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# A Decreased Positivity for CD90 on Human Mesenchymal Stromal Cells (MSCs) Is Associated with a Loss of Immunosuppressive Activity by MSCs

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**Biologic and clinical interest in human mesenchymal stromal cells (hMSC) has risen over the last years, mainly due to their immunosuppressive properties. In this study, we investigated the basis of immunomodulant possible variability using hMSC from different sources (amniotic membrane, chorion, and bone marrow from either healthy subjects or patients with hematological malignancies, HM) and having discordant positivity for several immunological markers. The CD90+ hMSC reduced lymphoproliferative response in phytohemagglutinin (PHA) activated peripheral blood mononuclear cells (PBMC) via sHLA-G and IL-10 up-modulation. On the contrary, hMSC showing a significantly lower expression for CD90 antigen, elicited a lymphoproliferative allogeneic response in PHA/PBMCs without any increase in soluble HLA-G and IL-10 levels. These data seems to suggest that CD90 molecule may be considered a novel predictive marker for hMSC inhibitory ability, and might cooperate with HLA-G molecule in regulating suppressive versus stimulatory properties of hMSC. These results may have clinical implication in either transplantation or in regenerative medicine fields.** © 2008 Clinical Cytometry Society

**Key terms: mesenchymal stromal cells; phenotypic markers on MSCs; CD90 antigen expression; in vitro T-cell response**

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Several studies indicate that human mesenchymal stromal cells (hMSCs) have unique immunological properties, because they are not immunogenic, they do not simulate alloreactivity, and they escape cytotoxic T and natural killer cell lysis activity (1,2), making them suitable for cellular therapy. Clinical data have shown that hMSCs could reduce the incidence and severity of graft-vs.-host-disease (GVHD) after allogeneic stem cell transplantation, and may be beneficial for the treatment of autoimmune diseases (3,4). Phenotypically, hMSC are identified by the absence of CD45, CD34, and other hematopoietic associated markers, whereas they are positive for CD105, CD73, CD90, CD59, CD29, and MHC-class I antigens and, in a lesser extent, for CD106, CD44, CD166, CD10.

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HLA-G antigens are nonclassic MHC class I molecules characterized by a limited polymorphism and a splicing mechanism that regulates membrane and soluble isoforms (HLA-G1-G7). Membrane and soluble HLA-G antigens (sHLA-G) showed tolerogenic functions against innate and adaptative cellular responses affecting NK and CD8+ T cells cytotoxicity and CD4+ T cells activities. Interleukin 10 (IL-10) is considered one of the main up-modulators of sHLA-G production (5,6) creating a feed-back loop interaction.

In a previous paper, we demonstrated the functional role for soluble HLA-G antigens in hMSC mediated immunomodulation (6). Our results showed a significant correlation between the presence of increased levels of sHLA-G and IL-10 in the MSC/peripheral blood mononuclear cell (PBMC)/phytohemagglutinin (PHA) culture supernatants and the lymphoproliferative inhibition. The data obtained demonstrated a significant increase in sHLA-G concentration in the culture supernatants of PHA-activated PBMC in the presence of MSCs able to mediate immune modulation. A significant negative correlation was found between the lymphoproliferative residual response and increases in sHLA-G levels in MSC/PBMC/PHA cocultures. On the other hand, sHLA-G concentrations in MSC/PBMC/PHA culture supernatants without lymphoproliferative inhibition were similar to the basal levels observed in PBMC/PHA cultures. Neutralizing experiments performed with monoclonal antibodies directed against HLA-G and IL-10 molecules confirmed the inhibitory ability of sHLA-G antigens. Furthermore, exogenous IL-10 induced sHLA-G molecule secretion by MSCs alone in a polymorphic way. The same results were obtained by Selmani et al. (7). They documented that MSC culture supernatants previously incubated with anti-HLA-G antibody failed to inhibit lymphocytes response in a mixed lymphocyte reaction.

However, we observed a variability in both lymphoproliferative inhibition and sHLA-G production among hMSCs with a different origin towards the same PBMCs response to PHA, and we hypothesized either the loss of hMSC inhibitory ability in relation to the increased *in vitro* culture passages or the existence of different hMSC cell types responsible for sHLA-G production. In fact, even though *in vitro* expanded hMSCs have been phenotypically defined positive for CD105, CD90, and CD73, we recently demonstrated the possible down-regulation and loss of Thy-1 (CD90) antigen expression on bone marrow (BM) derived hMSCs from haematological malignancies (HM) isolated and cultured under *in vitro* angiogenic conditions that resulted associated with high *in vitro* proliferative rate and other immunophenotypic variability (8,9).

On the basis of these findings, in this study we investigated the *in vitro* immunomodulatory ability of CD90-negative hMSCs subset from different sources, in the inhibition of PBMCs response to PHA, in relation to the production of sHLA-G and IL-10.

## METHODS

### Isolation of Human hMSCs from Different Sources

BM-derived human MSC (BM-hMSC) from five patients with new HM (acute lymphoblastic leukaemia no. 2 and myelodisplastic syndromes no. 3; mean age 45 ± 5 years, 2 males and 3 females) were isolated and cultured in parallel either in a DMEM-LG or in a medium for endothelial cell differentiation (EBM medium with EGM-2 SingleQuots; BioWhittaker, Cambrex Bio Science Walkersville) as previously reported (8). Amniotic membrane human MSCs from human term placenta (mean age: 33 years) was obtained from four healthy donors after informed consent. The amnion (AM-hMSC) and chorion (Cho-hMSC) were mechanically separated. Cell suspensions were plated in culture flasks and cultured as previously described (8,9). The PBMCs used in the various experiments were obtained from healthy donors after informed consent and as previously described (6). As controls, human BM samples were obtained from 10 healthy subjects (NS) (mean age 43 ± 7, 3 males/7 females) after informed consent.

### hMSCs/PBMC Cocultures

$8 \times 10^3$ hMSCs from the different sources were cocultured in quadruplicate in a 96-well cell culture cluster (Corning NY) in DMEM-LG, as previously described (6), in triplicate alone, with purified PHA 1 μg/ml (Murex, Dartford, UK) or supplemented with 20 ng/mL of recombinant IL-10 (PeproTech, Rocky Hill, NJ). MSCs/PBMC culture supernatants were collected and stored at −20°C pending ELISA analysis of sHLA-G and IL-10 levels (10).

### sHLA-G and IL-10 Detection by ELISA Assay

sHLA-G antigen concentrations were investigated in the different culture supernatants by enzyme immunosorbent assay, as reported in the Essen Workshop on sHLA-G quantification (10). As a control, PBMCs and hMSCs were cultured alone and with or without PHA. IL-10 concentrations were determined in triplicate of undiluted samples using the commercially available Human IL-10 BioSource Immunoassay Kit (Human IL-10 US; BioSource, Camarillo, CA) with a detection limit of 0.2 pg/ml.

The lymphoproliferative response was measured after 72 h culture by overnight incubation with 1 μCi/well of (methyl-$^3$H) Thymidine ($3 \times 7 \times 10^4$ Bq) (Amersham, Buckinghamshire, UK).

### Flow Cytometric Analysis of hMSCs

Primary culture-expanded hMSCs, isolated from different sources, were analyzed using a 4-color flowcytometric protocol. The expression of the different hMSC surface markers and in particular of CD90 (Thy-1) was analyzed at the first culture passage as previously described (8,9). A Facscalibur flow cytometer equipped with a four color option was used (Becton Dickinson).

Table 1
*Functional and Immunophenotypic Characteristics of MSCs from Different Sources*

| hMSC phenotypic and functional parameters | Immunosuppressive hMSC ($n$ = 12 samples) A | Stimulatory hMSC ($n$ = 11 samples) B | Statistical significance A vs. B ($P$ value) |
|---|---|---|---|
| Lymphoproliferative response[a] Mean% (range) | 56.6 ± 20 range (11–94) | 128.1 ± 19 range (102–163) | $P$ = 0.005 |
| Percentage of positivity for CD90 (Mean% range) | 93.2 ± 6 range (85–99) | 56.3 ± 19 range (32–80) | $P$ = 0.004 |
| [b]MFI for CD90 (calculated as peak channel) | 700 ± 71 range (597–789)<br>P/N ratio 4.6 ± 1.7 | 564 ± 125 range (447–815)<br>P/N ratio 2.7 ± 1.5 | $P$ = 0.05,<br>$P$ = 0.01 |
| CD90[b] MFI (calculated as mean values) | 717 ± 65 range (611–848)<br>P/N ratio 3.6 ± 0.5 | 612 ± 52 range (543–704)<br>P/N ratio 3.1 ± 0.6 | N.S. |
| CD90[b] MFI (calculated as median) | 701 ± 75 range (588–844)<br>P/N ratio 3.8 ± 0.6 | 601 ± 59 range (514–718)<br>P/N ratio 3.2 ± 0.7 | |
| CD90[c] NFI (calculated as net fluorescence for the median values) | 526 ± 69 range (448–609) | 320 ± 84 range (239–295) | $P$ = 0.001 |
| sHLA-G (ng/ml) | 8.4 ± 3 range (4–15) | 1.6 ± 2 range (0–4) | $P$ = 0.002 |
| IL-10 (pg/ml) | 91.7 ± 20 range (60–117) | 42.6 ± 4 range (40–45) | $P$ = 0.002 |

A. Data are expressed as mean percentage ± SD in the two groups (immunosuppresive vs. stimulatory MSCs).

B. Data are expressed as mean percentage ± SD in the two groups (immunosuppresive vs. stimulatory MSCs). Proliferation activity of PBL + PHA are considered as 100% and used as control.

[a]Lymphoproliferative response: compared to the basal proliferation detected in the PHA-activated PBMC culture (100%).

[b]MFI = mean fluorescence intensity of the positive events; for this purpose, the peak channel, median, and geometric mean were calculated for each sample and isotypic control.

[c]Net fluorescence intensity = (NFI), which was obtained by subtracting the MFI of the isotypic control from MFI of the sample (Data are expressed as mean percentage of positive cells. % (range)= percentage and range of positive cells).

P/N = ratio between MFI of positive cells (P) and MFI of isotypic control (N).

To optimize the analysis, nonviable cultured MSC were identified by uptake of 7-amino-actinomycin D (7-AAD) (Molecular Probes, Leiden, The Netherlands). The following panel of MoAbs was used: anti-CD45 (clone 2D1, APC), CD11c (S-HCL-3, PE), CD14 (MΦP9, PE), CD31 (WM-59, PE), CD36 (NL07, FITC), CD90 (5E10, FITC), CD59 (P282-H19, PE), CD184 (12G5, PE), CD166 (3A6, PE) (Becton-Dickinson-PharMingen, CA); anti-CD106, (1G1b1- PE) (Southern Biotechnology Associates); HLA-DR (Tu36, FITC,), CD 105 (SN6, PE), CD44 (MEM 85, FITC), HLA-ABC (Tu149, FITC), CD80 (MEM-233, PE), CD29 (MEM101A, PE) (Caltag Laboratories, CA), CD31 (CBL468F, FITC, Cymbus Biotechnology); CD34 (HPCA-2, Bdis, APC or PE-Cy7). The concentrations of McAbs were titrated towards a saturating titer, as assessed by flow cytometry analyses performed on MSCs obtained from healthy individuals. Negative controls with isotype matched nonrelevant MoAbs (mouse IgG1, IgG2a, IgM) were tested in all experiments. Cell positivity for the various immunological markers was expressed as percentage of positive cells.

Cell positivity for CD90 was expressed as follows:

1. percentage of positivity (using the standard marker approach).
2. Mean fluorescence intensity-linear units (MFI) of the positive events.
3. Net fluorescence intensity (NFI), which was obtained by subtracting the MFI of the negative control from MFI of the sample; for this purpose, the peak channel, median, and mean were calculated for each sample and isotypic control.
4. Positive/negative (P/N) ratio: ratio between MFI of sample' cells and that of isotypic control (Table 1).
5. Fluorescence minus one (FMO). This control staining uses all reagents except for the one of interest (CD90 in our samples) (11).

Autofluorescence, FMO, and isotypic control were used to establish the boundary between negative and positive fluorescent regions, and the results were expressed as the percentage of cells falling above the negative region for each antibody. When negative ad positive regions were separated, MFI and percentage of positivity was calculated on the positive region only. In case that only one region was present, MFI was calculated on the whole cell population.

### Statistical Analysis

By using the Pearson's test and nonparametric statistics (Wilcoxon test), we first compared the percentage of positivity for the various phenotypic markers, and in particular for CD90, expressed by hMSC from different sources, and IL-10, sHLA-G products levels. The hMSC immunosuppressive capacity was also compared.

## RESULTS

In this study, a multicolor flow cytometric protocol was used to characterize cultured hMSCs. It was based on the exclusion of CD45+ (known to be an hematopoi-

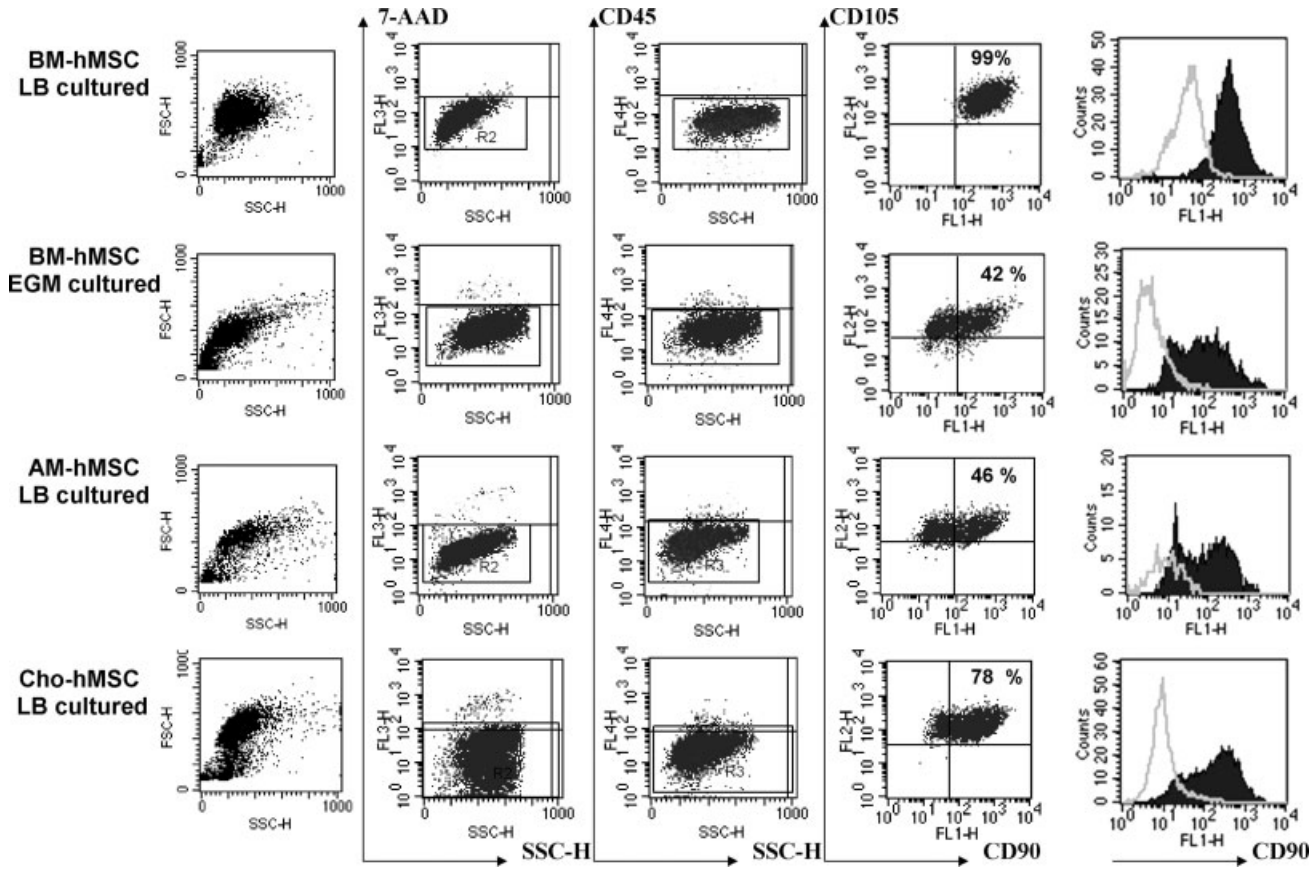


FIG. 1. A representative example of our multicolor flow cytometric protocol used for the assessment of BM-derived hMSCs is shown. The expression of CD90 and CD105 surface markers in gated CD45-7-AAD-hMSCs from different sources is reported. Briefly, the protocol was based on the use of 7-AAD (a nucleic acid staining compound, used for the recognition of dead and apoptotic cells) and CD45 (known to be an hematopoietic marker) in combination with several MSCs—associated lineage immunological markers. 7-AADneg (gate R2)/CD45neg (gate R3) MSCs were evaluated for CD90 and CD105 positivity. The percentage of double stained CD90/CD105 hMSCs is also shown. The histogram plot for CD90 antigen is given, in comparison with that of the isotypic control. Interestingly, AM-hMSC and Cho-hMSC displayed a significant lower proportion of viable CD90+/CD45neg MSCs. Culture conditions induced significant changes in CD90 antigen expression by hMSCs. FMO (fluorescence minus one) and isotypic control were used to establish the boundary between negative and positive fluorescent regions. LB = completed culture medium without cytokines. EGM = completed culture medium additioned with angiogenic cytokines.

etic marker) and 7-AAD+ (a nucleic acid staining compound, used for the recognition of dead and apoptotic cells) events. The mean percentage of 7AAD+ cells was comparable in patients and controls (9% ± 3 SD vs. 8% ± 3 SD). As far as the detection of contaminant hematopoietic cells within the stromal layers is concerned, the mean number of CD45+ cells observed in HM-MSCs at the first passage of culture (P1) was higher (5% ± 12 SD) than that of normal donors (1% ± 1 SD). Stromal layers with a % of CD45+ lower than 1% were used for the immunosuppressive tests.

As illustrated in Figure 1, more than 95% of BM-derived hMSCs that showed negativity for CD45 and 7-AAD resulted CD90+ (range 95–100%) in normal donors, whereas HM-MSC showed a much lower CD90 antigen expression (range from 32–75% within the examined samples) and, in a lesser extent, for CD105 (range from 56–81%), when isolated and cultured in the presence of angiogenic cytokines (Fig. 1). Interestingly, AM-hMSC and Cho-hMSC displayed constitutively the same immunophenotypic peculiarity (Fig. 1), even if cultured in a DMEM-LG completed classical basal medium without angiogenic cytokines (mean CD90 expression values from all examined AM-hMSC: range 38–96% and Cho-hMSC: range 63–91%). As reported in Table 1, hMSC, displaying a significant lower expression of CD90 (mean values from 3 AM-MSC, 5 HM-MSC treated with angiogenic cytokines, 3 Cho-MSC: 56.3 ± 19%; range 32–80) failed to induce immunosuppression when compared to hMSC with higher CD90 expression exerting an immunosuppressive function (1Cho-MSC, 1 AM-MSC, 10 NS-MSC: 93.2 ± 6%; range 85–99; $P = 0.004$). Interestingly, the CD90 expression could vary in relation to the culture passages and accordingly to the *in vitro* hMSC aging.

In contrast, the difference in CD105 antigen expression on hMSC from the various subject categories and sources was not significant.

Table 1 also shows the MFI, NFI, P/N ratio for CD90 staining, and FMO control staining.

Table 1 further showed that hMSC with a lower percentage of CD90 positivity displayed a diminished immunosuppressive cell activity on lymphocyte proliferation, even though CD90 low hMSCs conserved their clonogenic properties, and osteogenic, chondrogenic, and adipogenic differentiation capabilities, thus confirming their genuine MSC nature (data not shown) (1,8). Furthermore, a significant lower production of sHLA-G and IL-10 was evident in hMSC showing a loss of CD90 antigen expression (Table 1). Significant differences were observed in terms of NFI (net fluorescence intensity) between immunosuppressive CD90+ hMSC and stimulatory CD90low hMSC, while either culture condition or the increasing culture passages did not affect the immunodulatory properties of MSCs. An increasing autofluorescence of cultured hMSC after 4–5 passages was noticed. Interestingly, MFI values did not differ significantly between the two groups (immunostimulatory and suppressive MSCs); however these data refer to the positive events only, because in most cases a clear cut population of CD90 negative cells were detected.

The statistical analysis of the data revealed a negative correlation between CD90 expression and lymphoproliferative response to PHA activation (Pearson test $r = -0.73$) and a positive correlation between lower CD90 expression and decreased sHLA-G and IL-10 levels (Pearson test: $P = 0.51$ and $P = 0.72$, respectively).

FMO controls did not differ significantly from data obtained using the isotypic control.

MFI for FMO resulted 194 ± 25 SD (calculated as a mean), 182 ± 26 SD (calculated as median), 126 ± 36 SD (calculated as a peak channel), and 171 ± 27 SD (calculated as a geometric mean).

## DISCUSSION

The immunophenotypic profile of hMSC has been the object of several reports. However, in contrast with hematopoietic cells, the immunophenotype of *ex vivo* expanded hMSCs is far from being completely standardized and phenotypic characterization has been confined to single or dual staining analysis. Based on these considerations, we considered of great interest to develop guidelines to define of a standardized multicolor cytofluorimetric protocol which could allow an accurate detection and phenotypic characterization of hMSCs cultured in standard long-term culture conditions suitable for clinical application. In a previous paper, we provided evidence that multiparametric flow cytometry is essential for the establishment of a standardized protocol which allows to fully characterize hMSC and further identify various hMSC subsets and aberrant phenotypes (9). In the same paper, it was shown that HM patients may exhibit alteration in the expression of CD105, CD90, CD184, and HLA-DR molecules on hMSC.

In the present study, we investigated the basis of immunomodulant possible variability using hMSC from different sources (amniotic membrane, chorion, and BM) and having discordant positivity for several immunological markers. The data were correlated with IL-10 and sHLA-G production, taking into account the pivotal role played by soluble factors produced in the MSC/PBMC/PHA coculture microenvironment in the inhibition of T lymphocyte response. Among others, sHLA-G proved to be one of these molecules (6,12). Our previous results demonstrated a functional role for soluble HLA-G molecules in hMSC-mediated inhibition of the lymphoproliferative response to PHA, because neutralizing experiments showed that the addition of anti-HLA-G MoAb to MSC/PBMC/PHA combinations significantly restored the lymphoproliferative response (6). However, the percentages of restored lymphoproliferation ranged in the previous experiments from 69% to 80%, reinforcing the suggestion that other molecules such as tryptophan catabolites generated by indoleamine 2,3-dioxigenase (IDO) activity (13) could contribute to hMSC immune modulation mechanisms. Because we observed variability in both lymphoproliferative inhibition and sHLA-G production among hMSCs of different origins towards the same PBMC response, we hypothesized the involvement of other mechanisms in the complex immunomodulant pathway mediated by hMSC. Accordingly to our and other (14) previous studies, using multicolor flow cytometry and *in situ* immunocytochemical analysis, the inhibitory ability of hMSC seems to be related to the increased *in vitro* culture passages as well as by discordant positivity for HLA-DR molecule (9). In particular, in this study we examined the role played by different hMSC phenotypic subsets isolated from different tissue sources in inducing both lymphoproliferative inhibition and sHLA-G production.

In contrast with Soncini et al. (15,16) and in agreement with other authors (17–19), our data provide evidence that hMSC from human amniotic membrane, chorion, and BM from HM (treated with angiogenic cytokines) showing a significant decrease in CD90 surface molecule expression can elicit a lymphoproliferative allogeneic response in MSC/PBMC/PHA cultures without increase in culture supernatants of sHLA-G and IL-10 concentrations that resulted similar to the basal levels observed in PBMC/PHA culture controls.

Based on these observations, it can be stated that hMSCs with a depressed expression of CD90 antigens exhibit a diminished immunosuppressive cell activity on T cell proliferation.

Because we hypothesized the existence of possible different cell types responsible for sHLA-G production in the MSC/PBMC/PHA microenvironment, these results strengthen the relationship between hMSC activation, sHLA-G production, and Thy-1 expression, suggesting the Thy-1 molecule may be considered a marker implicated in the hMSC inhibitory ability.

The absence of CD90 molecules may be physiological in some embryonic tissues, and may occur in some pathological conditions, other than induced by *in vitro* culture conditions and/or cell aging. However, irrespective of the cause for this phenotypic abnormality, it can be associated with a progressive decrease in hMSC inhibitory function. These data seem to suggest that CD90 molecule

may be considered a novel predictive marker for hMSC inhibitory ability, and might cooperate with HLA-G molecule in regulating suppressive versus stimulatory properties of hMSC. However, further studies are needed to highlight the molecular signalling pathway that regulates HLA-G production-mediated immunosuppression.

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