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Mesenchymal stem cells expanded in human platelet lysate display a decreased inhibitory capacity on T- and NK-cell proliferation and function

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The use of fetal bovine serum (FBS) for the culture and expansion of mesenchymal stromal cells (MSCs) limits their possible clinical applications. Although some recent studies recommended substituting FBS with human platelet lysate (HPL) for the expansion of MSCs for clinical use, the functional capacity of the expanded cells has only been partially explored. 10% FBS and two other commercial FBS-containing media (MesenCult and MesenPro) were compared with 10% HPL-containing medium for their ability to support MSCs expansion and immunomodulation. We demonstrate that HPL sustained MSC proliferation and expansion in vitro. However, the cumulative cell numbers recovered were comparable with those obtained in MesenPro medium. Moreover, we show that HPL alters the expression of some relevant MSC surface molecules, namely the DNAM-1 ligands PVR and Nectin-2, the NKG2D ligand ULBP3, the adhesion molecules CD49d and αvβ3 and the fibroblast-associated protein. In addition, MSCs cultured in HPL displayed impaired inhibitory capacity on T-cell proliferation to alloantigen and NK-cell proliferation and cytotoxicity. Finally, they showed decreased constitutive PGE2 production while IL-6, IL-8 and RANTES secretion were upregulated. These results imply some limitations in the use of HPL for the expansion of MSCs to be used as immunomodulators in clinical applications.

Key words: Graft-versus-host disease · Hematopoietic stem cell transplantation · Human platelet lysate · Mesenchymal stromal cells · NK cells

Introduction

Human mesenchymal stromal cells (MSCs) have attracted major attention for their possible clinical use [1]. In addition to their tissue regenerative capacity, they have immune-modulatory properties for which they are used in the prophylaxis and treatment of GVH disease (GVHD) [2]. MSCs have been shown to prevent graft failure and to promote engraftment in hematopoietic stem cell transplantation (HSCT) [1, 2]. The expansion of MSCs for their clinical use has been done under different culture conditions, most of which were based on the addition of fetal bovine serum (FBS) as a supplement for different media [3]. Under these culture conditions, anti-FBS antibodies have been detected in most patients infused with MSCs cultured in the presence of FBS [4]. The identification of suitable culture conditions for optimal expansion and appropriate functional capability of MSCs still remains a crucial matter [5].

The recent interest in obtaining high numbers of MSCs for clinical use has led to the development of therapeutic protocols based on the non-transfusional use of hemocomponents such as

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autologous and allogeneic human serum or plasma, cord blood serum or human platelet lysate (HPL) [4]. HPL-containing media were recently described as possible substitutes for FBS-containing media for the expansion of MSCs for clinical use [6]. Indeed, HPL-containing media markedly increase proliferation of MSCs that displayed neither immunogenicity nor oncogenic potential [7].

Little is known about the effect of HPL-conditioned media on the functional properties of MSCs and hence on their efficacy in clinical application. Notably, HPL contains numerous bioactive molecules stored within the platelet organelles including adhesion molecules, coagulation factors, protease inhibitors and proteoglycans [8], as well as growth factors. These include platelet-derived growth factor (PDGF), basic fibroblast-derived growth factor (b-FGF), vascular endothelial growth factor (VEGF), insulin-like growth factor I (IGF-I) and transforming growth factor beta (TGF-β) [9] that may influence MSCs expansion and function [10].

In this context, some recent reports have addressed the influence of HPL on the generation and expansion of MSCs [10–12]. These studies mainly focused on MSC morphology, proliferation, differentiation, immunophenotyping, karyotyping and tumourogenicity [13, 14]. Telomerase activity, transformation and senescence have also been investigated due the rapid proliferation rate of MSCs [15, 16]. However, limited information is available on the influence of HPL on the immunoregulatory capacity of MSCs, which is a major property exploited for clinical application [17, 18]. Conflicting results have been obtained on the effect of HPL on mitogen-stimulated or alloantigen-induced T-cell proliferation [7, 19]. Various studies reported similar phenotypic and functional effects of MSCs expanded in HPL and in FBS-containing media [3, 20–22]. In addition, to our knowledge, there are no data addressing the effect of HPL on MSC–NK cell interactions. This information is important in view of the potent anti-leukemic effect exerted by allo-reactive NK cells in the HSCT setting.

In the present study, we show for the first time that HPL alters the expression of some functionally relevant MSC surface molecules which rendered them less susceptible to lysis by IL-2-activated NK cells. In addition, we show that HPL-expanded MSCs (HPL-MSCs) display a decreased ability to inhibit T-cell proliferation in response to alloantigen and NK-cell proliferation and cytotoxicity. Finally, a decrease in prostaglandin E2 (PGE2) production and increase in IL-6, IL-8 and RANTES secretion were detected in HPL-MSCs in comparison with MSCs cultured in FBS-containing media.

Results

Culture in HPL-containing media promotes strong MSC proliferation

The total cumulative numbers of MSCs expanded in 10% FBS (FBS-MSCs) and MesenCult (MesenCult-MSCs) were significantly lower than those obtained upon expansion in MesenPro (MesenPro-MSCs) or HPL-containing media (HPL-MSCs). Indeed, there was a much higher proliferation rate in HPL-MSCs and MesenPro-MSCs that was statistically significant in all passages (Fig. 1). The difference increased with each passage. For example, at passage (P) 6 (approximately after one month of culture), HPL-MSC and MesenPro-MSC numbers were more than 10 times higher than those obtained in 10% FBS-containing or MesenCult media. This high growth rate was maintained even at later passages (P10, data not shown). Major difference in the proliferation rate among the different culture conditions was also confirmed in three donors by tritiated (^{3}H) thymidine uptake experiments (data not shown). It is worth noting that standard RPMI 1640 medium supplemented with 2% FBS and 1% L-glutamine was used in comparison with the MesenPro condition. However, 2% FBS without additional growth factors failed to support MSC proliferation.

HPL-MSCs display a decrease of DNAM-1 and NKG2D ligands and increase of adhesion molecules

We next analyzed various surface molecules including conventional and non-conventional MSC markers. MSCs expanded from the different culture conditions used expressed HLA class I, CD29, CD73, CD90, CD105, CD166, CD146, CD44 and CD56 but not HLA-DR, CD14, CD34, CD45. No significant differences in the percentage of positive cells were detected with exception of CD56. HPL-MSCs had a higher percentage of CD56 expression (26%±19, mean±SD) than the other conditions (11.5%±3.5 FBS-MSCs, 8%±6 MesenCult-MSCs and 15%±12 MesenPro-MSCs, $p<0.05$) (Fig. 2). Cells expressing Nectin-2 and PolioVirus Receptor (PVR) (DNAM-1 ligands) were significantly lower in HPL-MSCs (74%±14 and 78%±6, respectively) as compared with cells expanded in MesenCult-MSCs (94%±4 and 93%±6)

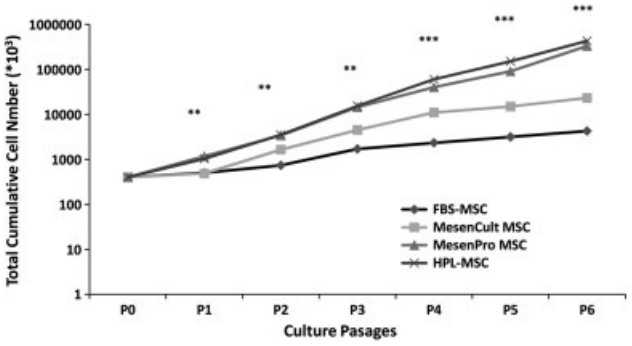

Figure 1. Proliferative capacity of MSCs under different culture conditions. Four hundred thousand MSCs were plated in 75 cm^2 flask (5000 cells/cm^2). After reaching ≥80% confluence, MSCs were harvested by trypsin, counted and re-plated for expansion at 3000 cells/cm^2. Total cumulative cell numbers are expressed as the mean of six experiments from time zero (P0) to P6. $^{**}p<0.01$ and $^{***}p<0.001$, two-way ANOVA with LSD post-hoc test revealed no significant difference between MesenPro-MSCs and HPL-MSCs as well as between FBS-MSCs and MesenCult-MSCs.

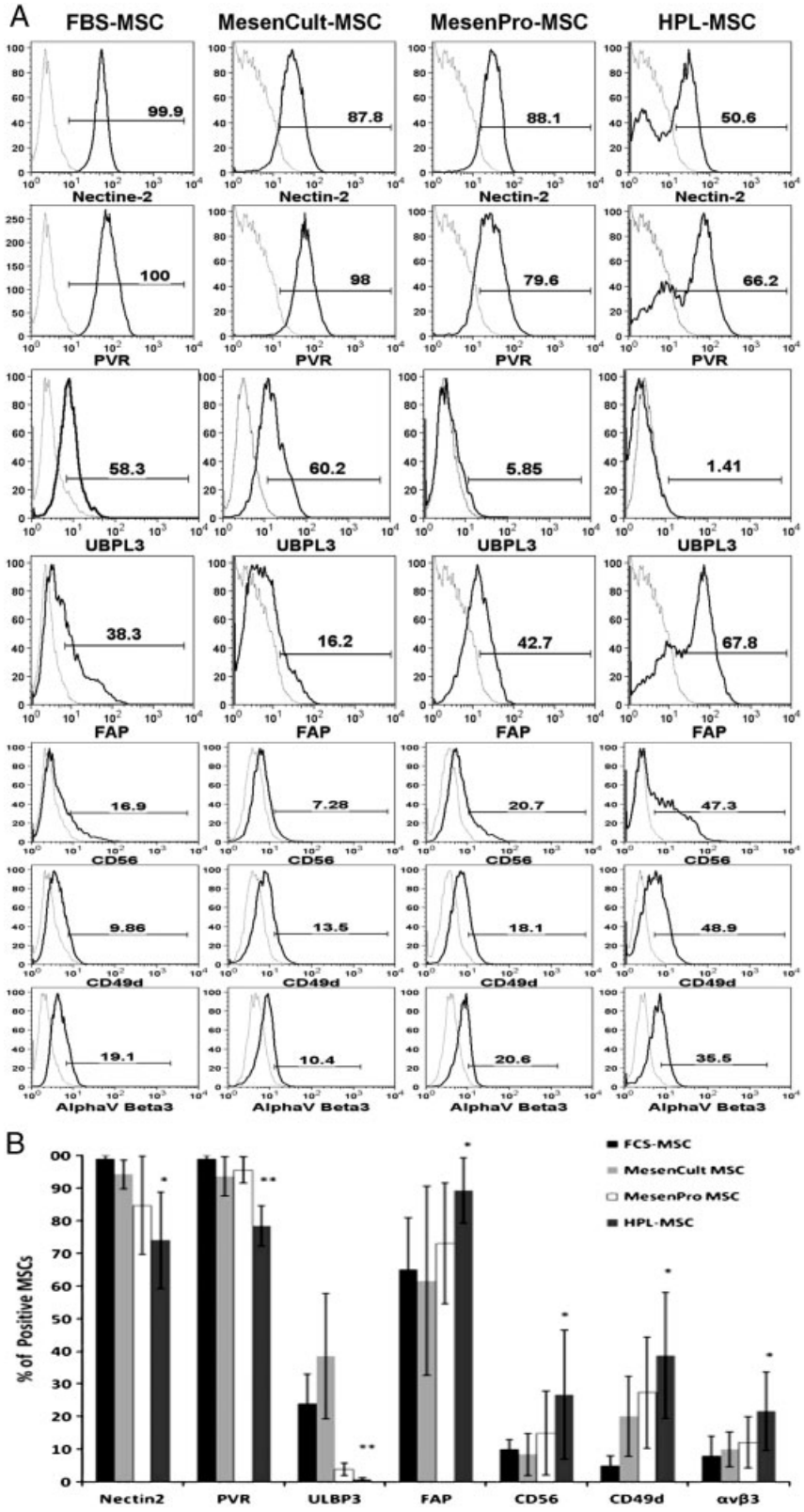

Figure 2. Effect of HPL as a culture supplement on MSC surface phenotype. Expression of surface molecules on MSCs cultured under different conditions was evaluated by flow cytometric analysis. (A) Surface expression of the indicated markers is shown for MSCs cultured in the presence of FBS, MesenCult, MesenPro and HPL. Data shown are from one experiment representative of seven performed on different MSCs populations, analyzed at P3 or P4. Numbers represent percentages of marker-positive cells (black histograms) with respect to negative control (grey histograms). (B) Data are expressed as the mean ± SD of the percentages of the surface marker expression in seven different experiments. $^{*}p<0.05$ and $^{**}p<0.01$ between HPL-MSCs and the other conditions using two-way ANOVA with LSD post-hoc test.

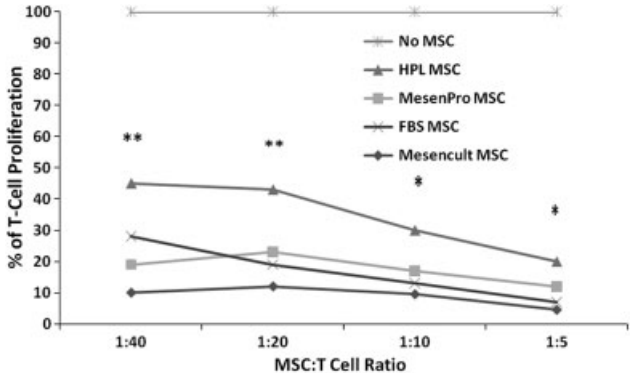

Figure 3. HPL-MSCs display a significantly reduced inhibitory capacity on T-cell proliferation in the MLR. Responder PBMCs (10^5) were incubated for 5 days with irradiated stimulator PBMCs (10^5) and different doses of irradiated third party MSCs (2.5×10^3, 5×10^3, 10×10^3, 20×10^3) cultured under different conditions. All experiments were performed in triplicate. Data are expressed as mean percentage of T-cell proliferation with respect to the control (100% proliferation in the absence of MSCs) from seven independent experiments. $^*p<0.05$ and $^{**}p<0.01$, two-way ANOVA with LSD post-hoc test revealed no significant difference between MesenCult-MSCs, MesenPro-MSCs and FBS-MSCs in all ratios analyzed.

and FBS-MSCs (99%±1 and 99%±1, $p<0.05$ and $p<0.01$, respectively). Expression of the NKG2D ligand ULBP-3 was almost absent in HPL-MSCs and MesenPro-MSCs, while it was expressed in FBS-MSCs and MesenCult-MSCs (24%±12 and 38%±19 respectively, Fig. 2).

We also investigated non-conventional MSC markers including CD340, CD349 (frizzled-9), CD271, stage-specific embryonic antigen (SSEA-4) and mesenchymal stem cell antigen-1 (MSCA-1). Although these markers showed differences in the surface expression among different culture conditions, none were statistically significant due to the high variability among donors and to the low expression under our culture conditions (data not shown). The expression of surface molecules involved in cellular interaction and homing such as fibroblast-associated protein (FAP) and the adhesion molecules CD49d and αvβ3 (CD51/61) was significantly higher in HPL-MSCs. MSCs displayed a stable phenotype over time. Indeed, no substantial change in the surface marker expression was detected from P3 to P10.

HPL-MSCs display a decreased inhibitory activity on T-cell proliferation in MLR

In order to assess whether the immune-modulatory capacity of ex-vivo expanded MSCs may be influenced by different culture conditions, differently expanded MSCs were evaluated for their capacity to inhibit T-cell proliferation in MLR. In seven independent experiments, MSCs consistently inhibited alloantigen-induced T-cell proliferation. However, significant differences existed in the degree of inhibition among MSCs expanded under different conditions. Figure 3 shows data obtained using different MSC/T-cell ratios. It is evident that HPL-MSCs had a significantly lower inhibitory capability than MSCs expanded under the other conditions at the ratios analyzed. The differences decreased at higher MSC/T-cell ratios. These results show that, in order to obtain an equivalent degree of inhibition, higher numbers of HPL-MSCs would be required.

HPL-MSCs display a low inhibitory activity on NK-cell functions while being protected from NK-cell lysis

In order to evaluate whether HPL-MSCs have a lower inhibitory effect also on NK-cell proliferation, MSCs expanded under different conditions were co-cultured with freshly separated NK cells at MSC:NK-cell ratio of 1:10 and 1:20 in the presence of 100 U/mL IL-2. As shown in Fig. 4A, only partial inhibition of NK-cell proliferation was detected with HPL-MSCs at both ratios tested. On the other hand, MSCs expanded under the other conditions significantly inhibited NK-cell proliferation. IL-2-induced NK-cell proliferation in the absence of MSCs was used as a control ($p<0.001$). We further investigated the inhibitory capability of HPL-MSCs on NK-cell cytotoxicity. As shown in Fig. 4B, HPL-MSCs had a lower inhibitory capacity than MSCs expanded under the other conditions.

We previously demonstrated a role for DNAM-1 and NKG2D receptors in the NK-cell mediated lysis of MSCs [23]. The finding that HPL-MSCs displayed decreased or absent surface expression of DNAM-1 ligands and NKG2D ligands suggested that HPL-MSCs could be, at least in part, resistant to NK-cell-mediated lysis. Accordingly, differently expanded MSCs were used as target cells and NK-cell degranulation was analyzed by CD107a staining. NK cells showed a lower degree of degranulation when HPL-MSCs were used as targets as compared with MSCs expanded under the other conditions (Fig. 4C). These data indicate a significantly lower susceptibility of HPL-MSCs to NK-cell-mediated lysis.

HPL alters the secretory profile of MSCs

In an attempt to further analyze whether different expansion conditions may influence the secretory cytokine profile of MSCs, we analyzed 16 different cytokines by a multiplex FACS analysis (see *Materials and Methods*). While MSCs cultured under different conditions produced no or very low amounts of the majority of cytokines examined, IL-6, IL-8 and RANTES were detectable in higher amounts in HPL-MSC cultures (Fig. 5A) suggesting an influence of the culture media on the secretory profile of MSCs. Notably, these differences may influence the immune-modulatory capacity of MSCs.

Since we have previously shown that MSC-mediated inhibition of NK-cell proliferation and cytotoxicity was dependent on PGE2, we further analyzed the amount of PGE2 secreted by MSCs cultured under different conditions. Supernatants were collected after 5 days from MSCs cultured either alone or together with NK cells or in MLR (Fig. 5B). HPL-MSCs displayed the lowest PGE2 secretion both when cultured alone and when co-cultured with

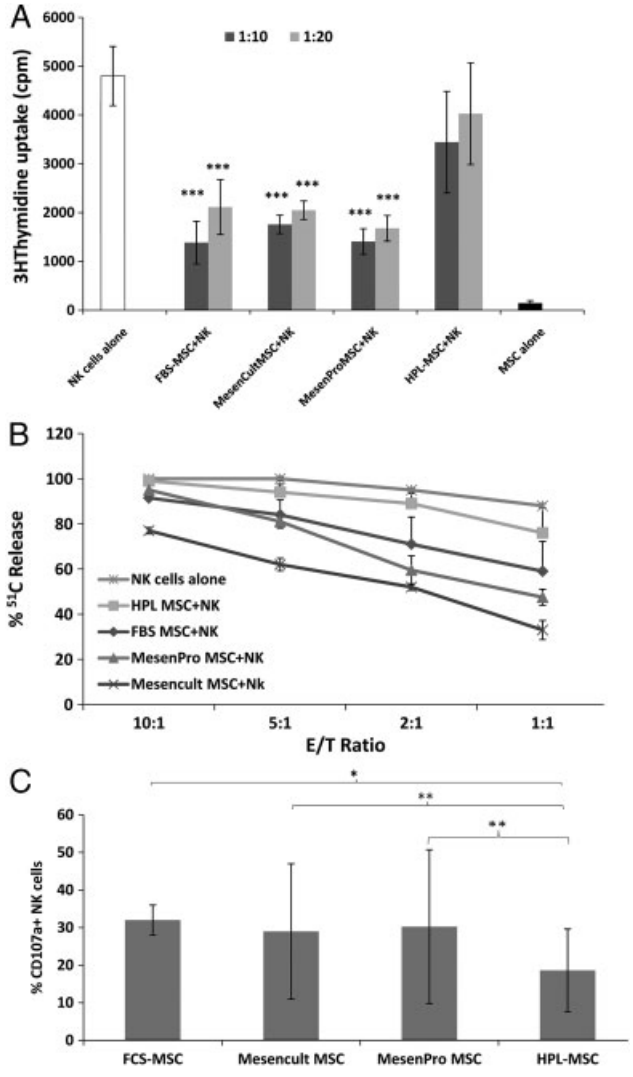

Figure 4. Effect of MSCs cultured in HPL on MSC–NK cell interactions. NK cells were cultured alone (control) or with allogeneic MSCs in the presence of 100 U/mL IL-2. (A) Proliferating NK cells were analyzed for ^{3}H-thymidine uptake at day 6 of culture in the absence (white bar) or in the presence of MSCs expanded under the indicated conditions (grey bars) or irradiated MSCs alone (black bar). MSCs cultured with NK cells at ratios of 1:10 and 1:20, respectively. ***$p<0.001$ compared with the control (NK cells alone), paired *t*-test. (B) Percentage ^{51}Cr release as a measure of the cytotoxic activity of cultured NK cells against the K562 tumor cell line was determined at different effector/target (E/T) ratios. NK cells were cultured with IL-2 for 6 days either alone or in the presence of differently expanded MSCs at a 10:1 ratio. (C) NK cells were used as effector cells against differently expanded MSCs at 4:1 ratio. Degranulation was measured as the percentage of CD107a expression on the surface of NK cells. Data are shown as mean ± SD of (A) 4, (B) 3 and (C) 8 experiments. *$p<0.05$, **$p<0.01$ and ***$p<0.001$, paired *t*-test.

NK cells. Supernatants obtained from NK cells cultured alone did not contain PGE2 while supernatants from MLR in the absence of MSCs contained low amounts of PGE2 (170 ± 45 pg/mL mean ± SD, data not shown). Surprisingly, HPL markedly augmented PGE2 secretion by MSCs in co-cultures as compared with the other conditions. In this context, it must be considered

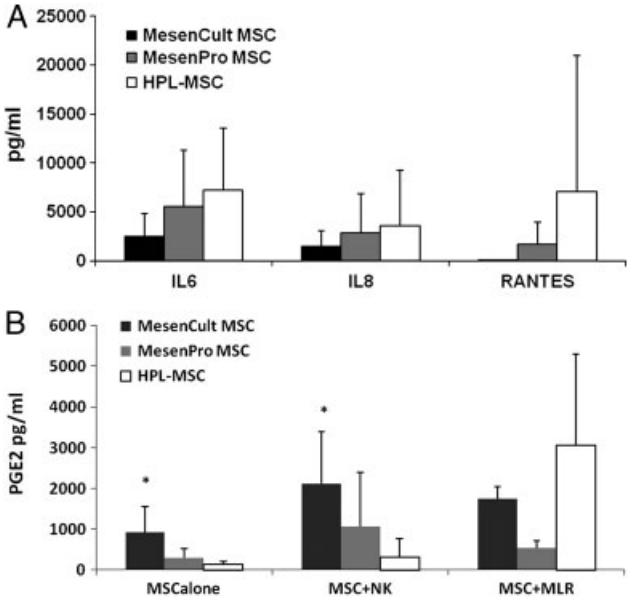

Figure 5. HPL alters the secretory profile of MSCs. (A) IL-6, IL-8 and RANTES production in 24 h culture supernatants by differently expanded MSCs was measured by flow cytometric multiplex bead-based analyte detection. (B) PGE2 concentration in 5-day culture supernatants of differently expanded MSCs cultured alone, with NK cells or in MLR was measured by ELISA. All data are expressed as the mean ± SD of four independent experiments. *$p<0.05$ between MesenCult-MSCs or HPL-MSCs and the other two conditions, two-way ANOVA with LSD.

that, as reported in previous studies [22, 24], PGE2 has a marginal role in the inhibitory mechanism of MSCs in MLR.

Discussion

Our data provide evidence that although culture in HPL-containing media greatly enhances the proliferative capacity of MSCs, the resulting cells display an altered phenotype and reduced capacity to inhibit T-lymphocyte and NK-cell proliferation. In addition, HPL-MSCs show an impaired inhibitory effect on NK-cell-mediated cytotoxicity. These data may have relevant implications on the use of HPL-MSCs to induce immunosuppression (e.g. to treat GVHD).

In order to obtain MSC numbers suitable for clinical use ($>2\times10^{6}$/kg) [4], considerable in vitro expansion is strictly necessary. In this context, HPL was revealed as an optimal supplement to reach sufficient numbers of MSCs in a relatively short time interval [3, 12, 25]. HPL is indeed rich in PDGF and b-FGF which are important growth factors for MSCs [26]. Yet, the cumulative cell numbers recovered were comparable with those obtained in MesenPro medium containing 2% FBS and other unknown supplements. In contrast, media containing 2% FBS alone (i.e. without additional growth factors) failed to support MSCs growth and survival (data not shown). Indeed, it is known that different combinations of growth factors affect MSC

expansion. The advantage of HPL in obtaining MSC expansion in vitro is related not only to the increased MSC proliferation, but also to the fact that, different from FBS, HPL does not induce antibody responses in the patients [21, 27, 28]. Limited information is available on the functional capability of HPL-MSCs. In this context, while their clinical application in regenerative medicine appeared particularly successful in different studies [29–33], their use in clinical protocols aimed at inducing immunosuppression revealed a limited effect. Thus, MSCs expanded in HPL had a limited effect in patients with steroid-resistant acute GVHD [1]. In addition, Lucchini et al. reported a reduced efficiency of HPL-MSCs in chronic GVHD [34]. In contrast, MSCs cultured in FBS-containing media mediated efficient immunosuppression [35–38].

These conflicting results could reflect an impaired immunemodulatory capacity of HPL-MSCs and prompted us to further investigate this issue. Indeed, we found that HPL-MSCs had an impaired inhibitory capacity on both T-cell and NK-cell proliferation. However, it is worth mentioning that at high MSC:T-cell ratios, similar inhibitory effects were observed by using MSCs expanded under different conditions. This may explain the conflicting results on the similar functional effects of MSCs expanded in HPL and in FBS-containing media reported by studies using high ratios [4, 11, 21, 27]. Whether these findings are relevant in vivo remains to be determined; however, since GVHD mostly reflects proliferation of donor-derived allogeneic T cells [39, 40], our data are consistent with the lower capacity to control GVHD.

The mechanisms by which HPL exerts its effect on MSC function remain to be clarified. A possible explanation is that HPL may affect the production of modulatory factors by MSCs. In this context, IL-6, a primary proinflammatory cytokine involved in the enhancement of APC function, cytotoxic lymphocyte activity and B-cell proliferation, may play a role. Indeed, higher amounts of IL-6 were detected in HPL-MSCs culture supernatants which may result in a possible stimulatory activity.

We have previously shown that MSCs inhibit NK-cell proliferation and function via the production of soluble factors [41]. In this setting PGE2 played a major role. Since HPL-MSCs produced lower levels of PGE2, this may be partially responsible for the lower inhibitory effect observed in NK-cell proliferation and function.

Based on our results, one may envisage a favorable scenario in haploidentical HSCT since NK cells are known to play a crucial role in the graft-versus-leukemia (GVL) effect [42–44]. In these patients, the infusion of HPL-MSCs to improve the engraftment, would not substantially interfere with the NK-cell proliferation and function.

Analysis of surface markers on MSCs expanded under different conditions showed no substantial variations in their expression and surface density. A remarkable exception, however, was represented by PVR and Nectin-2 (DNAM-1 ligands) and by ULBP3 (an NKG2D-ligand), the expression of which was decreased in HPL-MSCs. Since these molecules have been shown to play a crucial role in NK-cell-mediated lysis of MSCs [23], we investigated this point and found that HPL-MSCs were partially resistant to lysis. This finding may be relevant in HSCT. In particular, HPL-MSCs infused to improve engraftment would be resistant (at least in part) to NK-cell-mediated killing and could thus exert their beneficial effect in supporting hematopoiesis.

In conclusion, HPL-MSCs, in view of their limited capacity to inhibit T-cell and NK-cell proliferation and function, may be less effective in the control of immune-mediated pathologies, particularly GVHD. However, beside their usefulness in regenerative medicine, they may be of benefit in HSCT engraftment. Our data offer an interesting clue regarding possible functional differences in MSC output by different expansion protocols. Further insights into the mechanisms leading to the decreased inhibitory potential together with the understanding of the in vivo biological relevance of these in vitro observations are clearly required to better exploit the clinical use of HPL-MSCs.

Materials and methods

Samples were obtained after the ethical committee approval of the institutional review board of the Giannina Gaslini Institute, Genova, Italy, and informed consent was obtained from donors and patients' legal guardians in accordance with the Declaration of Helsinki.

HPL preparation

Platelet aphaeresis procedures were performed at the Gaslini Institute's transfusion centre from healthy donors using COBE Spectra (Gambro BCT, Lakewood, CO, USA). Products were frozen at -80°C until use. Twelve platelet aphaeresis products were collected in which the platelet concentration from each donor ranged between 2 and 3×10^9/mL. Subsequently, all aphaeresis collections were thawed at 37°C to obtain platelet-released growth factors. They were pooled in a single culture supplement and centrifuged at $900 \times g$ for 30 min to remove platelet bodies [10]. The supernatant was collected and submitted to three new rounds of centrifugation. The final supernatant was heparinised (5000 U.I, Mayne Pharma Srl-Napoli, Italy) to avoid clot formation, aliquoted and stored at -20°C.

MSC isolation and expansion

MSCs were derived from discarded bone fragments of paediatric patients undergoing orthopaedic surgery to correct major scoliosis. Boney tissues were mechanically flushed with medium to extrude cells without the use of enzymes. Cell suspension was harvested and centrifuged. Pellet was cultured at a concentration of 1×10^6 cells/mL in 25-cm^2 tissue-culture flasks in MesenCult

basal medium for human MSCs with human MSC stimulatory supplement (both from StemCell Technologies, Vancouver, BC, Canada) and incubated at 37°C in a 5% CO_2 humidified atmosphere. After 48–72 h, non-adherent cells were removed and fresh medium was added. Half the medium volume was replaced twice a week [45]. MSCs were cryopreserved at −80°C after 2–3 expansion passages to ensure depletion of monocytes/macrophages. Cells from all donors were then thawed for re-expansion under different conditions. Three FBS-containing media were used: standard RPMI 1640 medium (Biowhittaker, Cambrex, Verviers, Belgium) supplemented with 10% FBS (Biochrome, Germany), and 1% L-glutamine (both from Lonza, Belgium), MesenCult basal medium with human supplement containing 10% FBS (StemCell Technologies, Vancouver, BC, Canada) and MesenPro with low serum containing (2% FBS) growth supplement (Invitrogen, Carlsbad, CA, USA). 10% HPL in standard RPMI 1640 medium (Biowhittaker) was used as a growth supplement for the fourth condition. Four hundred thousand MSCs were incubated in 10 mL of the different media at 37°C, 5% CO_2 in a humidified atmosphere in 75 cm^2 flask (5000 cell/cm^2) to compare their expansion potential. After reaching ≥80% confluence, MSCs were harvested by trypsin/EDTA (Biowhittaker), counted and re-plated for expansion at 3000 cells/cm^2. After 3 or 4 passages in each culture condition, number of cells obtained, proliferation, morphology and cell surface phenotype were evaluated.

mABs and flow cytometric analysis

Immunophenotypic analysis was performed on seven different MSC donors expanded under different conditions. The following commercial mAbs were used: anti-CD29, anti-CD105-PE, anti-CD106 and anti-CD166 mAbs (Ancell, Bayport, MN, USA); anti-CD14, anti-CD34, anti-CD45, anti-CD44, anti-CD146 and anti-CD73 mAbs (BD Biosciences PharMingen); anti-integrin αvβ3 (CD51/61), anti-CD340, anti-CD349 (frizzled-9), anti-SSEA-4 and anti-MSCA-1 (Biolegend, CA, USA); anti-CD271 (Miltenyi Biotec, Bergisch Gladbach, Germany); anti-FSP (GeneTex,Inc); anti-FAP IgG1 (ATCC). Anti-ULBP1(IgG1), anti-ULBP2 (IgG1), anti-ULBP3 (IgG1) and anti-ULBP4 (IgG1) mAbs were purchased from R&D systems.

The following mAbs were produced in our laboratory: L-95 (IgG1, anti-PVR), L-14 (IgG2a, anti-Nectin-2). To evaluate the surface expression of HLA class I and HLA class II molecules, W632 and D1-12 (kind gift from R. Accolla) mAbs were used.

MSC phenotype was analyzed by single fluorescence analysis. Nearly 50 000–150 000 cells were first incubated with primary specific mAbs for 30 min at 4°C. Then, cells were washed and incubated with PE-conjugated AffiniPure F(ab′)2 goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for the indirect staining, or with the labeled mAbs for the direct staining. After 30 min at 4°C, cells were washed and resuspended in RPMI 1640 5% FBS. Samples were analyzed using the BD FACS-Calibur and CellQuest software. Comparative analysis was performed with FlowJo Version 7.2.5 (TreeStar, Ashland, OR, USA).

MLR

Peripheral blood mononuclear cells (PBMCs) from different healthy donors were obtained by Ficoll-Hypaque gradient centrifugation (specific gravity 1.077 g/mL; Lympholyte, Cedarlane, CA, USA) and resuspended in RPMI 1640 medium supplemented with 10% FBS [46]. Stimulator mononuclear cells and unrelated, third party, MSCs were irradiated at 30 Gy. MSCs derived from the different conditions described under "MSC isolation and expansion" were plated at different MSC:T-cell ratios (1:40–1:5) in flat-bottomed 96-well plates (Corning, New York, NY, USA) in RPMI medium supplemented with 10% FBS to avoid the effect of medium containing growth factors. Totally, 1×10^5 responders and 1×10^5 stimulators were added for 5 days. Proliferation was measured on day 6, after 18-h pulse with 1 μCi/well of ^{3}H-thymidine (Amersham Biosciences, Amersham, UK) [23]. Cells were harvested and ^{3}H-thymidine incorporation was measured using the MicroBeta Trilux Counter (Wallac, Turku, Finland). Each experiment was performed in triplicate.

NK-cell isolation

Fresh buffy coats were obtained from Gaslini Institute's transfusion center and NK cells were isolated by NK-cell Isolation Kit (Miltenyi Biotec), according to the manufacturer's instructions. Purity of NK cells, evaluated by flow cytometric analysis of $CD56^+CD3^-CD19^-$ lymphocytes, ranged from 94 to 96%.

NK-cell proliferation assay

To evaluate the effect of the MSC culture condition on the MSC-mediated inhibition of NK-cell proliferation, we used the ^{3}H-thymidine uptake method [23]. Differently cultured MSCs were plated in U-bottom 96-well plates and NK cells were added at 10:1 and 20:1 NK/MSC ratio. After co-culture for 5 days in RPMI supplemented with 10% FBS and 100 U/mL IL-2 to induce NK-cell proliferation, ^{3}H-thymidine was added for the last 18 h and uptake was measured as described under "MLR".

NK-cell cytotoxicity assay

Cytotoxicity assays were performed using the 4-h ^{51}Cr-release method [47]. Isolated NK cells were cultured in RPMI supplemented with 10% FBS and 100 U/mL IL-2 either alone (used as a control) or in the presence of the differently cultured MSCs at 10:1 and 20:1 NK/MSC ratios. Since NK cells grow in suspension and MSCs adhere to plastic, after 5 days NK cells were collected as the non-adherent layer and used as effectors in cytotoxicity

assays against K562 cell line. Target cells were labeled with 100 uCi (3.7 MBq) $^{51}Cr/10^6$ cells and plated at 5000 cells/microwell. The lytic potential of NK cells was assessed by plating cells at different effector/target (E/T) ratios.

NK-cell degranulation assay

Susceptibility of MSCs to NK-cell lysis was evaluated by the frequency of degranulating NK cells, measured by the expression of CD107a, (lysosome-associated membrane protein-1) [48]. Isolated NK cells were treated overnight with 100 U/mL IL-2, then incubated for 5 h with differently expanded MSCs at the effector/target ratios of 4:1 and 2:1. Anti-CD107a-FITC (BD Biosciences PharMingen) was added directly to the co-cultures. After 1 h incubation, Golgi Stop reagent (BD Biosciences PharMingen) was added and incubated for another 4 h. Cells were then stained with CD56-PE and CD3 APC (BD Biosciences PharMingen) and analyzed on a BD FACS Calibur.

PGE2 production by MSCs

Supernatants collected after 5 days of culture from differently expanded MSCs either alone or with NK cells or in MLR were used for PGE2 quantification by competitive ELISA technique using a commercially available ELISA kit (R&D Systems), according to the manufacturer's instructions. Concentrations were calculated by comparison with known standards with a lowest detection limit 39 pg/mL. All determinations were made in duplicate.

Multiplex growth factor analysis

Differently cultured MSCs were harvested and 40 000 cells re-plated in 24-well plate in 1 mL RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine. After 24 h, supernatants were frozen at $-80^{\circ}C$ until used. Multiplex bead-based analyte detection for IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17a IL-23, IFN-γ, TNF-α and β, MIP1α, ICAM1 and RANTES (Bender MedSystem Flowcytomix Vienna) was performed according to the manufacturer's instructions.

Statistical analysis

Statistical tests were performed using SPSS 16.0 (SPSS, Chicago, IL, USA). Data are represented as mean ± SD. Statistical differences were calculated using two-way analysis of variance (ANOVA) with the least significant difference (LSD) post-hoc correction test and *t*-test (paired *t*-test where applicable). Differences were considered significant at $p<0.05(^*)$, $p<0.01(^{**})$ or $p<0.001(^{***})$.

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Abbreviations: **FBS:** fetal bovine serum · **GVHD:** graft-versus-host disease · **HPL:** human platelet lysate · **HSCT:** hematopoietic stem cell transplantation · **MSC:** mesenchymal stromal cell · **PGE2:** prostaglandin E2 · **PVR:** PolioVirus Receptor

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