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Effect of inflammatory environment on equine bone marrow derived mesenchymal stem cells immunogenicity and immunomodulatory properties

L. Barrachina[a,b], A.R. Remacha[a], A. Romero[a,b], F.J. Vázquez[a,b], J. Albareda[a,c], M. Prades[a,d], B. Ranera[a], P. Zaragoza[a], I. Martín-Burriel[a], C. Rodellar[a,*]

[a] Laboratorio de Genética Bioquímica LAGENBIO, Universidad de Zaragoza, C/Miguel Servet, 177, 50013 Zaragoza, Spain
[b] Servicio de Cirugía y Medicina Equina, Hospital Veterinario, Universidad de Zaragoza, C/Miguel Servet, 177, 50013 Zaragoza, Spain
[c] Servicio de Cirugía Ortopédica y Traumatología. Hospital Clínico Universitario Lozano Blesa, Zaragoza, Avda. San Juan Bosco, 15, 50009 Zaragoza Spain
[d] Departament de Medicina i Cirugia Animal, Universidad Autónoma de Barcelona, Edifici H, UAB, 08193 Bellaterra, Barcelona, Spain

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ABSTRACT

Mesenchymal stem cells (MSCs) are being investigated for the treatment of equine joint diseases because of their regenerative potential. Recently, the focus mainly has addressed to their immunomodulatory capacities. Inflammation plays a central role in joint pathologies, since the release of proinflammatory mediators to the synovial fluid (SF) leads to the activation of enzymatic degradation of the cartilage. MSCs can modulate the local immune environment through direct or paracrine interaction with immune cells, suppressing their proliferation and re-addressing their functions. Proinflammatory molecules can induce MSC immunoregulatory potential, but they could also increase the expression of immunogenic molecules. Studying the effect of inflammatory environment on MSC immunomodulation and immunogenicity profiles is mandatory to improve cellular therapies. The aim of this study was to analyse the response of equine bone marrow MSCs (eBM-MSCs) to three inflammatory conditions. Equine BM-MSCs from three animals were exposed to: (a) 20% allogeneic inflammatory SF (SF); (b) 50 ng/ml of TNFα and IFNγ (CK50) and (c) 20 ng/ml of TNFα and IFNγ (CK20). After 72 h of exposure, expression of immunogenic and immunomodulation-related molecules, including cell-to-cell contact and paracrine signalling molecules, were analysed by RT-qPCR and flow cytometry. The gene expression of adhesion molecules was upregulated whereas MSC migration-related genes were downregulated by all inflammatory conditions tested. CK culture conditions significantly upregulated the expression of *COX-2*, *iNOS*, *IDO* and *IL-6*. *MHC-I* gene expression was upregulated by all conditions, whereas *MHC-II* was upregulated only after CK priming. The expression of *CD40* did not significantly change, whereas the ligand, *CD40L*, was downregulated in CK conditions. Flow cytometry showed an increase in the percentage of positive cells and mean fluorescence intensity (MFI) of the MHC-I and MHC-II molecules 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 might not be increased. This study provides a better understanding about the behaviour of eBM-MSCs inside the inflamed joint and constitutes a first step to improve MSC-based therapies for equine joint diseases.

* Corresponding author. Fax: +34 976 762949.
E-mail addresses: lbarrach@unizar.es (L. Barrachina), aremacha@unizar.es (A.R. Remacha), aromerol@unizar.es (A. Romero), pvazquez@unizar.es (F.J. Vázquez), albaredajorge@gmail.com (J. Albareda), marta.prades@uab.cat (M. Prades), beatrizranera@gmail.com (B. Ranera), pilarzar@unizar.es (P. Zaragoza), minma@unizar.es (I. Martín-Burriel), rodellar@unizar.es (C. Rodellar).

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1. 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 Committe, 2005). Current therapeutic strategies for DJD are focused on reducing pain, decreasing disability, and limiting deterioration of articular structures (Walker-Bone et al., 2000). 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 MSCs administration. This effect has been attributed to the anti-inflammatory and immunoregulatory properties of MSCs (Whitworth and Banks, 2014), suggesting that the MSC therapeutic mechanism may be due to an immunoregulation exerted by cells promoted by the environment (Kode et al., 2009; Meirelles Lda et al., 2009). This phenomenon seems to be a multifactorial process that requires both direct cell-to-cell contact and contact-independent paracrine signalling governed through different molecules such interleukin 6 (IL-6), indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide synthase (iNOS), prostaglandin E2 (PGE2), interleukin 10 (IL-10), or tumor necrosis factor-inducible gene 6 protein (TSG-6), among others. These molecules are involved in proliferation, differentiation, migration or apoptosis in different immune cells (Ma et al., 2014), varying between species (Ren et al., 2009). The expression of several molecules related to paracrine and cell-contact mechanisms, like chemokines and adhesion molecules, is regulated in MSCs by pro-inflammatory molecules such interferon γ (IFNγ), especially in combination with TNFα, interleukin 1α (IL-1α) or IL-1β (Ren et al., 2008). This fact suggests that for a full regulatory function of MSCs, MSC activation or licensing through exposure to an inflammatory environment might be needed (Renner et al., 2009; Cuerquis et al., 2014).

Inflammatory synovial fluid (SF) contains variable amounts of several inflammatory molecules that may alter MSC function and characteristics. Some studies in equine and human species have reported that changes in the composition of inflammatory molecules of the SF might affect the expression and production of paracrine signalling molecules by MSCs, acting as inductors of their immunomodulatory potential (Leijs et al., 2012; Vézina Audette et al., 2013). However, the stimulus exerted by SF could be heterogeneous because it depends on the variable amounts of the different mediators, which is defined by the SF inflammatory level (Leijs et al., 2012). *In vitro*, the combination of definite doses of INFγ with another proinflammatory cytokine, such TNFα, has demonstrated a change in the immunoregulatory abilities of MSCs, inducing the expression or the secretion of anti-inflammatory and regulatory factors and suppressing T cell proliferation in co-culture experiments (Cuerquis et al., 2014; Paterson et al., 2014). However, an increase of the immunogenicity has also been observed due to this priming (Chan et al., 2006, 2008), indicating a possible limitation to use allogeneic primed MSCs. The enhancement of MSC immunoregulatory properties, without detriment of their immune-evasive status, could improve the MSC therapeutic efficacy and allow their allogeneic use (Ankrum et al., 2014).

Despite the recent findings, little is known about the effects and behaviour of MSCs when they are IA administered within an injured joint. Pre-existing joint inflammation may impact the secretome of MSCs altering their therapeutic efficacy (Roberts et al., 2011), therefore, a deep understanding of the changes induced in MSC immunogenicity and immunoregulatory abilities by an inflammatory synovial environment is needed. Since immunoregulatory potential of MSCs seems to be crucial for their therapeutic potential, stimulating this ability could be of major interest in order to improve the benefits of regenerative medicine. In consequence, know how the synovial environment affects administered MSCs, and the way in which we can stimulate them to enhance their immunoregulatory potential, will be the key for developing effective joint regenerative treatments.

The aim of the present study was to assess the effect of different inflammatory stimuli on eBM-MSCs immunoregulatory ability and immunogenicity, studying the expression of immunogenic and immunomodulation-related molecules. Firstly, the influence of allogeneic inflammatory SF on eBM-MSCs was investigated, and subsequently, the effect of priming eBM-MSCs with a combination of the two pro-inflammatory molecules IFNγ and TNFα, was tested at two different doses. SF and CK inflammatory conditions were chosen according to previous studies (Ren et al., 2008; Leijs et al., 2012; van Buul et al., 2012; Vézina Audette et al., 2013; Zimmermann and McDevitt, 2014). This work contributes to understand the effects of inflammatory exposure on eBM-MSCs, as a previous step to enhance their use *in vivo* for equine joint diseases.

2. Materials and methods

2.1. Experimental design

Three repeat experiments were designed to investigate the *in vitro* response of eBM-MSCs to different inflammatory stimuli:

Experiment 1 (SF). The culture medium was supplemented with 20% of equine allogeneic inflammatory SF.

Experiment 2 (CK50). The culture medium was supplemented with pro-inflammatory cytokines: IFNγ (50 ng/ml) and TNFα (50 ng/ml).

Experiment 3 (CK20). The culture medium was supplemented with pro-inflammatory cytokines: IFNγ (20 ng/ml) and TNFα (20 ng/ml).

Equine BM-MSCs ($n = 3$) were cultured in triplicate for each inflammatory condition for 72 h. Subsequently, the expression of molecules related to immunoregulation and immunogenicity was analysed by flow cytometry and RT-qPCR. Controls in triplicate were run in each experiment using the same eBM-MSCs ($n = 3$) cultured with control media.

2.2. Animals

Four geldings (age: 6–12 years; weight: 450–500 kg,) were used in this study, named as 057, 059, 060 and 063. Animals 057, 059 and 060 were patients from the Veterinary Hospital of the University of Zaragoza determined to be healthy based on clinical and hematologic examination. Biological samples (BM or SF) were obtained with owner consent and according to local animal welfare reg-