentiated MSCs induced proliferation of allogeneic T-cells in vitro (Shi et al., 2010; Ryan et al., 2014) and local and systemic T-cell memory response in vivo (Ryan et al., 2014). Moreover, while allogeneic administration of undifferentiated MSCs did not show adverse effects and even therapeutic benefit was elicited, administration of allogeneic pre-differentiated cells (Xia and Cao, 2013) or MSC differentiation in vivo after injection (Huang et al., 2010; Gu et al., 2015) led to immune rejection associated to MHC-I and MHC-II induction. Thus, previous studies suggest that allogeneic MSCs would go from a regulatory status (undifferentiated) towards an immunogenic one after differentiation, only providing short-term therapeutic benefit since differentiated cells would be rejected. This immunogenicity increase might be not only due to increased MHC expression but also related to a loss of MSC immunomodulatory ability (Ryan et al., 2014). In addition, different results observed in vitro and in vivo might be also explained by the potential influence of the in vivo microenvironment. As proinflammatory cytokines may upregulate MHC expression in undifferentiated MSCs (Barrachina et al., 2016), inflammatory conditions might also play a role in the immunogenicity of differentiated MSCs in vivo (Yang et al., 2017).

Summarizing, current knowledge about how differentiation influences MSC immunogenicity presents some contradictions but evidences that MSC immune properties could be affected, warranting further research in this area. The present study

provides preliminary data about the changes induced by differentiation in the immunogenic profile of eBM-MSCs. Gene expression of *MHC-I* and *MHC-II*, but not *CD40* and *CD80*, was increased after tri-lineage differentiation of eBM-MSCs, which might influence their immunogenicity and, subsequently, their allogeneic use, either if differentiation may occur in vivo after administration or whether equine MSCs are previously differentiated for a tissue engineering approach. Implications of these findings for the allorecognition of differentiated eBM-MSCs needs to be further addressed, as well as the possible role of the in vivo microenvironment and the effect of differentiation on immunomodulatory ability of eBM-MSCs, since MSC immune properties play a key role in the development of effective and safe cell therapies.

Conflict of interest statement

The authors declare that they have no competing interests. None of the authors has any financial or personal relationships that could inappropriately influence the content of the paper.

Role of the funding source

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

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Table 1.- Primers used for gene expression by RT-qPCR. GenBank accession numbers of the sequences used for primers design. Primers (F: Forward and R: Reverse) and length of the amplicon in base pair (bp). Genes were grouped in agreement with the functions and implications of encoded molecules.

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
IMMUNOGENICITY-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
CD80	Krampera 2006	F: CAGGAAAGTTGGCTCTGACCA R: TCTCCATTGTGATCCTGGCTC	135
OSTEOGENIC MARKERS			
ALP	XM_001504312	F: GATGGCCTGAACCTCATCGA R: AGTTCGGTCCGGTTCCAGAT	92
RUNX2	XM_001502519.3	F: CTCCAACCCACGAATGCACTA R: CGGACATACCGAGGGACATG	80
ADIPOGENIC MARKERS			
LPL	XM_001489577	F:TGTATGAGAGTTGGGTGCCAAA R:GCCAGTCCACCACAATGACAT	70
PPARγ	XM_001492411	F:TGCAAGGGTTTCTTCCGGA R:GCAAGGCATTTCTGAAACCG	104
CHONDROGENIC MARKERS			
ACAN	AF019756	F: CTACGACGCCATCTGCTACA R: ACCGTCTGGATGGTGATGTC	96
COL2A1	XM_005611082.1	F: TTAGACGCCATGAAGGTTTTCTG R: CTCTTGCTGCTCCACCAGTTCT	101

Figure legends

Figure 1.- Tri-lineage differentiation induction was confirmed by specific staining (**A**) and gene expression (**B**). Alizarin red staining of eBM-MSCs differentiated into osteoblasts (magnification 20x) (**A.1**); Oil red O staining of eBM-MSCs differentiated into adipocytes (magnification 4X) (**A.2**) and Alcian Blue staining of pellets from eBM-MSCs undergoing chondrogenic differentiation (maginification 10X) (**A.3**). Results from gene expression are expressed as Mean ± S.E.M (n=4) fold change of differentiated eBM-MSCs over undifferentiated eBM-MSCs (control) for osteogenic (**B.1**), adipogenic (**B.2**) and chondrogenic (**B.3**) markers. ALP, Alkaline phosphatase; RUNX2, Runt-related transcription factor 2; LPL, Lipoprotein lipase; PPARγ, Peroxisome proliferator-activated receptor γ; COL2A1, Collagen type II alpha I; ACAN, Aggrecan; * = $p<0.05$.

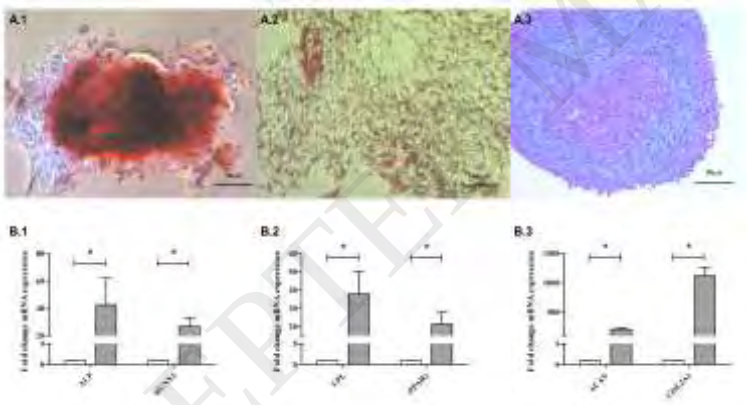

Figure 2.- Gene expression data are reported as Mean ± S.E.M (n=4) fold change of osteogenic, adipogenic and chondrogenic differentiated eBM-MSCs over undifferentiated eBM-MSC (control) for the immunogenic markers MHC-I (**A**), MHC-II (**B**), CD40 (**C**) and CD80 (**D**). MHC-I, major histocompatibility complex type I; MHC-II, major histocompatibility complex tipe II; CD40, cluster of differentiation 40; CD80, cluster of differentiation 80; * = $p<0.05$; ** = $p<0.01$