

# Immunosuppressive Effects of Mesenchymal Stem Cells: Involvement of HLA-G

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**Introduction.** Mesenchymal stem cells (MSCs) possess unique immunomodulatory properties. They are able to suppress allogenic T-cell response and modify maturation of antigen-presenting cells. Their role in the treatment of severe graft versus host disease has been reported. The underlying molecular mechanisms of immunosuppression are currently being investigated. Histocompatibility locus antigen (HLA)-G is a nonclassical major histocompatibility complex class I antigen with strong immune-inhibitory properties.

**Methods.** We studied the role of HLA-G on MSC-induced immunosuppression. The expression of HLA-G on human MSCs cultured alone and in mixed lymphocytes reaction (MSC/MLR) was analyzed.

**Results.** We found that HLA-G can be detected on MSCs by real-time reverse-phase polymerase chain reaction, immunofluorescence, flow cytometry ( $52.4 \pm 3.6\%$ ), and enzyme-linked immunosorbent assay in the supernatant ( $38.7 \pm 5.2$  ng/mL). HLA-G protein expression is constitutive and the level is not modified upon stimulation by allogenic lymphocytes in MSC/MLR. The functional role of HLA-G protein expressed by MSCs was analyzed using the 87G anti-HLA-G blocking antibody in a MSC/MLR. We found that blocking HLA-G molecule significantly raised lymphocyte proliferation in MSC/MLR ( $35.5\%$ ,  $P=0.01$ ).

**Conclusion.** Our findings provide evidences supporting involvement of HLA-G in the immunosuppressive properties of MSCs. These results emphasize the potential application of MSCs as a relevant therapeutic candidate in transplantation.

**Keywords:** Mesenchymal stem cell, Immunosuppression, Mixed lymphocytes reaction, Histocompatibility locus antigen-G, Graft versus host disease.

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Experimental evidence and preliminary clinical studies have demonstrated that human bone marrow stromal cells, referred as mesenchymal stem cells (MSCs), have an important immunomodulatory function in the management of allogeneic hematopoietic stem cell (HSC) transplantation (1). Injection of MSCs could cure severe graft versus host disease (GVHD) (2, 3) and promote hematopoietic recovery (4, 5). Furthermore, MSC injection in immunocompetent baboons prolonged skin allograft survival (6).

MSC-mediated inhibition of immune response is a complex mechanism that involves changes in the maturation of antigen-presenting cells (7) as well as suppression of differentiation and function of monocyte derived dendritic cells (DCs) (8). Furthermore, MSCs altered the cytokine secretion profile of naïve DC, effector T cells, and natural killer cells (NKs) and modified the proinflammatory TH1 profile towards TH2 anti-inflammatory profile. These properties could be useful for the prevention and treatment of GVHD and the inhibition of graft rejection (9).

MSCs exert profound immunosuppression by inhibiting T-cell proliferation in response to various stimuli in vitro (10). They induce regulatory immunosuppressive lymphocytes (9) and CD8 apoptosis (11). MSCs inhibit cell cycle progression (12) and CD4 allo-proliferation (10). This immunosuppressive effect of MSCs is mediated through several inducible soluble factors, such as transforming growth factor- $\beta$  (10), hepatocyte growth factor (10), interleukin-10 (IL-10) (7), prostaglandin E2 (PGE2) (9), and indoleamine 2,3-dioxygenase (IDO) (13). In these studies, a partial reversion of the MSC inhibitory effect on T-cell proliferation was demonstrated.

Natural processes allow fetal allografts to evade from rejection by the mother. This phenomenon is partially dependant on histocompatibility locus antigen (HLA)-G molecules (14). HLA-G is a nonclassical major histocompatibility complex (MHC) class I, which is expressed in both membrane-bound and soluble isoforms. Both of them can display tolerogenic properties via interaction with inhibitory receptors on DC, NK, and T cells. Soluble HLA-G exerts an immunosuppressive effect by inducing apoptosis of CD8+ T cells and down modulating CD4+ T cells proliferation. Membrane-bound HLA-G protein has been shown to inhibit NK cells and T cell-mediated cytotoxicity, to suppress proliferation of allo-specific CD4+ T lymphocytes and to induce TH2 cytokine profile. For review, see (15). Furthermore, a good correlation between HLA-G expression and graft acceptance has been evidenced (16).

Production of HLA-G protein by adult MSCs had never been demonstrated. We investigated whether HLA-G was expressed by MSCs and contributed to MSC-mediated inhibition of immune response in vitro. We report here that MSCs express HLA-G protein and demonstrate that HLA-G is involved in MSC-mediated lymphocytes proliferation inhibition.

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## MATERIALS AND METHODS

### Isolation and Culture of Human Bone Marrow (BM) MSCs

BM cells were obtained after informed consent of patients undergoing total hip replacement surgery and were used in accordance with the procedures approved by the human experimentation and ethic committees of Hospital St Antoine (France). Ten to twenty milliliters of BM were harvested in  $\alpha$ -minimum essential medium (MEM; Invitrogen, Cergy, France) supplemented with heparin. Total cells were isolated from bone fragments after two rounds of mechanical extraction/sedimentation. The recovered cells were centrifuged and resuspended in culture medium. Nucleated cells were counted after red cells lysis by acetic acid. Then, the nucleated cells were plated at 50,000 cells/cm<sup>2</sup> in  $\alpha$ -MEM supplemented with 10% fetal calf serum (research-grade FCS, Hyclone, Perbio, France), 1% L-glutamine, 1% penicillin streptomycin, and 1 ng/mL betaFGF (Sigma, France) referred as FCS- $\alpha$ MEM. FCS- $\alpha$ MEM corresponds to the culture techniques used in clinics (17). To study the importance of culture conditions for HLA-G protein expression,  $\alpha$ -MEM medium supplemented with platelet lysate from platelet-rich plasma (PRP- $\alpha$ MEM) was used in comparison with FCS- $\alpha$ MEM. For the culture in PRP- $\alpha$ MEM, the cells were plated at 2.10<sup>5</sup> cells/cm<sup>2</sup> in  $\alpha$ -MEM, 1% L-glutamine, 1% penicillin streptomycin, heparin 2U/mL, and 5% of platelet lysate from PRP as previously published (18). Culture flasks were incubated at 37°C with 5% CO<sub>2</sub> in humidified atmosphere. After 72 hr, nonadherent cells were removed, and the medium was replaced twice a week until the 90% of confluence was reached. Then, cells were detached using 0.25% trypsin (Stem Cell Technologies), and passaged up to passage 4 (P4). The cells were characterized by phenotypic analysis, and ability to differentiate into adipocytic, chondrocytic and osteocytic lineages as previously described (19).

### Antibodies, Flow Cytometry, and Enzyme-Linked Immunosorbent Assay (ELISA)

At each passage, the cells were characterized using monoclonal antibodies specific for CD105 (SEROTEC, France), CD73 (BD Pharmingen, France), and CD45 (Beckman Coulter, France) conjugated with FITC, PE, and PC5, respectively. Acquisitions and data analysis were performed using the flow cytometer FACScalibur (BD Biosciences) and CELLquest software (Becton Dickinson). For HLA-G analysis we used the mouse anti HLA-G1/G5 MEMG/9 FITC antibody (Exbio, Praha, Czech Republic) at 1/500 final concentration. For all analysis isotypic controls were systematically included. Intracellular staining was performed using the Cytofix/cytoperm kit (BD Biosciences) according to the recommended conditions. To detect soluble HLA-G molecule in culture supernatant we performed an ELISA coated with the same MEMG/9 antibody, according to the manufacturer's instructions (Exbio, Praha, Czech Republic). Functional assessment of HLA-G activity was performed using (87 G) anti HLA-G blocking antibody (Exbio, Praha, Czech Republic, 10-437-C100).

### Semiquantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared from MSCs using the Trizol method according to the manufacturer's instructions (Invitrogen, Paisley, Scotland). A total of 1  $\mu$ g of DNase treated RNA was transcribed into cDNA using 200 units of SuperScript II reverse transcriptase (Invitrogen, Groningen, Netherlands) and 150 ng of random primers (Invitrogen). PCRs were performed in duplicate using the SYBR green Master Mix according to the manufacturer's instructions (Applied Biosystems, Foster City CA USA). *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, access number NM002046) was used as a reference gene. *GAPDH* gene forward GAAGGTGAAGGTCG-GAGTC and reverse primer GAAGATGGTGATGGGATTTC and *HLA-G* genes (access number X17273) forward primer ACCATCCCCATCAGGTATC and reverse primer ACCG-CAGCTCCAGTGACTACA were designed on Primer Express™ software (Applied Biosystems). Primers sequences were checked for theoretical target genes specificity on BLAST2. The specificity of PCR products was checked with melting temperature dissociation software (Applied Biosystems). We used universal RNA control (Clontech Universal Reference Total RNA, Clontech) to prepare standard curve. Then sample quantity is determined from the standard curve. We calculated the average of HLA-G value from six healthy donors in duplicate using equation of standard curve.

### Immunofluorescence

MSCs were cultured on Labtek chamber slide system (Nunc) up to 80% confluence. After three washes in phosphate-buffered saline 1 $\times$ , the cells were fixed for 20 min in 4% paraformaldehyde. Permeabilization and blocking were done with 0.3% Triton X-100, 1% BSA, and 10% SVF in phosphate-buffered saline 1 $\times$  during 45 min at room temperature. MSCs and positive control were incubated with the 87G (Exbio, Praha, Czech Republic) primary antibody (1/50) in 300  $\mu$ L of the same buffer without Triton X-100 for 4 hr at room temperature. After washing, cells were incubated with the secondary fluorescent antibody diluted at 1/100 for 1 hr. To visualize nuclei, slides were mounted with 10  $\mu$ L DAPI anti fading and visualized on microscope.

### Peripheral Blood Mononuclear Cells (PBMCs) Proliferation Assay

Human PBMCs from two different donors were isolated from heparinized blood by gradient centrifugation on a Ficoll solution (density 1.077 g/mL, Biochrom, Germany) at 400g for 20 min at room temperature. Stimulator PBMCs were treated by mitomycin at 25  $\mu$ g/mL for 30 min at 37°C (Sigma, Isle d'Abeau, France). Cell count and viability were assessed by trypan blue dye exclusion, and then used directly in mixed lymphocyte reaction (MLR).

Human MSCs were plated in triplicate at passage 2 onto U-bottomed 96-well plates at 10<sup>5</sup> cells/mL in 100  $\mu$ L of FCS- $\alpha$ MEM and were allowed to adhere to the plate for 1 to 2 hr. Human responder (10<sup>5</sup> PBMCs) and an equal number of stimulator PBMCs were added to wells in 100  $\mu$ L of RPMI 1640 (Invitrogen, Cergy, France) 10% inactivated FCS (Sigma, France). Cultures were incubated at 37°C in 5% CO<sub>2</sub>.

for 5 days and then pulsed with thymidine for the final 18 hr (1  $\mu$ Ci per well, Amersham Pharmacia). Thymidine incorporated in DNA was recovered on filters, counted, and expressed in count per minute. Donors are different for MSCs, responder or stimulator PBMCs.

### HLA-G Functional Assay

To test the ability of anti HLA-G blocking antibody (87G) to restore lymphocyte proliferation, the same procedures were followed with addition of 3  $\mu$ g/well of 87G antibody (concentration of 1 mg/mL), as previously described (20), on the first day of the MSC/MLR cultures (9 MSC donors). We performed an antibody titration for optimal concentration at 3, 6, 9, and 12  $\mu$ L per well. We confirmed that the dose of 3  $\mu$ L was optimal and the upper doses (6, 9, 12  $\mu$ L) did not increase the restoration.

### Statistical Analysis

The statistical analysis was performed with the statistical package for the social sciences (SPSS Institute, Chicago, IL) version 10. Statistical significance was calculated using *t* test analyses. Significance was set at  $P < 0.05$  (\*). All values were expressed as the mean and SEM (standard error of the mean). Twenty different MSC samples were used to evaluate the difference in the percentage of intracellular and membrane bound HLA-G in MSC/MLR. As well, lymphocyte proliferation in the presence or absence of HLA-G blocking antibody was compared from nine individually acquired MSC samples. Finally, the percentage of HLA-G positive MSC was determined through up to four successive in vitro passages from six independent samples.

## RESULTS

### MSC Characteristics

Primary human MSCs were generated from adherent fraction of bone marrow of healthy donors. At each passage the percentage of CD105, CD73 and the absence of the hematopoietic marker CD45 were analyzed. At the end of the second passage MSCs were negative for hematopoietic antigens CD45 (0.96%) and they expressed antigens known to be present in MSCs: CD73 (80.45  $\pm$  6.27%) and CD105 (97.98  $\pm$  5.5%). The immune suppressive properties of the studied populations were assayed in MLR. An inhibition of 56  $\pm$  4.5% of the PBMC proliferation was observed. We have also verified that MSCs retain their capacity to differentiate into adipocytic, os-

teoblastic and chondroblastic lineages, using adequate media (data not shown) as previously described (19).

### MSCs Expression of the HLA-G Molecule

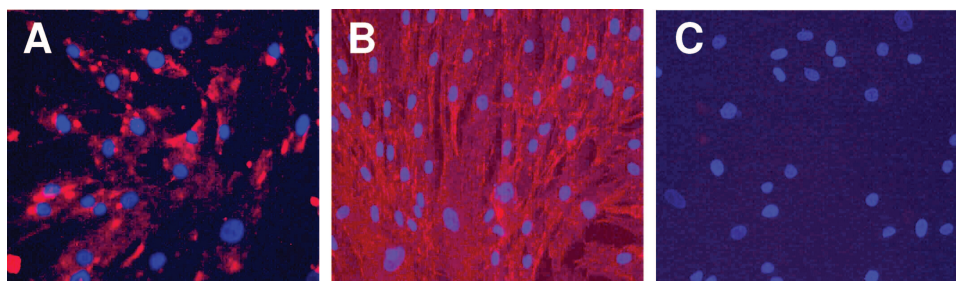
RT-PCR was used to analyze the expression of mRNA *hla-g* transcripts by MSCs. A total of 10 ng of total RNA of MSCs expressed 0.03  $\pm$  0.01 ng of HLA-G transcripts. To analyze the presence of HLA-G protein on MSCs, we used the specific 87G antibody, which recognizes the soluble and membrane-bound isoforms of the native molecule, and performed immunofluorescence. As shown in Figure 1A, HLA-G molecule was readily detectable on human MSCs. Figure 1B and 1C show positive (fetal tissue) (15) and negative control (isotype-matched mouse monoclonal antibodies), respectively.

Presence of HLA-G molecule on MSCs was quantified by flow cytometry using the specific MEMG/9 antibody on 16 different healthy donors. Extracellular and intracellular staining were used to study membrane-bound and intracytoplasmic HLA-G protein. A mean value of 52.4  $\pm$  3.6% of HLA-G-positive MSCs (Fig. 2A) was detected using intracellular staining. A low percentage of 13.7  $\pm$  1.3% HLA-G-positive MSCs (Fig. 2B) was detected using membrane-bound HLA-G staining. The immunostaining specificity was verified using lymphocytes from healthy donors as negative control; no extracellular or intracellular HLA-G molecule was detected (data not shown).

To quantify soluble HLA-G in culture supernatants, we performed an ELISA on 14 different MSC donors using the same MEMG/9 antibody. A mean value of 38.7  $\pm$  5.2 ng/mL HLA-G proteins was detected in MSC culture supernatants. The expression of HLA-G varied with the length of culture: successive passage showed a decrease of HLA-G positive cells (Fig. 3).

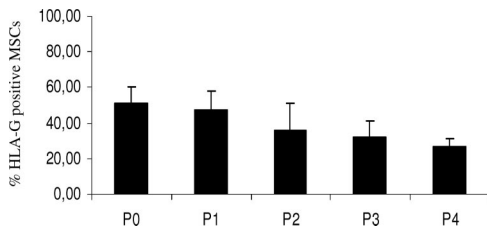
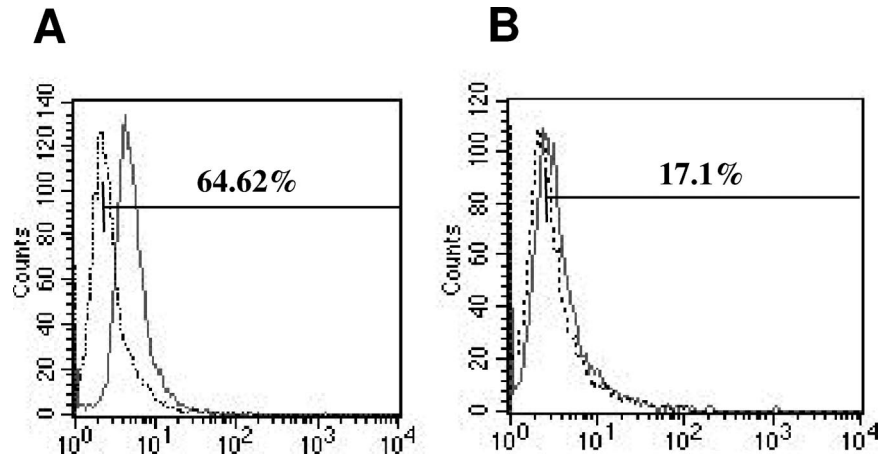
### Percentage of HLA-G-Positive MSCs and Culture Conditions

To test whether HLA-G production was dependant of the MSC culture conditions, a medium supplemented with platelet lysate obtained from PRP (PRP- $\alpha$ -MEM) was used (18) and compared with FCS-enriched medium (FCS- $\alpha$ -MEM). Using flow cytometry, we found no difference in the percentages of MSCs expressing CD105 or CD73 antigens. In both culture conditions, intracellular HLA-G is readily detectable in a large percentage of the MSC population (Table 1). HLA-G membrane-bound molecule was found on a low



**FIGURE 1.** Detection of HLA-G on MSCs with immunofluorescence. (A) MSCs and (B) fetal tissue (positive control) were incubated with 87G antibody then a secondary rhodamine-labeled antibody. (C) Negative control: MSCs incubated with the rhodamine-labeled secondary antibody. Red color shows intracytoplasmic HLA-G protein expressed by positive cells. Blue color evidenced DAPI staining.

**FIGURE 2.** Flow cytometry study of HLA-G-positive MSCs. HLA-G-positive MSCs were evidenced using the specific MEMG/9 antibody (dark line). An isotypic mAb was used as a control (dotted line). Cytometric traces show the level of fluorescence (x-axis) and the number of cells (y-axis). (A) HLA-G intracellular staining (64.62%), (B) HLA-G cell surface expression (17.1%). Experiments were performed using MSCs from 16 donors; shown are typical profiles from one donor. The percentage of HLA-G-positive cells was calculated after the subtraction of isotypic control by CELLquest software.



**FIGURE 3.** HLA-G production by MSCs decreases during passages. MSCs from six different donors were cultured in FCS- $\alpha$ -MEM. Mean percentages of intracellular HLA-G-positive MSC of six different donors at all passages were obtained by flow cytometry analysis using MEM/G9 specific antibody. Successive passages (P) showed a decrease in percentage of HLA-G-positive MSCs.

percentage of both MSC-cultures. The percentage of intracellular HLA-G-positive cells is significantly higher in PRP- $\alpha$ MEM than in FCS- $\alpha$ MEM cultures, with a p value of 0.023 (Table 1).

**HLA-G Molecule Is Implied in the Inhibition Induced by MSCs of Allogenic PBMC Proliferation**

To test the role of HLA-G expressed by MSCs on lymphocyte proliferation, we analyzed the modifications in HLA-G expression in MSC/MLR as compared with MSC cultures alone. Flow cytometry experiments performed on MSCs from four different donors showed no statistical change in the percentages of membrane-bound or intracytoplasmic HLA-G-positive MSCs in MSC/MLR when compared with MSC cultures alone (Fig. 4A). In the same experimental conditions, no HLA-G-positive PBMC was detected in MLR (Fig. 4B). Soluble HLA-G in the supernatant of the cultures was quantified with ELISA. Results showed that the level of soluble HLA-G in supernatants of MSC/MLR culture was (40.6 $\pm$ 7.1 ng/mL) and of MSC alone was (38.7 $\pm$ 5.2 ng/mL). The difference between both types of cultures was not statistically significant (P=0.89). The level of soluble HLA-G detected by ELISA could be linked to the high percentage of intra-cellular HLA-G positive cells detected by flow cytometry.

No change in HLA-G production could be detected upon allogenic stimulation (MSC/MLR). However, the role

**TABLE 1.** HLA-G production is dependant on culture conditions

	FCS- $\alpha$ -MEM	PRP- $\alpha$ -MEM	P value
Mean percentage intracellular staining (SEM)	45.03 $\pm$ 4.06	61.34 $\pm$ 4.61	P=0.023 (*)
Mean percentage extracellular staining (SEM)	15.32 $\pm$ 0.87	22.51 $\pm$ 2.39	P=0.4 (NS)

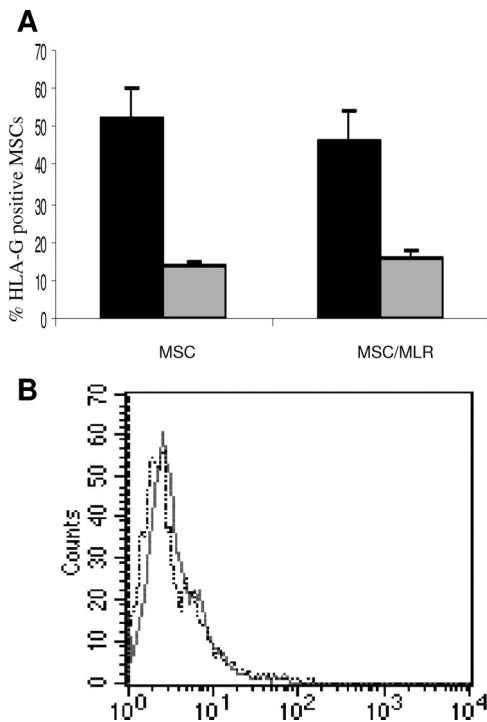
MSCs were cultured in FCS- $\alpha$ -MEM or PRP- $\alpha$ -MEM medium. Mean percentage of intracellular HLA-G-positive MSCs analysis by flow cytometry from different donors (n=6). \* P<0.023.

of HLA-G as a possible mediator of MSC immunosuppressive effect cannot be excluded. To test the functional role of the HLA-G protein expressed by MSCs, the specific neutralizing antibody 87G was used in MSC/MLR (MSC/MLR+87G). Allogenic PBMC proliferation was taken as a reference of 100% proliferation. The mean percentage of PBMC proliferation was significantly greater in MSC/MLR+87 G (65.9 $\pm$ 7%) when compared with MSC/MLR (44 $\pm$ 4.5%, P=0.01, Fig. 5). The MSC induced inhibition of allogenic lymphocyte proliferation was alleviated by 87G antibody blockade with a mean value of 35.5%. These results suggest that HLA-G could be a novel T-cell inhibitory effector produced by human MSCs.

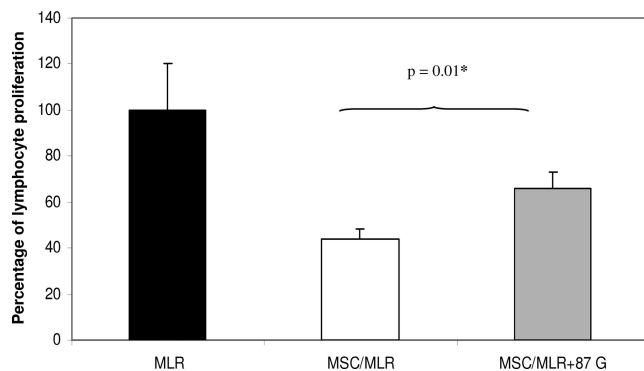
**DISCUSSION**

In this work, we studied the expression of the immunotolerogenic molecule HLA-G by MSCs and studied its implication toward the MSC immunosuppressive effects. We analyzed HLA-G expression at messenger RNA and protein level. This is the first report showing that human adult bone marrow MSCs express HLA-G as detected by RT-PCR, immunofluorescence, flow cytometry and ELISA.

Our results confirm the presence of *hla-g* transcripts in adult MSCs (21, 22). Gotherstrom et al. (22) have already reported detection of HLA-G protein by western blot in fetal MSCs, however not in adult MSCs. This later result differs from our report. This could be due to the differences in experimental design or culture conditions. Indeed in vitro we demonstrated (Table 1) that MSC culture in FCS- $\alpha$ MEM or



**FIGURE 4.** The percentage of HLA-G-positive MSCs is not modified in MSC/MLR. HLA-G-positive cells were detected by flow cytometry using MEMG/9 antibody. (A) Mean percentage of intracellular (black histogram) and membrane-bound HLA-G (gray histogram) in MSC/MLR as compared with MSCs alone (n=4). No statistical change of HLA-G expression in MSC/MLR was observed. (B) Negative control: Representative flow cytometry data of the percentage of intracellular HLA-G on allogeneic PBMCs alone (dotted line) and after 5-day coculture (dark line).



**FIGURE 5.** MSC tolerogenic effects are mediated in vitro via secretion of HLA-G. Tritiated thymidine (<sup>3</sup>HTdR) incorporation was measured as a marker of PBMC proliferation and data were presented as percent change in incorporated <sup>3</sup>HTdR in presence or absence of 87G (PBMC+PBMC in absence of MSCs=100%). The <sup>3</sup>HTdR incorporation test indicates that addition of MSCs significantly inhibited the proliferative response. The inhibition was partially restored in presence of 87G HLA-G blocking Antibody. Percent of restoration was calculated as follows: (MSC/MLR+87G-MSC/MLR)/(MLR-MSC/MLR)×100. Results represent the Mean±SEM of 9 separate experiments performed in triplicate.

in PRP-αMEM could induce significant differences in the percentage of HLA-G-positive MSCs.

Human MSCs are multipotent unspecialized cells with a capacity for self-renewal and differentiation into multiple cell lineages. These stem cell characteristics could explain the expression of HLA-G molecules, which is found on numerous fetal tissues such as cytotrophoblast, hematopoietic progenitors (23) and fetal MSCs (22). This is in accordance with our results. Indeed, a decrease of the number of HLA-G-positive cells was observed over time using intracellular staining. Whether the observed decrease is linked with senescence of the cell remains to be studied (24).

It has been shown that co-culture of nonhuman primate or human MSCs with peripheral blood lymphocytes from allogeneic donors did not stimulate their proliferation in vitro (6, 25). This effect could be mediated through constitutive HLA-G expression by MSCs. Hereby, we demonstrated that, with two different antibodies (MEMG/9 and 87G), MSCs expressed constitutively the strong immune-inhibitory HLA-G molecule, which could explain the escape of immune recognition of MSCs by allogeneic peripheral blood lymphocytes.

MSCs can exert profound immunosuppression by inhibiting T-cell proliferation in response to various stimuli in vitro (10, 25). In vivo, injection of MSCs leads to prolonged allograft survival in non-human primate (6) and murine models (26). A possible mechanism to explain the MSC inhibitory effect was a veto cell-like activity (27). Indeed, in vitro veto cells inhibit CTL function against their own antigens, but not against third-party allogeneic cells. This contrasts with reported inhibition of allogeneic lymphocytes cytotoxicity and proliferation by MSCs (6, 10). A veto cell like activity is in agreement with the extensive data demonstrating engraftment and detection of MSCs (17, 28, 29) in various species. The constitutive expression of HLA-G by MSCs could support the veto cell-like activity, which plays an important role in their low immunogenicity.

It has been shown that human MSCs in cultures can mediate suppression of lymphocytes proliferation by several secreted factors such as hepatocyte growth factor, transforming growth factor-β (10) and IL-10 (30). However blocking these factors with antibodies does not completely reverse the MSC-mediated immunosuppression. Recently, the role of tryptophan catabolizing enzyme IDO has been suggested to play a role in the MSC-mediated immunosuppression (13). IDO is not constitutively expressed in MSCs but depends on their activation by lymphocytes or exogenous IFN-γ. This could explain the inhibition of allogeneic lymphocytes but could not explain the low immunogenicity and the engraftment of MSCs in allogeneic recipients. More recently, PGE2 which modulates a wide variety of immune cell functions in vitro (31), has been suggested to play a role in the MSC-mediated immunosuppression (30). However, using PGE2 synthesis inhibitors negated 70% of MSC's inhibitory effect (9). The overall data suggest the possibility of other anticipated molecules.

MSCs inhibit CD4+ T cells proliferation and DC maturation. They induce regulatory immunosuppressive cells, cell cycle arrest, and TH2 cytokines profile. It has been demonstrated that HLA-G exerts the same immunosuppressive effects (15), which could indicate that HLA-G expressed by MSCs may be responsible for much of human MSC-mediated

immunomodulatory effects. We studied the implication of HLA-G in the inhibitory effect mediated by MSCs. We evaluated the percentage of HLA-G-positive cell expression (flow cytometry) and the level of HLA-G in culture supernatant (ELISA) in MSC/MLR compared with cultured MSCs alone. We could not detect a statistically significant difference in HLA-G expression in both assays. However, this does not exclude the effective role of HLA-G in MSC-mediated suppressive effect and could indicate an optimal operational concentration of HLA-G expressed by MSCs. This is supported by our results: we have shown that with a neutralizing HLA-G antibody it was possible to partly counterbalance MSC-immunosuppressive effects. Hereby we demonstrated that HLA-G is a tolerogenic molecule constitutively expressed in cultured MSCs alone or in MSC/MLR.

Restoration of lymphocytes proliferation was consistently demonstrated in MSC/MLR using allogenic PBMCs from different donors. The significant restoration of lymphocytes proliferation in presence of HLA-G neutralizing antibody suggests that HLA-G may be partly responsible for human MSC-mediated immunomodulatory effects in vitro.

The constitutive expression of HLA-G protein, along with other reported inhibitory factors expressed by human MSCs, corroborates the effectiveness of MSCs in the management of GVHD (2). Our results showing that MSCs can limit lymphocytes response via HLA-G secretion could offer one explanation for this beneficial effect. It has been demonstrated that HLA-G secretion was associated with a better heart and liver graft acceptance (16, 32). A careful evaluation of the harmful immunomodulatory effects of MSCs is needed with regard to minimal residual disease and tumor escape in vivo. MSCs open new insights in the prevention and treatment of graft rejection in tissue and organ transplantation.

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### REFERENCES

- Lazarus HM, Koc ON, Devine SM, et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. *Biol Blood Marrow Transplant* 2005; 11: 389.
- Le Blanc K, Rasmusson I, Sundberg B, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004; 363: 1439.
- Ringden O, Uzunel M, Rasmusson I, et al. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* 2006; 81: 1390.
- Koc ON, Gerson SL, Cooper BW, et al. Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. *J Clin Oncol* 2000; 18: 307.
- Almeida-Porada G, Flake AW, Glimp HA, Zanjani ED. Cotransplantation of stroma results in enhancement of engraftment and early expression of donor hematopoietic stem cells in utero. *Exp Hematol* 1999; 27: 1569.
- Bartholomew A, Sturgeon C, Siatskas M, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol* 2002; 30: 42.
- Beyth S, Borovsky Z, Mevorach D, et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood* 2005; 105: 2214.
- Jiang XX, Zhang Y, Liu B, et al. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* 2005; 105: 4120.
- Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005; 105: 1815.
- Di Nicola M, Carlo-Stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002; 99: 3838.
- Plumas J, Chaperot L, Richard MJ, Molens JP, Bensa JC, Favrot MC. Mesenchymal stem cells induce apoptosis of activated T cells. *Leukemia* 2005; 19: 1597.
- Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood* 2005; 105: 2821.
- Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 2004; 103: 4619.
- Rouas-Freiss N, LeMaout J, Moreau P, Dausset J, Carosella ED. HLA-G in transplantation: a relevant molecule for inhibition of graft rejection? *Am J Transplant* 2003; 3: 11.
- Carosella ED, Moreau P, LeMaout J, LeDiscorde M, Dausset J, Rouas-Freiss N. HLA-G molecules: From maternal-fetal tolerance to tissue acceptance. *Adv Immunol* 2003; 81: 199.
- Lila N, Carpentier A, Amrein C, Khalil-Daher I, Dausset J, Carosella ED. Implication of HLA-G molecule in heart-graft acceptance. *Lancet* 2000; 355: 2138.
- Fouillard L, Bensidhoum M, Bories D, et al. Engraftment of allogeneic mesenchymal stem cells in the bone marrow of a patient with severe idiopathic aplastic anemia improves stroma. *Leukemia* 2003; 17: 474.
- Doucet C, Ernou I, Zhang Y, et al. Platelet lysates promote mesenchymal stem cell expansion: A safety substitute for animal serum in cell-based therapy applications. *J Cell Physiol* 2005; 205: 228.
- Francois S, Bensidhoum M, Mouiseddine M, et al. Local irradiation not only induces homing of human mesenchymal stem cells at exposed sites but promotes their widespread engraftment to multiple organs: A study of their quantitative distribution after irradiation damage. *Stem Cells* 2006; 24: 1020.
- Lila N, Rouas-Freiss N, Dausset J, Carpentier A, Carosella ED. Soluble HLA-G protein secreted by allo-specific CD4+ T cells suppresses the allo-proliferative response: A CD4+ T cell regulatory mechanism. *Proc Natl Acad Sci U S A* 2001; 98: 12150.
- Nasef A, Chapel C, Mazurier C, et al. Identification of IL-10 and TGF- $\beta$  transcripts involved in inhibiting T Lymphocyte proliferation during cell contact with human mesenchymal stem cells. *Gene Expression*, in press.
- Gotherstrom C, West A, Liden J, Uzunel M, Lahesmaa R, Le Blanc K. Difference in gene expression between human fetal liver and adult bone marrow mesenchymal stem cells. *Haematologica* 2005; 90: 1017.
- Menier C, Rabreau M, Challier JC, LeDiscorde M, Carosella ED, Rouas-Freiss N. Erythroblasts secrete the nonclassical HLA-G molecule from primitive to definitive hematopoiesis. *Blood* 2004; 104: 3153.
- Bonab MM, Alimoghaddam K, Talebian F, Ghaffari SH, Ghavamzadeh A, Nikbin B. Aging of mesenchymal stem cell in vitro. *BMC Cell Biol* 2006; 7: 14.
- Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol* 2003; 57: 11.
- Chung NG, Jeong DC, Park SJ, et al. Cotransplantation of marrow stromal cells may prevent lethal graft-versus-host disease in major histocompatibility complex mismatched murine hematopoietic stem cell transplantation. *Int J Hematol* 2004; 80: 370.
- Potian JA, Aviv H, Ponzio NM, Harrison JS, Rameshwar P. Veto-like activity of mesenchymal stem cells: Functional discrimination between cellular responses to alloantigens and recall antigens. *J Immunol* 2003; 171: 3426.

28. Chapel A, Bertho JM, Bensidhoum M, et al. Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrome. *J Gene Med* 2003; 5: 1028.
29. Bensidhoum M, Chapel A, Francois S, et al. Homing of in vitro expanded Stro-1- or Stro-1+ human mesenchymal stem cells into the NOD/SCID mouse and their role in supporting human CD34 cell engraftment. *Blood* 2004; 103: 3313.
30. Rasmuson I, Ringdén O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms. *Exp Cell Res* 2005; 15: 33.
31. Harris SG, Padilla J, Koumas L, Ray D, Phipps RP. Prostaglandins as modulators of immunity. *Trends Immunol* 2002; 23: 144.
32. Creput C, Durrbach A, Charpentier B, Carosella ED, Rouas-Freiss N. [HLA-G: immunoregulatory molecule involved in allograft acceptance]. *Nephrologie* 2003; 24: 451.