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IMMUNOMODULATORY PROPERTIES OF PORCINE, BONE MARROW-DERIVED MULTIPOTENT MESENCHYMAL STROMAL CELLS AND COMPARISON WITH THEIR HUMAN COUNTERPART

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Abstract

Thanks to their immunonodulatory properties, multipotent mesenchymal stromal cells (MSCs) are a promising strategy for preventing/reducing the risk of graft rejection after hematopoietic cell and solid organ transplantation. We have previously demonstrated that porcine MSCs (pMSCs) can be isolated from bone marrow and display similar morphology and differentiative capacity as compared to human MSC (hMSCs). In this study, we investigated the *in vitro* immunomodulatory properties (namely the ability to suppress lymphocyte proliferation in response to phytohemagglutinin and the cytokine production in the culture supernatants) of pMSCs from six Large White 6-month old piglets. Similarly to hMSCs, pMSCs reduced the phytohemagglutinin-induced lymphocyte proliferation. High levels of IL-6 were found in culture supernatants, whereas IL-10 and TGF-β were not detectable. In conclusion, *ex vivo* expanded pMSCs share selected biological/functional properties with hMSCs. pMSCs may be used in *in vivo* models to investigate novel approaches of prevention of graft rejection in solid organ transplantation.

Key words: Mesenchymal stromal cells, pig, animal model, *ex vivo* expansion, solid organ transplantation.

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Abbreviations: hMSCs: human multipotent mesenchymal stromal cells ; **PBMCs**: peripheral blood mononuclear cells; **PHA**: phytohemagglutinin; **pMSCs**: porcine MSCs.

INTRODUCTION

Multipotent mesenchymal stromal cells (MSCs) contribute to the creation of bone marrow (BM) microenvironment and play a crucial role in the development and differentiation of the lympho-hematopoietic system (7,10,14). Due to their immunomodulatory and anti-inflammatory properties, MSCs are currently eliciting great interest for clinical application in the treatment of immune-mediated disorders, such as graft-versus-host disease (GvHD) and graft rejection occurring after allogeneic hematopoietic cell transplantation (HCT) (11,9,2). MSCs may also play a role in the definition of novel therapeutic strategies for prevention/mitigation of allograft

rejection in the setting of solid organ transplantation (SOT) (8,12).

The implementation of MSC-based cellular therapy approaches in large animals could be useful to clarify the biological mechanisms by which MSCs exert their immunosuppressive and anti-inflammatory functions. In particular, in the context of both HCT and SOT, a large animal model could allow to address still unsolved questions related to the use of MSCs, such as effective cell dosage and timing, cell survival, trafficking and homing after administration. For this purpose, the standardization of methodologies for *ex vivo* isolation and expansion of MSCs from large animals, such as pigs, as well as a definition of their biological and functional characteristics, is warranted.

We have recently demonstrated that porcine MSCs (pMSCs) can be readily isolated from BM and expanded *ex vivo* (6). In the current study, we characterized pMSC functions by investigating their *in vitro* immunomodulatory properties and cytokine secretion profile. pMSC functional properties were compared with those of their human counterpart (hMSCs).

MATERIALS AND METHODS

BM aspirates and peripheral blood mononuclear cells (pPBMCs) were obtained from six Large White 6-month old pigs (mean weight 24±5 Kg). All procedures performed on the animals were approved by the local Animal Care and Use Committee. pMSC were isolated from all 6 BM aspirates, expanded *ex vivo*, characterized by flow cytometry and assessed for their differentiation capacities, according to previously reported methods (6,3).

After written informed consent, human PBMCs (hPBMCs) and BM-derived human MSCs (hMSCs) were obtained from six healthy volunteers (median age 27 years, range 21-36) (3,4) and employed for comparison with pMSCs. Both pMSCs and hMSCs were cultured in complete medium: DMEM-LG (Gibco Invitrogen, Paisley, UK), supplemented with gentamicin 50 µg/mL (Gibco Invitrogen) and 10% fetal calf serum (FCS) (Mesenchymal Stem Cell Stimulatory Supplements, StemCell Technologies, Vancouver, Canada).

In order to investigate the *in vitro* immunomodulatory properties of MSCs, pPBMCs from all six animals were cultured with (stimulated culture) or without (unstimulated culture) phytohemagglutinin (4 µg/ml) (PHA-L; Boehringer, Mannheim, Germany), either in the absence or in the presence of pMSCs. Irradiated (30 Gy) autologous or allogeneic pMSCs were seeded in triplicate in flat-bottom 96 wells microplates (Corning Costar, Celbio) to achieve final pMSC:pPBMC ratios of 1:2, 1:20, 1:200, 1:1000 per well and allowed to attach overnight before adding 10^5 pPBMCs per well. Cultures were performed in RPMI 1640 medium (Gibco-BRL, Life Technologies, Paisley, UK) supplemented with 10% FCS (Euroclone, Celbio, Milan, Italy) with or without PHA. After a 3-day incubation at 37°C in a humified, 5% CO_2 atmosphere, ^{3}H thymidine (^{3}HTdR 0.5 µCi/well; Amersham, Buckinghamshire, UK) was added to the wells for the last 21 hours of culture; thymidine incorporation was measured by standard procedures, and results were expressed as stimulation index (SI): cpm stimulated/cpm unstimulated, where cpm means counts per minute. The effect of hMSCs on the proliferation of PHA-stimulated hPBMCs, as well as the xenogeneic combinations (pMSCs with hPBMCs and vice versa), were tested for comparison.

The concentration of porcine interleukin-6 (pIL-6), pIL-10 and transforming growth factor-β (pTGF-β) in supernatants collected from the PHA-stimulated proliferation assays, both in the autologous and allogeneic porcine settings, was quantified by commercially available ELISA using a polyclonal antibody specific for porcine cytokines (Quantikine, R&D Systems, Minneapolis), according to the manufacturer's instructions.

Statistical Analysis

The non parametric Mann-Whitney test for independent samples was performed for the comparison of population doubling (PD) and inhibition of PBMC proliferation to PHA. P values less than 0.05 were considered to be statistically significant.

RESULTS

As initially reported for four animals (6), pMSCs were successfully isolated and expanded *ex vivo* from all six pigs. The cells showed the typical spindle-shape morphology and differentiation potential as compared with their human counterpart expanded under the same culture conditions (see also Figure 1 A, B, C). Moreover, pMSCs resulted positive for CD90, CD29 and CD105, whereas contamination with hematopoietic cells was no longer detectable starting from P1 (CD45 and CD11b: <5% positive cells; data not shown).

The proliferative capacity of pMSCs was evaluated in terms of PD from passage 1 (P1) to P4, and compared to hMSCs. The mean PD for single passage was 2.9 (range 3.0-3.6) for six pMSC samples (cumulative PD = 11.7; see Figure 1D), whereas PDs of hMSCs expanded from six healthy donors was significantly lower (mean PD for single passage: 2.3; range 1.4-2.9; cumulative PD = 10.0; P=0.001, see Figure 1D). The median time to reach 80% confluence for all passages from P1 to P4 was 7 days (range: 6-10) and 6 days (range: 5-11) for pMSCs and hMSCs (6 donors); respectively (P=ns). Porcine MSCs derived from two pigs were continuously cultured *in vitro* until reaching P16 (pig N. 2) and P24 (pig N. 5), respectively. At these late time-points, cultures were terminated to perform sampling and data analysis. MSCs from both pigs maintained their typical spindle-shaped morphology and their immune-phenotype

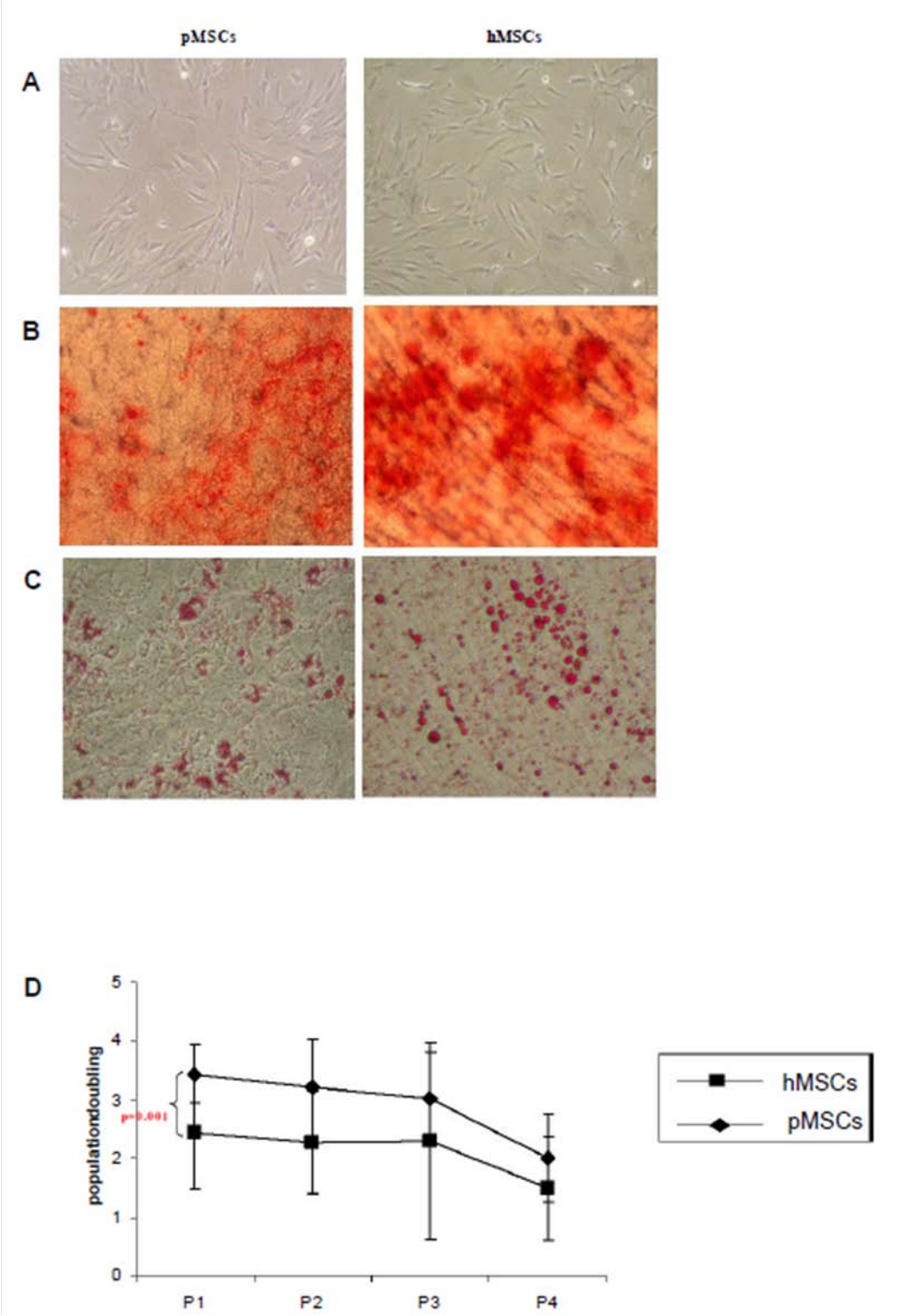

Figure 1. Characterization of *ex vivo* expanded porcine multipotent mesenchymal stromal cells (pMSCs) derived from bone marrow (BM) in comparison with their human counterparts; pMSCs are from one of the pigs employed in our study, while hMSCs are from a healthy control. **(A)** Morphology of pMSCs from pig N.3 at passage 2 (P2), in comparison to that of BM-derived human MSCs (hMSCs) from a healthy donor at passage P2. **(B)** Osteogenic differentiation capacity of pMSCs from pig N.6 at P3 (left panel), as demonstrated by calcium deposition stained by Alzarin Red. Osteogenic differentiation of BM-derived hMSCs from a healthy donor at passage P3 is reported for comparison (right panel). Magnification 20X. **(C)** Adipogenic differentiation capacity of pMSCs from pig N.6 at P3 (left panel), as shown by the formation of lipid droplets stained with Oil Red. Adipogenic differentiation of BM-derived hMSCs from a healthy donor at passage P3 is reported for comparison (right panel). Magnification 20X. **(D)** Population doublings (PDs, calculated as log10(N)/log10(2), where N=cells harvested/cells seeded) of pMSCs from passage (P) 1 to P4, in comparison to that of BM-derived hMSCs. Results are expressed as the mean (+SD) calculated from data obtained from the six pigs and six healthy donors.

remained unchanged throughout the entire culture period (data not shown).

Concerning the immunomodulatory functions of pMSCs, in the porcine autologous setting (*i.e.* pPBMCs/pMSCs from the same pig), PHA-stimulated proliferation of pPBMCs was reduced by up to 48% ± 22% (maximum inhibition at a MSC: PBMC ratio of 1:2, corresponding to a SI of 42 P=0.07 in comparison to basal response to PHA) by the addition of pMSCs (see Figure 2A). The inhibition of proliferation was lost at a MSC: PBMC ratio of 1:1.000. Also in the porcine allogeneic setting (where pPBMCs and pMSCs were obtained from independent animals), pMSCs inhibited the proliferation of PHA-stimulated pPBMCs with maximum inhibition at a MSC: PBMC ratio of 1:2 (59% ± 15%, corresponding to a SI of 18; P=0.05 in comparison to basal response to PHA) (see Figure 2B). The findings in the animal setting parallel well the inhibitory effect induced by hMSCs on hPMBCs both in the autologous (hPBMCs/hMSCs from the same healthy donor; maximum inhibition at ratio 1:2 = 74% ± 14%, corresponding to a SI of 6; P=0.06 in comparison to basal response) and allogeneic (hPBMCs/hMSCs from unrelated healthy donors; maximum inhibition at ratio 1:2 = 36% ± 11%, corresponding to a SI of 31; P=0.05 in comparison to basal response) settings as shown in Figure 2C and 2D, respectively. The xenogeneic setting was also evaluated: pMSCs were tested with PHA-stimulated hPBMCs and hMSCs were used in co-culture with PHA-stimulated pPBMCs. Porcine MSCs were capable of inhibiting the proliferation of PHA-stimulated hPBMCs (maximum inhibition at a MSC:PBMC ratio 1:20 = 78% ± 16%, corresponding to a SI of 56; P=0.07 in comparison to basal response; Fig 2E); similarly PHA-stimulated proliferation of pPBMCs was reduced by the addition of hMSCs (maximum inhibition at MSC:PBMC ratio 1:20 = 37% ± 9%, corresponding to a SI of 61; P=0.07 in comparison to basal response; Fig 2F). Thus, pMSCs display the ability to inhibit polyclonally-induced lymphocyte proliferation both in an autologous and allogeneic setting, as well as when tested against human lymphocytes in a manner similar to that of hMSCs. Moreover, we did not find any effect of MSCs on apoptosis of PBMCs exposed to PHA. The cytokine production pattern in culture supernatants collected at the end of the pPBMC proliferation assays, both in the autologous and allogeneic porcine settings, showed a strong increase in pIL-6 levels after pMSC addition (see also Table 1 for details), whereas IL-10 and TGF-β were undetectable in all the experimental conditions tested. The increase in pIL-6 levels induced by the addition of pMSCs was dose-dependent (see Table 1).

DISCUSSION

We have previously demonstrated that the well-established methods for harvesting, culturing and *ex vivo* expanding hMSCs can be successfully reproduced in pigs and that pMSCs and hMSCs have similarities in terms of morphology, immunophenotype, and differentiation potential (6). We now document that pMSCs have the capacity to inhibit the proliferation of pPBMCs in both the autologous and allogeneic settings. Noteworthy, pMSCs were effective also in a xenogeneic *in vitro* model. Altogether, these data represent preliminary conditions for performing preclinical studies in pigs aimed at testing the potential role of MSCs in modulation of alloreactivity (13). Indeed, large animals are more similar phylogenetically to the human beings, thus representing a more suitable model to address still unsolved issues related to MSC trafficking, homing, secretion of soluble factors and mechanisms of action relevant for their therapeutic effect.

In a very recent paper (5), pMSCs failed to inhibit *in vitro* PHA-stimulated, as well as alloantigen-induced, pPBMC proliferation. We did not investigate the proliferation of pPBMCs in response to allogeneic stimuli, while we only tested the effect of pMSCs on a response induced by a polyclonal activator such as PHA. The discrepancy between the results reported by Brunswig-Spickenheier *et al* (5) and those we obtained in this study might be explained by the different experimental conditions employed. In particular, the method of pMSC isolation from BM and the concentrations of PHA utilized were different; moreover, in our experiments MSCs were not irradiated and our method for measuring lymphocyte proliferation was based on incorporation of ^{3}H thymidine, instead of Ki67 antigen staining (5). As also shown by Brunswig-Spickenheier *et al* (5), we found in the culture supernatants high levels of pIL-6, a multifunctional cytokine regulating immune response, inflammation and hematopoiesis. This finding is also in agreement with results obtained using hMSCs previously published by our own

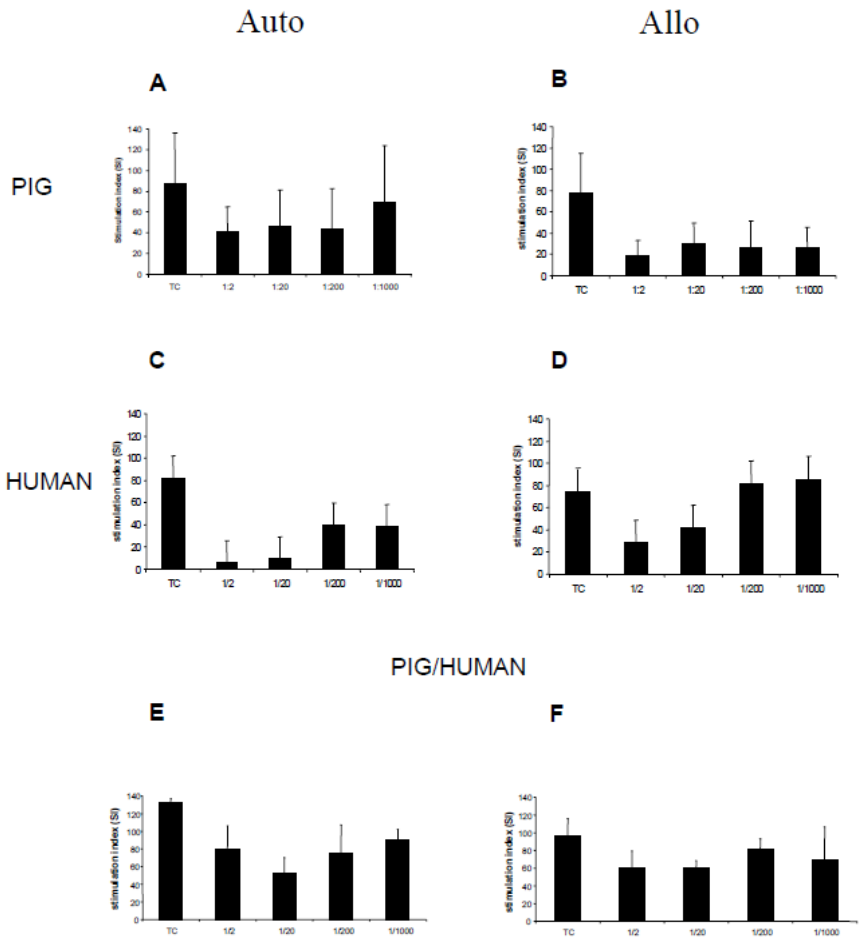

Figure 2. Proliferation of porcine PBMCs (pPBMCs; A and B) and human PBMCs (hPBMCs; C and D), stimulated with phytohemagglutinin (PHA), in the presence of autologous (Auto) and allogeneic (Allo) pMSCs or human MSCs (hMSCs). Results are expressed as stimulation index (SI): cpm stimulated/cpm unstimulated, where cpm is counts per minute. Each bar represents the percentage of proliferation of 100.000 PBMCs in the absence (TC) and in the presence of decreasing numbers of MSCs (MSC:PBMC ratios 1:2; 1:20; 1:200; 1:1,000). The cpm values at each cell concentration were normalized to the cpm of PBMCs without MSCs in each experiment. Each bar represents the mean ± SD of multiple experiments (4 to 6 MSC samples, each point being in triplicate). **A)** pPBMCs/pMSCs, autologous setting; **B)** pPBMCs/pMSCs, allogeneic setting; **C)** hPBMCs/hMSCs, autologous setting; **D)** hPBMCs/hMSCs, allogeneic setting; **E)** hPBMCs/pMSCs; **F)** pPBMCs/hMSCs.

Table 1. Levels of porcine interleukin-6 (pIL-6) in culture supernatants collected at the end of the phytohaemagglutinin (PHA) proliferation assays in the autologous (AUTO) and allogeneic (ALLO) porcine settings, at the different pMSC:pPBMC ratios. TC is the condition with 100.000 PBMCs alone. The values are expressed as median and range of four tested pigs and reported as pg/ml.

MSC:PBMC ratio	Setting AUTO		Setting ALLO	
	TC	PHA	TC	PHA
No MSC	22 (5-845)	294 (258-1442)	14 (0-254)	234 (230-1785)
1:2	2900 (2593-24241)	81330 (79840-615780)	3040 (2632-5834)	75190 (64830-105760)
1:20	960 (919-6495)	7300 (5378-24530)	1600 (1271-2313)	19310 (15505-27098)
1:200	720 (17-1068)	1840 (718-5248)	50 (0-996)	4050 (3721-4103)
1:1000	28 (17-158)	203 (150-1578)	163 (0-222)	760 (172-2081)

group (1) and others (11) and confirms the complex network of interaction of MSCs with cells involved in immune responses.

In conclusion, our study provides evidence that pMSCs inhibit lymphocyte proliferation, this supporting the use of pigs to experimentally investigate the potential role of MSCs in novel immunomodulatory approaches aimed at controlling/preventing allograft rejection.

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