

# blood

2008 111: 1327-1333  
Prepublished online Oct 19, 2007;  
doi:10.1182/blood-2007-02-074997

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# Mesenchymal stem cells inhibit natural killer–cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2

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**Recently, a number of clinical trials used either mesenchymal stem cells (MSCs) or natural killer (NK) cells in an attempt to improve the effectiveness of hematopoietic stem cell transplantation (HSCT). In view of the relevant role of both MSCs and NK cells in HSCT, we have recently explored the result of possible interactions between the 2 cell types. We found that activated NK cells could kill MSCs,**

**whereas MSCs strongly inhibited interleukin-2 (IL-2)–induced NK-cell proliferation. In this study, we further analyzed the inhibitory effect exerted by MSCs on NK cells. We show that MSCs not only inhibit the cytokine-induced proliferation of freshly isolated NK cells but also prevent the induction of effector functions, such as cytotoxic activity and cytokine production. Moreover, we show that this inhibi-**

**tory effect is related to a sharp down-regulation of the surface expression of the activating NK receptors NKp30, NKp44, and NKG2D. Finally, we demonstrate that indoleamine 2,3-dioxygenase and prostaglandin E2 represent key mediators of the MSC-induced inhibition of NK cells. (Blood. 2008;111:1327-1333)**

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## Introduction

In recent years, mesenchymal stem cells (MSCs) have become a promising tool for novel therapeutic approaches aimed at inhibition of the immune responses.<sup>1-3</sup> In particular, MSCs may be used to prevent/suppress graft-versus-host disease (GVHD) in patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT)<sup>4</sup> or for the treatment of certain autoimmune diseases.<sup>5,6</sup> So far, results of phase 1 trials have revealed the feasibility of MSC isolation, in vitro expansion, and infusion with no reports of major adverse reactions.<sup>7,8</sup> The first in vitro evidences of an effective MSC-mediated immunoregulatory activity rapidly evolved into clinical use of these cells in novel protocols of adoptive immunotherapy. Therefore, it is particularly important to clarify the mechanism(s) underlying the inhibitory effect exerted by MSCs on immunocompetent cell populations.

Natural killer (NK) cells are major effector cells of the innate immunity and are generally thought to play a fundamental role in antiviral and antitumor responses.<sup>9,10</sup> As first described by Ruggeri and colleagues<sup>11,12</sup> and subsequently confirmed by other groups,<sup>13</sup> donor-derived NK cells would be responsible for eradication of leukemic cells in acute myeloid leukemia (AML) patients who received haploidentical HSCT. Remarkably, such a graft-versus-leukemia (GVL) effect was evident only in donor-recipient couples in which a killer immunoglobulin–like receptor (KIR)/KIR-ligand mismatch was present. Therefore, after selection of the most suitable donor, NK cells could be used in novel HSCT-associated immunotherapeutic strategies either as cells originating directly from transplanted CD34<sup>+</sup> hematopoietic precursors or as mature NK cells that had been highly purified from peripheral blood and infused intravenously.

MSCs and NK cells have been shown to interact in vitro.<sup>14-17</sup> The outcome of this interaction may depend on the state of NK-cell activation and/or on the cytokines present in the milieu. Thus, it may result in altered cell function and/or survival of either one or the other cell type. We previously described that the cytokine-induced proliferation of freshly isolated, resting NK cells is highly susceptible to MSC-mediated inhibition.<sup>14</sup> We asked whether such inhibitory effects could be exerted also on NK-cell effector functions, such as cytotoxic activity and cytokine production. These NK-cell functions are regulated by a series of surface receptors that can transduce either inhibitory or activating signals.<sup>18-20</sup> Exposure of resting NK cells to activating cytokines, such as interleukin-2 (IL-2), induces either de novo expression or increase of surface density of the activating receptors NKp44, CD69, NKp30, and NKG2D. As the levels of surface expression of activating NK receptors are positively correlated with NK-cell function,<sup>21,22</sup> we analyzed whether MSCs could exert an inhibitory effect on the IL-2–induced upregulation of the major activating receptors. Thus, in parallel with the phenotypic analysis, we performed functional studies aimed at the definition of possible correlations between receptor expression and the functional state of NK cells that had been cultured in IL-2 either in the presence or in the absence of MSCs. Moreover, because little is known about the mechanism(s) underlying the MSC-mediated inhibitory effect, we have further investigated the role of indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), and transforming growth factor- $\beta$  (TGF- $\beta$ ) on the MSC-mediated regulation of NK-cell activity, because these factors have been proposed as possible mediators.<sup>23-28</sup>

Submitted February 21, 2007; accepted October 10, 2007. Prepublished online as *Blood* First Edition paper, October 19, 2007; DOI 10.1182/blood-2007-02-074997.

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## Methods

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

### Isolation and culture of MSCs

MSCs were derived from discarded bone tissues of pediatric patients who underwent surgery to correct major scoliosis. Samples were obtained after ethics committee approval and informed consent. Bone marrow (BM) cells were plated in 25-cm<sup>2</sup> tissue culture flasks at a concentration of 10<sup>6</sup> cells/mL in Mesencult basal medium supplemented with MSC stimulatory supplements (both from StemCell Technologies, Vancouver, BC). After a 24-hour incubation at 37°C in a 5% CO<sub>2</sub> humidified atmosphere, nonadherent cells were removed, and the adherent fraction was cultured in fresh medium. For further expansion passages of confluent cells, MSCs were detached by treatment with trypsin/ethylenediaminetetraacetic acid solution (BioWhittaker, Cambrex, Belgium) and replated in 75-cm<sup>2</sup> tissue culture flasks. MSCs were used in the experiments only after 2 to 4 expansion passages to ensure depletion of monocytes/macrophages. MSCs were assessed by cytofluorometric analysis for the expression of the typical markers CD105, CD106, CD166, and CD29, and the absence of the hematopoietic markers CD45, CD34, and CD14.

### Isolation and culture of NK cells

NK cells were isolated from fresh buffy coats obtained from the Gaslini Institute's transfusion center. Immediately upon obtaining the blood, separation of NK cells was performed by negative selection using the RosetteSep method (StemCell Technologies), according to the manufacturer's instructions. Purity of NK cells, evaluated by cytofluorometric analysis of CD56<sup>+</sup> CD3<sup>-</sup> CD19<sup>-</sup> lymphocytes, ranged from 95% to 98%. Purified NK cells were cultured for up to 7 days in RPMI 1640 (BioWhittaker) 10% fetal calf serum (FCS) supplemented with 100 U/mL IL-2 (Proleukin; Chiron, Emeryville, CA) to obtain short-term activated polyclonal NK cells. For coculture experiments, NK cells were plated with irradiated MSCs at 4:1 NK/MSC ratio, which has been demonstrated to be a suitable cell ratio to obtain MSC-mediated inhibition of NK-cell proliferation.<sup>14</sup> A series of MSC populations derived from different donors was used in allogeneic combination with NK cells. To analyze the involvement of soluble factors (ie, IDO, PGE<sub>2</sub>, TGF- $\beta$ ) in the immunoregulatory phenomenon, we used specific inhibitors: 1 mM 1-methyl-tryptophan (1-M-Trp; Sigma-Aldrich, St Louis, MO), an inhibitor of IDO activity; 5  $\mu$ M NS-398 (Cayman Chemicals, Irvine, CA), an inhibitor of PGE<sub>2</sub> synthesis; and 10  $\mu$ g/mL anti-TGF- $\beta$  (R&D Systems, Minneapolis, MN) neutralizing monoclonal antibody (mAb). Analysis of PGE<sub>2</sub> in culture supernatants was measured at different time points of 24 hours, 48 hours, and 72 hours of NK-MSC cocultures with and without the inhibitor NS-398. This was done by a competitive enzyme-linked immunosorbent assay (ELISA) technique using a commercially available ELISA kit (R&D Systems, Oxford, United Kingdom), according to the manufacturer's instructions. Concentrations were calculated by comparison with known PGE<sub>2</sub> standards with a lowest detection limit of 39 pg/mL, and all determinations were made in duplicates.

### Monoclonal antibodies and cytofluorometric analysis

The following mAbs were used in this study: the anti-CD56-PC5, anti-NKp46-PE, anti-NKp30-PE, anti-NKp44-PE, anti-CD16-FITC, anti-NKG2D-PE, anti-NKG2A-PE, and anti-KIR2DL1/S1-PE mAbs were purchased from Immunotech-Coulter (Marseille, France). The anti-CD3-FITC, anti-CD14, anti-CD19-PE, anti-CD34, anti-CD45, anti-CD45-PerCP, anti-CD56-PE, anti-CD122-PE, anti-CD132-PE, anti-KIR2DL2/L3/S2-PE, and anti-IFN- $\gamma$ -PE mAbs were provided by BD Biosciences Pharmingen (San Diego, CA). The anti-CD29, anti-CD69-PE, anti-CD105-PE, anti-CD106, and anti-CD166 mAbs were from Ancell (Bayport, MN).

Cytofluorometric analysis of NK cells was performed by triple color staining. Briefly, purified NK cells were stained with fluorochrome-conjugated mAbs and incubated for 30 minutes at 4°C. Then, unbound reagents were removed by washing, and cells were resuspended in RPMI 1640 5% FCS and analyzed on a FACSCalibur (Becton Dickinson, Mountain View, CA). Expression of different markers was assessed by gating on CD56<sup>bright</sup>CD16<sup>-</sup> or CD56<sup>dim</sup>CD16<sup>+</sup> subsets or on all CD56<sup>+</sup> cells. To compare the surface densities of NK receptors among NK cells cultured in different conditions, we calculated the mean ratio fluorescence intensity (MRFI); that is, the ratio between mean fluorescence intensity (MFI) of cells stained with the selected mAb and that of unstained cells (negative control).

MSC phenotype was analyzed by indirect single fluorescence analysis. MSCs were first incubated with primary marker-specific mAbs for 30 minutes at 4°C. Then, cells were washed and incubated with phycoerythrin (PE)-conjugated AffiniPure F(ab')<sub>2</sub> goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). After 30 minutes, cells were washed and resuspended in RPMI 1640 5% FCS before the analysis.

### Cytotoxicity assays and culture of target cells

Cytotoxicity assays were performed using the 4-hour <sup>51</sup>Cr-release method. Purified NK cells derived from different donors were cultured in IL-2 either alone or in the presence of MSCs. After 6 days, NK cells were collected and used as effectors in the cytotoxicity assays. Notably, NK cells did not contain any significant contamination of MSCs (growing as adherent cells). A series of different cell types was used as targets; that is, immature dendritic cells (iDCs), MM6 and THP-1 leukemia cell lines, and SKNBE and HTLA-230 neuroblastoma cell lines (HTLA-230 was a kind gift from Dr E. Bogenmann, Children's Hospital, Los Angeles, CA). Target cells were labeled with 100  $\mu$ Ci (3.7 MBq) <sup>51</sup>Cr/10<sup>6</sup> cells and plated at 5000 cells/V-bottom microwell. The lytic potential of NK cells was tested by plating cells at different effector-to-target (E/T) ratios.

To generate monocyte-derived iDCs, peripheral blood mononuclear cells obtained from buffy coats by ficoll density centrifugation were plated in 75-cm<sup>2</sup> tissue culture flasks at a concentration of 2  $\times$  10<sup>6</sup> cells/mL and incubated at 37°C in 5% CO<sub>2</sub> humidified atmosphere. After 1 hour and 30 minutes, nonadherent cells were removed, and the monocyte-enriched and adherent fraction was cultured in RPMI 1640 10% FCS supplemented with 50 ng/mL granulocyte-macrophage colony-stimulating factor and 20 ng/mL IL-4 (Peprotech, London, United Kingdom). After 7 days, cells were assessed for their iDC phenotype by cytofluorometric analysis and used in the cytotoxicity assays. Tumor cell lines were maintained in culture in RPMI 1640 10% FCS.

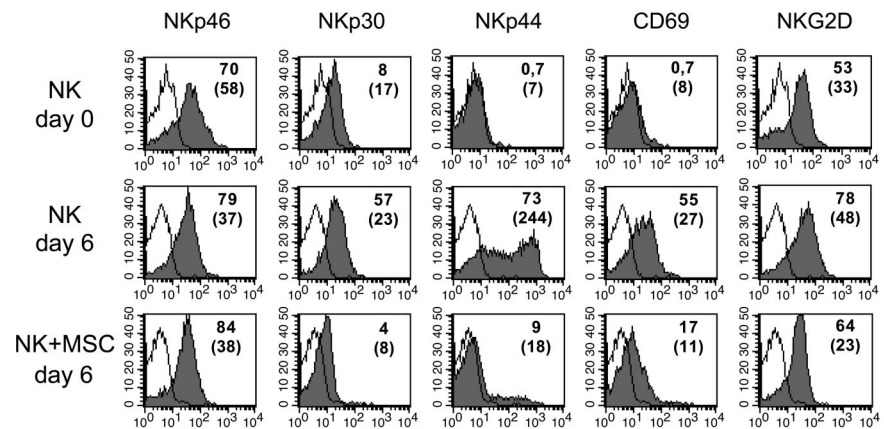
### Cytokine production

To analyze interferon- $\gamma$  (IFN- $\gamma$ ) production by NK cells, we used the intracytoplasmic detection method. Polyclonal NK cell populations were used in the experiments after 6-day culture with 100 U/mL IL-2 either in the presence or in the absence of MSCs. NK cells were stimulated with FO-1 melanoma cells in medium with 100 U/mL IL-2 at an E/T ratio of 8:1 in V-bottom 96-well plates. The negative control for cytokine production was represented by NK cells plated in medium with 100 U/mL IL-2 without FO-1 melanoma cells. To inhibit IFN- $\gamma$  secretion, Golgi Stop (Becton Dickinson) was added at the beginning of stimulation. After 5 hours, cells were harvested, and surface and intracellular stainings were performed. Briefly, cells were incubated with anti-CD56-PC5 mAb for 30 minutes at 4°C. For intracellular staining, NK cells were fixed and permeabilized with Cytofix/Cytoperm solution (Becton Dickinson), then incubated with anti-IFN- $\gamma$ -PE mAb for 30 minutes at 4°C, washed, and resuspended in phosphate-buffered saline 2% FCS for cytofluorometric analysis.

### Proliferation assays

To evaluate the MSC-mediated inhibition of NK-cell proliferation and a possible involvement of IDO, PGE<sub>2</sub>, and TGF- $\beta$  in the suppressive effect, we used the <sup>3</sup>H-thymidine uptake method. Freshly isolated NK cells were plated at 2  $\times$  10<sup>5</sup> cells/well in U-bottom 96-well plates either alone or in the

**Figure 1. MSCs alter IL-2-induced upregulation of activating NK receptors.** Expression of Nkp46, Nkp30, Nkp44, CD69, and NKG2D on freshly isolated NK cells derived from a representative donor and on the same NK cells after 6-day culture in IL-2 in the presence or in the absence of MSCs. Cells were analyzed by gating on CD56<sup>+</sup> lymphocytes. Gray profiles represent expression of activating receptors; open histograms represent negative control. Numbers indicate percentages of positive cells; numbers in parentheses represent MFI.



presence of  $5 \times 10^4$  irradiated MSCs at an NK/MSC ratio of 4:1. In some wells, inhibitors of IDO, PGE2, and TGF- $\beta$  (ie, 1-M-Trp, NS-398 and anti-TGF- $\beta$  mAb), used singularly or in combination, were added to NK-MSC cocultures at appropriate concentrations. After culture for 6 days with 100 U/mL IL-2 to induce proliferation of NK lymphocytes, cells were pulsed for 18 hours with  $^3\text{H}$ -thymidine at 1  $\mu\text{Ci}$  (0.037 MBq)/well (Amersham Biosciences, Amersham, United Kingdom) and then harvested.  $^3\text{H}$ -thymidine incorporation was measured using the Plate Chameleon Multilabel Counter (Hidex, Turku, Finland). In some experiments, NK cell proliferation also was evaluated with the 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution method, as previously described.<sup>14</sup>

### Statistical analysis

For intergroup comparisons, the statistical significance was assessed by the Mann-Whitney *U* test with the Bonferroni correction. A *z* test has been performed to compare the combined observed mean effect of 1-M-Trp and NS-398 to the expected mean value calculated for an additive model. All calculations were performed using Prism software package (release 3.00; GraphPad Software, San Diego, CA). A *P* value of less than .05 (\*), less than .01 (\*\*), or less than .001 (\*\*\*) was considered statistically significant.

## Results

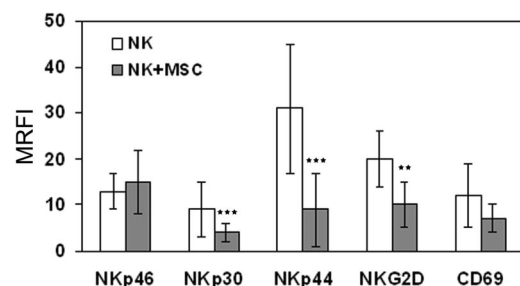
### MSCs inhibit the cytokine-induced expression of activating NK receptors

To investigate whether MSCs could affect the surface expression of activating or inhibitory receptors on NK cells, experiments were performed in which the NK-cell phenotype was analyzed by triple-fluorescence cytofluorometric analysis both in freshly isolated NK-cell populations (Figure 1, NK day 0) and in NK cells that had been cultured for 6 days in 100 U/mL IL-2 either in the absence (NK day 6) or in the presence of MSCs (NK + MSC day 6). As shown in Figure 1 (a representative experiment), after culture in IL-2, NK cells displayed increased expression of the activating NK receptors Nkp30 and NKG2D, whereas Nkp46 was not significantly modified. Moreover, the Nkp44 receptor and the activation marker CD69 (absent in fresh NK cells) were expressed de novo in these IL-2-cultured NK cells. When NK cells were cultured in the presence of MSCs, no upregulation of Nkp30, Nkp44, or NKG2D could be detected. On the other hand, no significant decrease of the surface density of Nkp46 and CD69 occurred. Similarly, no changes in the expression of the human leukocyte antigen (HLA) class I-specific inhibitory receptors KIRs and CD94/NKG2A were detected (data not shown). Notably, the surface density of activating NK receptors may vary among NK-cell populations derived from different donors. To assess whether these variations may have

an impact on the MSC-induced downregulation of NK-receptor expression, the statistical significance of phenotypic data was evaluated. Thus, we analyzed the results obtained in 10 independent experiments performed using NK cells derived from different individuals. Figure 2 shows the results of this analysis, represented as means plus or minus SD MRFI of surface Nkp46, Nkp30, Nkp44, NKG2D, and CD69 in IL-2-activated NK cells cultured alone versus the same cells cultured with MSCs. It is evident that MRFI values of surface Nkp30, Nkp44, and NKG2D were significantly lower in NK cells cultured with MSCs with respect to NK cells cultured alone ( $3.5 \pm 2.3$ ,  $9.5 \pm 8.0$ , and  $10.5 \pm 5.2$  vs  $9.4 \pm 5.9$ ,  $30.6 \pm 13.8$ , and  $19.6 \pm 5.6$ ;  $P < .001$ ,  $P < .001$ ,  $P < .01$ , respectively). We also evaluated the expression of other surface molecules, such as 2B4 (CD244), IL-2R $\gamma$  chain (CD132), CD16, and CD56 (not shown). In agreement with Sotiropoulou et al,<sup>15</sup> we found that MSCs inhibited the surface expression of 2B4 and CD132, whereas they did not affect the expression of CD16 molecules. In contrast, we could not detect any significant decrease of the levels of CD56 in either the CD56<sup>bright</sup>CD16<sup>-</sup> or the CD56<sup>dim</sup>CD16<sup>+</sup> NK cell subsets.

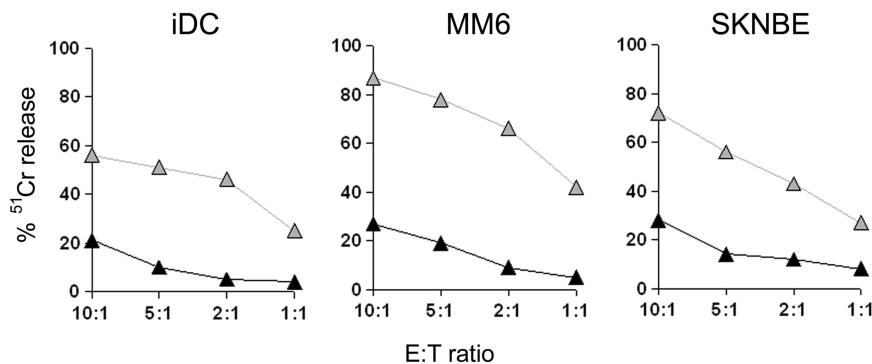
### Impaired cytotoxic activity of NK cells after coculture with MSCs

The finding that MSCs could inhibit the expression of activating receptors on the surface of NK cells was suggestive of a possible impairment of cytotoxic activity known to involve engagement of triggering receptors. To evaluate a possible MSC-mediated inhibitory effect on the lytic potential of NK cells, we performed cytolytic assays in which different NK-cell populations derived from different donors were used as effectors after short-term



**Figure 2. MSC-mediated modulation of NK-cell phenotype.** Expression of activating NK receptors on the surface of IL-2-activated NK cells cultured for 6 days alone (□) or in the presence of MSCs (■). Results are represented as mean plus or minus SD of MRFI ("Monoclonal antibodies and cytofluorimetric analysis") obtained from 10 independent experiments. \*\**P* < .01; \*\*\**P* < .001.





**Figure 3. MSCs inhibit NK-mediated cytotoxic activity.** Cytotoxic activity of one NK-cell population cultured with IL-2 for 6 days either alone (gray symbols) or in the presence of MSCs (black symbols) before the cytolytic assay against iDCs or SKNBE or MM6 tumor cell lines. In case of iDCs, NK cells were autologous with target cells. Data are representative of 5 different experiments, in which NK-cell populations derived from 5 different donors were used.

culture with 100 U/mL IL-2 either in the presence or in the absence of MSCs. As target cells, we used MM6 or THP-1 leukemia cell lines and SKNBE or HTLA-230 neuroblastoma cell lines. We also used NK-susceptible iDCs in both autologous and allogeneic combinations as target cells. NK cells cocultured with MSCs compared with NK cells cultured alone displayed decreased cytolytic activity. Figure 3 shows the results of a cytotoxicity assay in which NK cells derived from a representative donor were used as effectors against MM6 or SKNBE tumor cell lines or autologous iDCs. It is of note that when cultured in IL-2 alone, NK cells could efficiently lyse all targets. On the contrary, when effector cells were cultured in the presence of MSCs, a strongly reduced killing capability was detected. Similar results were obtained by using other target cells, including THP-1 and HTLA-230 cell lines or iDCs derived from different donors. These data suggest that the MSC-mediated downregulation of activating receptors on the surface of NK cells correlates with impaired cytotoxic activity.

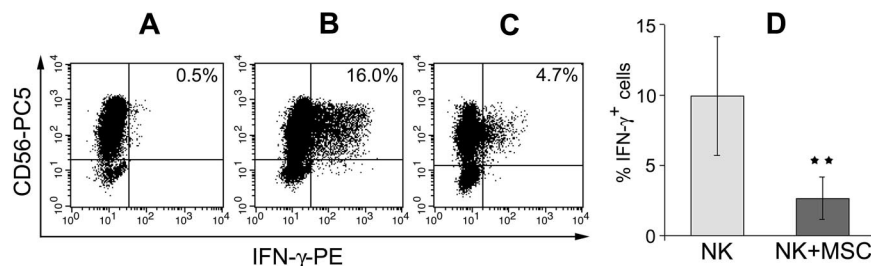
#### MSCs inhibit IFN- $\gamma$ production by NK cells

Together with cytolytic activity, cytokine production is another main NK-cell function. Therefore, to better characterize the inhibitory effect exerted by MSCs, we also analyzed the cytokine production by NK cells after interaction with MSCs. To this aim, freshly isolated NK-cell populations derived from different donors were cultured with 100 U/mL IL-2 either in the presence or in the absence of MSCs. After 6 days, NK cells were collected and incubated with FO-1 cells at an 8:1 cell ratio, respectively, in the presence of GolgiStop. After 5 hours, cells were collected and stained with anti-CD56-PC5 mAb. Then, cells were fixed and permeabilized, and intracytoplasmic staining was performed using an anti-IFN- $\gamma$ -PE mAb to detect cytokine production. As shown in Figure 4B, NK cells cultured in IL-2 alone produced IFN- $\gamma$  as a

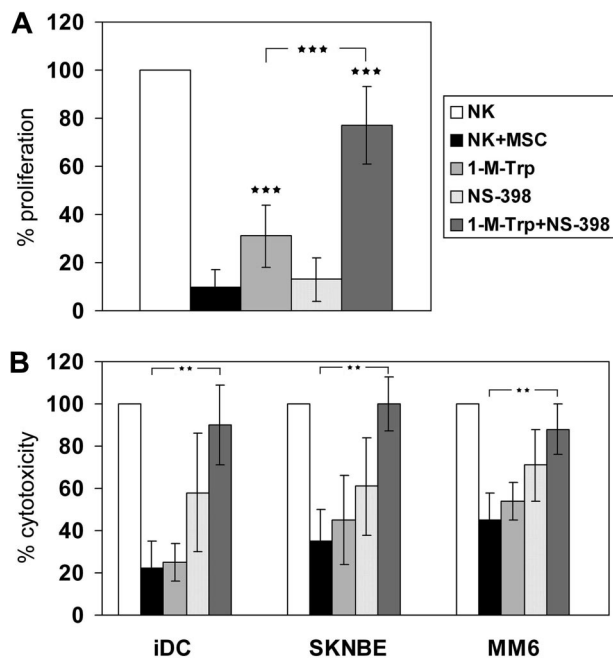
consequence of the interaction with FO-1 cells, whereas the same cells cultured in the presence of MSCs (Figure 4C) were much less responsive to stimulation. Data obtained from 6 independent experiments (mean  $\pm$  SD of IFN- $\gamma$ -positive cells:  $9.9 \pm 4.2$  of NK cells vs  $2.7 \pm 1.5$  of NK cells cultured with MSCs) performed using NK-cell populations derived from different donors (Figure 4D) were analyzed, and a statistical significance was observed ( $P < .01$ ). These results indicate that the inhibitory effect exerted by MSCs on NK cells can affect different aspects of NK-cell activation and function, ranging from proliferation to cytotoxic activity and cytokine production.

#### Role of soluble factors in the immunoregulatory activity of MSCs

To investigate the mechanisms underlying the MSC-mediated inhibition of NK-cell function, we studied the role of soluble factors possibly involved in this phenomenon. Because there is growing evidence that IDO, PGE2, and TGF- $\beta$  may represent relevant mediators of MSC-mediated inhibition,<sup>23-25</sup> we performed coculture experiments in the presence or in the absence of inhibitors of these soluble factors. These included 1-M-Trp, an inhibitor of IDO enzymatic activity; NS-398, an inhibitor of PGE2 synthesis; and a neutralizing anti-TGF- $\beta$  monoclonal antibody. Freshly isolated NK cells were cultured in the presence of IL-2 either alone or with MSCs. Inhibitors of IDO, PGE2, and TGF- $\beta$  were added to the wells of NK-MSC cocultures (see "Methods"). In some experiments, inhibitors were also used in combination to assess their possible additive (or synergistic) effects. After 6 days, NK cells were analyzed for their proliferative capacity in a <sup>3</sup>H-thymidine uptake assay. As shown in Figure 5A, NK cells underwent proliferation upon IL-2-induced activation. As already reported,<sup>14</sup> when NK cells were cultured in the presence of MSCs, a



**Figure 4. Impaired cytokine production of NK cells after interaction with MSCs.** After culture for 6 days with IL-2 in the presence or in the absence of MSCs, NK cells were cocultured with FO-1 melanoma cell line (as stimulator) at an E/T ratio of 8:1. After 5 hours of culture in the presence of 100 U/mL IL-2 and GolgiStop, cells were collected. Both surface and intracytoplasmic stainings were performed with anti-CD56-PC5 and anti-IFN- $\gamma$ -PE mAbs, respectively. Then, NK cells were analyzed by flow cytometry by acquiring  $5 \times 10^4$  events in the lymphocyte region. (A) Unstimulated NK cells represent negative control. (B,C) NK cells, cultured alone or in the presence of MSCs, respectively, stimulated with FO-1 cells. Numbers indicate percentages of double (CD56 and IFN- $\gamma$ ) positive cells. (D) Means plus or minus SD of percentages of IFN- $\gamma$ -positive NK cells (upon interaction with the NK-susceptible FO-1 cells) obtained in 6 independent experiments performed using different NK-cell populations after culture in IL-2 either in the presence or in the absence of MSCs. \*\* $P < .01$ .



**Figure 5. Role of IDO and PGE2 in the MSC-mediated inhibition of NK-cell proliferation and cytotoxic activity.** Freshly isolated NK cells were cultured with IL-2 either alone or in the presence of MSCs. In some wells, coculture of NK cells and MSCs was set in the presence of inhibitors, namely, 1-M-Trp (1 mM, inhibitor of IDO enzymatic activity) and NS-398 (5  $\mu$ M, inhibitor of PGE2 synthesis), used singularly or in combination. To evaluate NK-cell proliferation (A), after 6 days, cells were pulsed with  $^3$ H-thymidine for 18 hours, and then  $^3$ H-thymidine incorporation was measured after harvesting. Results are expressed as percentage of proliferation of NK cells cultured with MSCs, in the presence or in the absence of inhibitors of IDO or PGE2, with respect to NK cells cultured alone (100%). Bars represent mean plus or minus SD of 9 independent experiments. NK-mediated cytotoxic activity (B) was analyzed after 6-day culture using the  $^{51}$ Cr-specific release method. Data represent the percentage of lysis mediated by NK cells cultured with MSCs in the presence or in the absence of IDO and/or PGE2 inhibitors, with respect to NK cells cultured alone (100%). Bars represent means plus or minus SD obtained from 5 independent experiments. The E/T ratio used was 5:1 for all targets analyzed. \*\* $P < .01$ ; \*\*\* $P < .001$ .

strong inhibition of cell proliferation was observed. The addition of 1-M-Trp counteracted, at least in part, the inhibitory effect exerted by MSCs ( $P < .001$ ), whereas neither NS-398 nor anti-TGF- $\beta$  mAb (data not shown) had any substantial effect. Remarkably, however, the simultaneous blocking of IDO and PGE2 could almost completely restore the NK-cell proliferation ( $P < .001$  for NK + MSC treated with 1-M-Trp and NS-398 vs both NK + MSC and NK + MSC treated with 1-M-Trp), whereas other combinations of inhibitory compounds were ineffective (data not shown). The combined observed effect of 1-M-Trp and NS-398 (evaluated in terms of absolute cpm) was significantly greater than the mean expected value calculated for an additive model (mean expected value: 43.7, SD:11.0;  $P < .01$ ). These results suggest that both IDO and PGE2 are involved in the MSC-mediated inhibition of NK-cell proliferation and seem to exert a synergistic effect (note that NS-398 alone is ineffective). These data were further confirmed by using the CFSE dilution method to evaluate cell proliferation (data not shown). In addition, direct assessment of PGE2 in culture supernatants showed that MSCs produce PGE2 constitutively, as previously described<sup>24</sup>; however, its levels increased several-fold ( $P < .01$ ) upon culture with NK cells (which do not produce IFN- $\gamma$ ; data not shown). The increase of PGE2 production was abolished when the PGE2 inhibitor NS-398 was added to the culture. Table 1 shows the individual values with the mean and SD of 6 experiments performed in which the supernatants were

**Table 1. Production of PGE2 by MSCs cultured alone or cocultured with NK cells (in the presence or in the absence of the PGE2 inhibitor NS-398)**

Experiment no.	MSC	NK plus MSC	NK plus MSC plus NS-398
1	11250	36000	1000
2	11250	37500	1500
3	3600	23000	45
4	3600	20000	40
5	1000	16500	2500
6	1000	120000	2700
Mean	4090	42167	1298
SD	4208	39078	1157

Data refer to the levels of PGE2 expressed as pg/mL. The levels of PGE2 were evaluated in supernatants after 72 hours of culture.

collected after 72 hours of culture. We also investigated the role of IDO and PGE2 in the inhibition of NK-mediated cytotoxicity. To this aim, cytotoxicity experiments were performed in which iDCs or different leukemia or neuroblastoma cell lines were used as targets. Polyclonal NK-cell populations, derived from different donors, were used as effectors. These cells had been cultured for 6 days either alone or with MSCs in the presence or in the absence of specific inhibitors. Figure 5B shows the results of 5 independent experiments using iDCs, SKNBE, or MM6 as target cells. When NK cells were cultured in the presence of MSCs, their capability of killing target cells was markedly reduced in comparison with that of NK cells cultured alone. In contrast to results obtained in proliferation assays, no significant restoration of NK-cell function was detected using the PGE2 inhibitor NS-398 or the IDO inhibitor 1-M-Trp. However, also in the case of cytotoxic activity, virtually complete restoration was observed in cultures in which IDO and PGE2 inhibitors were used simultaneously ( $P < .01$ ). These data further confirm that IDO and PGE2 may exert a synergistic effect in the immunosuppressive activity mediated by MSCs.

## Discussion

In the present study, we provide evidence that MSCs can exert a profound inhibitory effect on NK-cell function, because they can suppress not only the IL-2-induced cell proliferation, but also the generation of cytolytic activity and the production of cytokines. In addition, we show that the MSC-mediated inhibition primarily reflects the activity of the soluble factors IDO and PGE2.

Recently, it has been reported that MSCs can exert an inhibitory effect not only on the proliferation of resting NK cells, but also on cytotoxic activity and IFN- $\gamma$  production.<sup>15,24</sup> Here we report a more accurate analysis of MSC-induced inhibition of NK cell function and define the molecular basis of such inhibitory effect. First, we show an inhibition of the surface expression of Nkp30 and NKG2D activating NK receptors that are involved in NK-cell activation and target cell killing. In addition, no surface expression of the Nkp44 activating receptor (absent in resting NK cells and expressed on cell activation) occurred in NK cells cultured with MSCs. MSCs sharply inhibited the NK-mediated cytotoxic activity. In contrast to data reported by another group,<sup>15</sup> inhibition was documented not only in cytolytic assays against HLA class I<sup>+</sup> but also against HLA class I<sup>-</sup> target cells, such as SKNBE and HTLA-230 neuroblastoma cell lines. This apparent discrepancy is likely to reflect the type of HLA class I<sup>-</sup> tumor lines used as target cells. Thus, those target cells that are highly susceptible to

NK-mediated lysis are not only HLA class I<sup>+</sup> but also express multiple ligands for different triggering NK receptors.<sup>29</sup> However, various HLA class I<sup>+</sup> tumors may express a limited number of such ligands, thus being less susceptible to NK-mediated lysis. Lysis of these tumors was strongly inhibited by MSCs.

Another relevant result of our present study is the evidence that MSCs inhibit NK-cell function via the production of soluble factors, including IDO and PGE2. Moreover, we provide evidence that these mediators may act synergistically. This observation is particularly evident in the inhibition of NK-cell proliferation, in which the use of the PGE2 inhibitor NS-398 alone was virtually ineffective, whereas its simultaneous use with the IDO inhibitor 1-M-Trp completely restored NK-cell proliferation. A possible interpretation of this finding might be suggested by experiments performed in dendritic cells.<sup>30</sup> In these cells, PGE2 has been shown to induce the *de novo* expression of IDO mRNA. In these experiments, TNF- $\alpha$  was involved in the functional activation of IDO. Therefore, synthesis of IDO in MSCs may be induced both directly (by the exposure of MSCs to IFN- $\gamma$ ) and indirectly, upon autocrine stimulation of cells by PGE2. It is conceivable that in NK-MSC interactions, IFN- $\gamma$  and TNF- $\alpha$ , secreted by NK cells upon activation (eg, promoted by IL-2), could induce production of PGE2 in MSCs which, in turn, may further promote the synthesis and activity of IDO (in combination with IFN- $\gamma$  and TNF- $\alpha$ ). Experiments to precisely define the molecular mechanism(s) involved are in progress in our laboratory.

MSCs were originally shown to exert a strong inhibitory effect on T-cell activation and function.<sup>23-25,31-33</sup> More recently, inhibition also has been observed on dendritic cells (DCs),<sup>34-36</sup> B cells,<sup>37</sup> and NK cells.<sup>14-16</sup> In this context, we reported that MSCs can block the IL-2-induced proliferation of fresh peripheral blood NK cells. This inhibitory effect on different cells involved in the immune response, primarily on T lymphocytes, offered an important clue for the use of MSCs as a tool to treat (or prevent) GvHD. Thus, the use of MSCs may become a common approach in BM transplantation not only for their possible beneficial effect on the engraftment of hematopoietic stem cells,<sup>8,38,39</sup> but also for their immunosuppressive properties.<sup>4</sup> On the other hand, NK cells have been shown to play a central role in the successful outcome of haploidentical BM transplantation to treat AML.<sup>40</sup> NK cells derived from the HSCs of the donor can exert a direct GVL effect, provided they express KIRs that do not recognize one or more HLA class I alleles of the patient. Our recent study on NK-MSC interactions not only provided evidence of a strong MSC-mediated antiproliferative effect on NK cells but also demonstrated that IL-2-activated NK cells can efficiently kill both allogeneic and autologous MSCs. Killing reflects the fact that MSCs are characterized by low levels of HLA class I antigens and also express several ligands recognized by activating NK receptors. In the present study, NK cells and

MSCs were derived from different donors (because MSCs were obtained from the BM of pediatric patients, from whom it was not possible to obtain sufficient numbers of fresh NK cells). However, as mentioned above, the results of the interaction between NK cells and autologous or allogeneic MSCs were undistinguishable. Therefore, it is conceivable that also in an autologous setting MSCs would inhibit NK-cell effector function. These data should be taken into account in designing novel protocols of adoptive immunotherapy in which both MSCs and NK cells may be infused into the patient to improve the clinical outcome of HSCT. Indeed, the adoptive transfer of activated NK cells could potentially kill MSCs if these are infused shortly before or simultaneously with NK cells. In addition, MSCs could inhibit NK-cell proliferation and function. In conclusion, our present study clearly shows that in addition to inhibiting NK-cell proliferation, MSCs markedly suppress major NK-effector functions, such as cytolytic activity and cytokine production. Taken together, these effects may result in a profound inhibition of NK-cell activities. This could have negative effects on the NK-mediated GVL, particularly in the haploidentical HSC transplantation setting. However, it is obvious that further confirmation of the relevance of our *in vitro* findings will require suitable *in vivo* studies in animal models.

## Acknowledgments

We thank Dr Angela Pistorio for the invaluable help in statistical analysis of data.

This work has been supported by grants awarded by Associazione Italiana per la Ricerca sul Cancro (AIRC), Istituto Superiore di Sanità (ISS), Ministero della Salute; Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR); FIRB-MIUR progetto cod. RBNE017B4C; Fondazione Compagnia di San Paolo, Torino, Italy; and European Union FP6, LSHB-CT-2004-503319-AI-loStem. The European Commission is not liable for any use that may be made of the information contained.

## Authorship

Contribution: G.M.S. designed and performed the research and wrote the paper; A.C. performed the research; H.A. performed the research and analyzed the data; F.B. provided selected samples; M.C.M. provided financial support and analyzed the data; L.M. designed the research, analyzed the data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no conflict of interest.

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