Mesenchymal stromal cells of the bone marrow and natural killer cells: cell interactions and cross modulation

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Received: 8 September 2017 / Accepted: 9 January 2018 / Published online: 19 January 2018
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Abstract

Bone marrow-derived mesenchymal stromal cells (BM-MSCs) are multipotent progenitor cells that have shown promise for several different therapeutic applications. As they are able to modulate the function of several types of immune cells, BM-MSCs are highly important in the field of cell-based immunotherapy. Understanding BM-MSC-natural killer (NK) cell interactions is crucial for improving their therapeutic efficiency. Here, we observed that the type of NK cell-activating cytokine (e.g., IL-2, IL-12, IL-15 and IL-21) strongly influenced the outcomes of their interactions with BM-MSCs. The expression patterns of the ligands (CD112, CD155, ULBP-3) and receptors (LAIR, NCR) mediating the cross-talk between BM-MSCs and NK cells were critically modulated following co-culture. BM-MSCs partially impaired NK cell proliferation but up-regulated their secretion of IFN- γ and TNF- α . As they are cytotoxic, activated NK cells induced the killing of BM-MSCs. Indeed, BM-MSCs triggered the degranulation of NK cells and increased their release of perforin and granzymes. Interestingly, activated NK cells induced ROS generation within BM-MSCs that caused their decreased viability and reduced expression of serpin B9. Collectively, our observations reveal that BM-MSC-NK cell interactions may impact the immunobiology of both cell types. The therapeutic potential of BM-MSCs will be significantly improved once these issues are well characterized.

Keywords BM-MSCs · NK cells · Immunomodulation · Cell interaction

Introduction

Mesenchymal stromal cells (MSCs) are multipotent progenitor cells present in nearly all tissues. MSCs were first identified within the bone marrow (BM) stroma, where they showed

the potential not only to support hematopoietic stem cells (HSCs) but also to differentiate into various mesodermal cell lineages (Friedenstein et al. 1974b; Friedenstein et al. 1974a; Prockop 1997). In addition to their tissue repair and regeneration abilities (Panetta et al. 2009), MSCs display immunoregulatory effects towards both innate and adaptive immune cells. The immunomodulatory potential of BM-MSCs has been successfully shown by delaying the development of acute graft versus host disease (GVHD), a critical issue following hematopoietic stem cell transplantation (HSCT) (Dunavin et al. 2017), and prolonging the viability of allogeneic skin grafts (Bartholomew et al. 2002). During the early reconstitution phase post-HSCT, the antitumor activities of natural killer (NK) cells are of particular significance in the graft-versus-leukemia (GVL) response, which is essential to prevent disease progression and leukemia relapse (Verneris 2013). The biology of NK cells is tightly regulated by a set of cell surface receptors (activating or inhibiting) and diverse pro-inflammatory cytokines (IL-2, IL-12, IL-15, IL-18 and IL-21) (de Rham et al. 2007). NK-activating receptors, including DNAM-1, NKG2D, NKP46, NKP44 and NKP30, interact with their cell target ligands, such as MHC class I-related A

Mehdi Najar and Mohammad Fayyad-Kazan are co-first authors with equal contribution.

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Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12079-018-0448-4>) contains supplementary material, which is available to authorized users.

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and B molecules (MICA/B), UL16-binding proteins (ULBPs), the poliovirus receptor (PVR) and Nectin-2 (Wu et al. 1999; Moretta et al. 2001; Moretta and Moretta 2004; Joyce and Sun 2011). The engagement of inhibitory receptors, such as LAIR-1, KIR2DL1/2 and LT2, with their ligands, such as collagen and HLA-A/B/C/G, prevents NK cell activation and ensures tolerance to healthy tissues (Solana et al. 2007; Joyce and Sun 2011). Once activated, NK cells become cytotoxic and pro-inflammatory by releasing perforin and granzymes, as well as secreting IFN- γ and TNF α , respectively (Biron et al. 1999; Trapani and Smyth 2002). Contrasting results concerning NK cells and MSCs have been previously reported (Sotiropoulou et al. 2006; Spaggiari et al. 2006; Lupatov et al. 2017). In the present study, we demonstrated that the crosstalk between BM-MSCs and NK cells has important effects on their respective immunologic profiles and behaviors. Remarkably, the outcomes of these effects were strongly dependent on the type of cytokines used to activate NK cells. Further, we demonstrate for the first time that activated NK cells induced ROS generation within BM-MSCs, which caused their reduced viability and expression of serpin B9. Thus, a deeper understanding of these issues will allow the design of more effective treatment strategies for HSCT.

Materials and methods

Ethical considerations

The present study was performed in accordance with the Declaration of Helsinki (1964) and was approved by the local ethical committee of the “Institut Jules Bordet” (Belgium). All samples were obtained from healthy donors who provided informed written consent.

BM-MSCs

BM was harvested from the sternum or iliac crest of seven healthy volunteers. Mononuclear cells (MNCs) were isolated from bone marrow aspirates by density-gradient centrifugation (Linfosep; Biomedics) and washed in Hank's buffered salt solution (HBSS; Lonza). MNCs were seeded at a cell density of 2×10^4 cells/cm² in low-glucose DMEM (DMEM-LG; Lonza) supplemented with 15% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 0.5% (v/v) antibiotic/antimycotic solution (all from Life Technologies). Cells were incubated at 37 °C in a 5% CO₂ humidified atmosphere. After 48 h, non-adherent cells were removed by washing, and the medium was changed twice per week. When subconfluency (80–90%) was achieved, adherent cells were harvested using TrypSelect (Lonza) and expanded by replating at a lower density (200 cells/cm²). The cell morphology was determined by phase contrast microscopy (100X

magnification) using an inverted fluorescence microscope (Leica, Diegem, Belgium). BM-MSCs were defined following the International Society for Cellular Therapy (ISCT) criteria (Dominici et al. 2006).

Immune cells

Mononuclear cells from peripheral blood (PB) samples were obtained by density-gradient centrifugation (LinfoSep, Biomedics, Madrid, Spain), washed in Hank's Balanced Salt Solution (HBSS, Lonza Europe, Verviers, Belgium) and seeded at 2×10^4 cells/cm² in Dulbecco's Modified Eagle's Medium with low glucose (DMEM-LG, Lonza) supplemented with 15% fetal bovine serum (FBS, Sigma–Aldrich, Bornem, Belgium), 2 mM L-glutamine and 50 U/ml penicillin (both from Lonza). Primary NK cells, CD3⁺ cells and CD14⁺ cells were purified from PBMCs using an NK Cell Isolation Kit, a CD3 or CD14 microbeads, respectively, based on MACS system technology (Miltenyi Biotec GmbH, Bergisch, Germany) following the manufacturer's instructions. As assessed by flow cytometry, the purity of the isolated cells was always greater than 95%.

For PBMC activation, a cocktail of phytohemagglutinin-P (PHA-P) (5 μ g/ml; Sigma) and IL-2 (10 ng/ml; R&D Systems) was used. For NK cell activation, IL-2 (10 ng/ml; R&D Systems), IL-15 (20 ng/ml; R&D Systems), IL-12 (20 ng/ml; R&D Systems) or IL-21 (30 ng/ml; eBioscience) were assessed.

Co-culture of BM-MSCs and immune cells

BM-MSCs from passage two (P2) were plated at 5×10^4 cells/cm² in a flat-bottomed 24-well plate. Following overnight adherence, 25×10^4 PBMCs, NK cells, CD3⁺ cells or CD14⁺ cells were added to MSCs (cell ratio of 5:1), and the culture was maintained for five days in RPMI-1640 medium supplemented with 10% FBS. Of note, NK cells and all immune cells were pre-activated with different cytokines and then added to the MSCs.

Cell viability

Trypan blue exclusion assays (Lonza) and 7 amino-actinomycin (7-AAD) staining assays (BD Biosciences) were used to examine the effects of immune cells on BM-MSCs viability. Following co-culture with the indicated immune cells, the supernatants were removed, adherent cells were washed with PBS (Lonza), harvested using TrypSelect (Lonza) and centrifuged. The cell pellets were suspended and stained with 0.4% trypan blue solution (Lonza). Cell viability was calculated with the following formula: % viable cells = $[1.00 - (\text{Number of blue cells} \div \text{Number of total cells})] \times 100$. BM-MSCs viability was also determined using the BD

Via-Probe™ viability staining solution (7-AAD). Following the centrifugation step, cells were first stained with an anti-CD45-VioBlue (Miltenyi)-labeled monoclonal antibody to exclude immune cells and then stained with 7-AAD solution. Absolute volumetric cell counting was performed using a MACSQUANT® flow cytometer.

Lactate dehydrogenase assay

The CytoTox96 Non-Radioactive Cytotoxicity Assay kit (Promega Benelux) was used to measure the cytotoxic activity of NK cells. Upon cell lysis, lactate dehydrogenase (LDH), a stable cytosolic enzyme, was released and quantitatively measured using an appropriate plate reader. Effector (E) cells (NK cells) and target (T) cells (BM-MSCs) were co-cultured for 4 h at a cell ratio of 5:1. Supernatants were then collected and incubated with the substrate mix for 30 min. The reaction was stopped by adding 1 M acetic acid. LDH release was measured in triplicate for each E:T ratio. Spontaneous LDH release was determined by incubating either the effector or target cells alone. Target Maximum LDH release was determined by adding the lysis solution (0.9% (v/v) Triton X-100). The cytotoxicity (%) was calculated for each E:T ratio according to the following formula: $100\% \times [(\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}) / (\text{Target Maximum} - \text{Target Spontaneous})]$. In some experiments, antibody mediated blocking assays of the interaction of CD112 and CD155 with CD226 were performed by using 10 µg/mL of monoclonal anti-human CD226 antibody (R&D Systems).

Flow cytometry phenotyping

BM-MSC-mediated expression of NK ligands was determined by flow cytometry using the following human monoclonal antibodies: CD112-PE (BioLegend Europe, San Diego, CA, USA), CD155-PE (BioLegend Europe, San Diego, CA, USA), and ULBP3-PE (R&D Systems Europe, Abingdon, United Kingdom).

The expression patterns of natural cytotoxicity receptors (NCRs) exhibited by NK cells was assessed by flow cytometry using the following human monoclonal antibodies: CD226 (DNAM-1)-APC (MACS, Auburn, CA, USA), CD314 (NKG2D)-APC, CD335 (NKp46)-PE, CD336 (NKp44)-PC5 and CD337 (NKp30)-PE (all from Beckman Coulter, Marseille Cedex, France).

The expression of leukocyte-associated Ig-like receptor (LAIR) by NK cells was analyzed by flow cytometry using the following human monoclonal antibodies: CD305 (LAIR-1)-PerCP/Cy5.5 (BioLegend Europe, San Diego, CA, USA) and CD306 (LAIR-2)-FITC (Sino Biological, Inc., Beijing, China).

NK cell proliferation

To assess NK cell proliferation during co-culture with BM-MSCs, we used the carboxyfluorescein diacetate-succinimidyl ester (CFDA-SE) labelling assay (CellTrace™ CFSE Cell proliferation kit, Invitrogen, Molecular Probes, Eugene, OR, USA) following the manufacturer's instructions. Briefly, activated NK cells were labelled with 5 µM CFDA-SE dye (10 min at 37 °C) prior to incubation with BM-MSCs. After 5 days, NK cells were collected from the co-cultures, and their proliferation was determined by flow cytometry analysis of their CFSE fluorescence.

Enzyme-linked immunosorbent assay

The protein levels of IFN-γ (R&D Systems; Minneapolis, USA; sensitivity less than 8.0 pg/mL), TNFα (R&D Systems; Minneapolis, USA; sensitivity of 1.6 pg/mL), perforin (Abcam; France; sensitivity less than 40 pg/mL), and granzymes A and B (BioVendor; Germany; sensitivity of 0.4 pg/mL and 0.2 pg/mL, respectively) in the cell culture supernatants were measured using ELISA techniques following the manufacturers' guidelines.

NK cell degranulation assay

The BD FastImmune CD107a-APC (BD Biosciences, San Jose, CA, USA) reagent is designed to assess the surface expression of CD107a by flow cytometry. CD107a, a lysosomal-associated membrane protein (LAMP-1) residing in cytolytic granule membranes, is usually used to identify the degranulation activity of cytolytic immune cells. Briefly, following co-culture with BM-MSCs, NK cells were collected and incubated for 4 h at 37 °C in the presence of CD107a-APC reagent.

Measurement of ROS

The production of ROS in BM-MSCs following co-culture with NK cells was measured using the 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) probe (Sigma). 2',7'-DCF-DA represents a cell-permeable non-fluorescent probe that upon oxidation is de-esterified intracellularly and converted to highly fluorescent 2',7'-dichlorofluorescein (DCF). After co-culture, BM-MSCs were collected and stained with 15 µM DCF-DA probe for 15 min at 37 °C according to the manufacturer's guidelines. BM-MSCs were then washed with PBS (Lonza) and subjected to a flow cytometric analysis of DCF fluorescence intensity as a readout of ROS production. In some experiments, BM-MSCs were pre-treated or not during 1 h with 10 mM of the ROS inhibitor N-acetyl-L-cysteine (NAC, Sigma) before H₂O₂ treatment (100 µM) or co-culture with IL-2-activated NK cells.

Expression of serpins

Following co-culture with NK cells, BM-MSCs cells were collected, fixed and permeabilized using FIX&PERM® reagents according to the manufacturer's instructions (Invitrogen). MSCs were intracellularly stained via incubation with monoclonal antibodies targeting alpha-1-antitrypsin (Serpina1-FITC; Abcam), antithrombin III (Serpina1; Abcam) or serpinB9 antibody (Abcam). Cells were then stained with PE-conjugated goat anti-mouse F(ab')₂ (Dako) and analyzed by flow cytometry.

Flow cytometry analysis

Following cell labelling, the data were acquired and analyzed on a MacsQuant analyzer (Miltenyi Biotec).

Statistical analysis

Data are presented as the means $\pm$ SEM of seven independent experiments and analyzed using paired Student's *t*-tests. *P*-Values < 0.05 (*), < 0.01 (**), and < 0.001 (***) were considered significant.

Results

ISCT compliance of BM-MSCs

BM-MSCs were defined according to the ISCT guidelines (Krampera et al. 2013). In our cultures, the cells displayed a fibroblast-like shape and capacity to adhere to plastic. They were also able to differentiate into osteoblasts, chondrocytes and adipocytes, confirming their multilineage potential. In terms of phenotype, they were positive ($>95\%$) for CD73, CD90 and CD105, but negative ($<5\%$) for CD14, CD19, CD34, CD45 and HLA-DR.

BM-MSCs are killed by activated PBMCs

When activated by a cocktail of PHA/IL-2, PBMCs triggered the death of BM-MSCs. Using phase contrast microscopy, cultures in the presence of activated, but not un-activated, PBMCs showed the absence of BM-MSCs with typical fibroblastic-like morphology (Fig. 1a). Consistently, the trypan blue exclusion assay demonstrated that the viability of BM-MSCs was significantly altered (from 100% to $36 \pm 8.4\%$) by the presence of activated PBMCs (Fig. 1b).

NK cells are responsible for BM-MSC death

To determine which immune cell population from activated PBMCs was involved in triggering their death, BM-MSCs

were co-cultured with all PBMCs, PBMCs lacking CD3, CD14 or CD56 cell populations or immuno-selected CD3⁺, CD14⁺ or CD56⁺ cell fractions (Fig. 2). PHA/IL-2-activated-PBMCs caused a significant reduction in the percentage of viable BM-MSCs (from 100% to $39 \pm 2.9\%$). Depletion of CD3⁺ and CD14⁺ cell populations did not ameliorate the viability of BM-MSCs. Consistently, neither purified CD3⁺ nor CD14⁺ cell fractions caused a decrease in the percentage of viable BM-MSCs. These observations indicate that these immune cells do not appear to account for BM-MSC cell death. Intriguingly, activated PBMCs lacking the CD56 cell population were not efficient in killing BM-MSCs. Therefore, purified CD56⁺ cell fractions induced a substantial decrease in the percentage of viable BM-MSCs (from 100% to $20 \pm 3.8\%$), confirming that NK cells are primarily involved in BM-MSC death.

NK cytokine activation differently induces BM-MSC death

Pro-inflammatory cytokines differentially triggered NK cell-mediated killing of BM-MSCs. Observing the cultures using phase contrast microscopy, we noted that BM-MSCs were less important in the presence of NK cells activated by IL-2, IL-12 or IL-15, but not IL-21 (Fig. 3a). Under the same conditions, a substantial reduction in the percentage of viable BM-MSCs was observed following co-culture with IL-2-, IL-12- and IL-15-activated NK cells (from 100% to $28.1 \pm 0.6\%$, $46.6 \pm 0.8\%$ and $34.6 \pm 1.5\%$, respectively) and to a lesser extent with IL-21-activated NK cells (from 100% to $63.8 \pm 4.5\%$) (Fig. 3b). The 7-AAD assay results corroborated these results, as $75.6 \pm 1.7\%$, $56 \pm 1.7\%$, $67.6 \pm 2.5\%$ and $22 \pm 2.1\%$ of BM-MSCs died following co-culture with IL-2-, IL-12-, IL-15- and IL-21-activated NK cells, respectively (Fig. 3c).

Activated NK cells induce cytotoxicity in BM-MSCs

Interestingly, activated NK cells induced cytotoxicity in BM-MSCs, as demonstrated via the measurement of LDH. A striking cytotoxic response was detected in BM-MSCs co-cultured with IL-2-, IL-12-, IL-15- or IL-21-activated-NK cells ($61.1 \pm 3.6\%$, $51.84 \pm 3.7\%$, $43.6 \pm 2.4\%$ and $32.7 \pm 2.7\%$ cytotoxicity, respectively) versus un-activated NK cells ($4.8 \pm 1.1\%$) (Fig. 3d). Furthermore, antibody mediated blocking assays of the interaction of CD112 and CD155 with CD226 has not rescued BM-MSCs from NK cell mediated cytotoxicity (Fig. 3e).

NK ligand expression by BM-MSCs

The constitutively high expression of CD112 (Nectin-2) and CD155 by BM-MSCs ($84.3 \pm 1.5\%$ and $92.3 \pm 1.5\%$, respectively) showed no significant changes following co-culture with either un-activated ($91.3 \pm 3.2\%$ and $91.3 \pm 1.5\%$,

Fig. 1 BM-MSCs are killed by activated PBMCs. BM-MSCs were cultivated for 5 days either alone or in the presence of un-activated or PHA/IL-2-activated PBMCs. **a** Morphology of BM-MSCs (100× objective). **b** Cell viability measured by trypan blue exclusion assay.

The values reported represent the means $\pm$ SEM of seven independent experiments, each performed in triplicate. *** $p < 0.01$ vs. MSCs; Paired Student's *t*-test

Fig. 2 NK cells are responsible for BM-MSc death. BM-MSCs were cultivated for 5 days either alone or in the presence of PHA/IL-2-activated PBMCs, PHA/IL-2-activated PBMCs lacking CD3, CD14 or CD56 populations, or IL-2-activated purified CD3⁺, CD14⁺ or CD56 cell fractions. Cell viability was measured by trypan blue exclusion assay. The percentage of viable BM-MSCs was calculated, and the values reported represent the means $\pm$ SEM of seven independent experiments, each performed in triplicate. * $p < 0.05$; *** $p < 0.001$ vs. MSCs; Paired Student's *t*-test

respectively) or IL-21-activated NK cells ($84.3 \pm 2.1\%$ and $84.3 \pm 1.1\%$, respectively). For CD112 and CD155 expression by BM-MSCs, a slight reduction was observed in the presence of IL-12-activated NK cells ($70.3 \pm 1.5\%$ and $83.3 \pm 2.5\%$, respectively), and a more striking decrease was observed in the presence of IL-2-activated NK cells ($28.6 \pm 3.5\%$ and $33 \pm 3.6\%$, respectively) and IL-15-activated NK cells ($19 \pm 2.6\%$ and $25 \pm 1.7\%$, respectively) (Fig. 4a and b). ULBP3 expression did not show any significant changes in all tested conditions (Fig. 4c).

NK cell expression of NCR

The expression profile of natural cytotoxicity receptors (NCRs) differed in a manner dependent on the type of cytokine used to activate NK cells, as well as on the presence or absence of BM-MSCs (Table 1).

In the absence of BM-MSCs, CD226 (DNAM-1) expression by un-activated NK cells ($48.4 \pm 2.4\%$) was clearly increased in IL-2- and IL-21-activated-NK cells ($82 \pm 2.5\%$ and $79.8 \pm 2.5\%$, respectively) and to a lesser degree in IL-12- and

Fig. 3 NK cytokine activation differentially induces cytotoxicity in BM-MSCs. BM-MSCs were cultivated for 5 days either alone or in the presence of un-activated or IL-2-, IL-12-, IL-15- or IL-21-activated NK cells. **a** Morphology of BM-MSCs (100× objective). **b** BM-MSC viability as measured by trypan blue exclusion assay. **c** BM-MSC death, as determined by 7 amino-actinomycin (7-AAD) staining assay. **d** Lactate Dehydrogenase (LDH) assay for measuring cellular toxicity. **e** Antibody

mediated blocking assays of the interaction of CD112 and CD155 with CD226 during the LDH assay by using 10 µg/mL of monoclonal anti-human CD226 antibody (R&D Systems). The percentage of cytotoxicity was calculated as described in the Materials and Methods section, and the values reported represent the means $\pm$ SEM of seven independent experiments, each performed in triplicate. $^{**}p < 0.01$ vs. MSCs; Paired Student's *t*-test

IL-15-activated-NK cells ($72.2 \pm 3.2\%$ and $69.8 \pm 3.4\%$, respectively). Following co-culture with BM-MSCs, CD226 expression prominently decreased in IL-2- and IL-21-activated NK cells ($53.3 \pm 2.1\%$ and $44.6 \pm 4.5\%$, respectively). On the other hand, co-culture of BM-MSCs with IL-12-activated NK cells modestly reduced CD226 expression ($61.6 \pm 1.5\%$) and had no significant effects on IL-15-activated NK cells ($73 \pm 3.6\%$) (Table 1).

Before co-culture with BM-MSCs, CD314 (NKG2D) expression in un-activated NK cells ($27.4 \pm 2.1\%$) was substantially increased in IL-2-, IL-15- and IL-21-activated-NK cells ($55.2 \pm 3.5\%$, $51.4 \pm 2.7\%$ and $49.4 \pm 2.1\%$, respectively), whereas only a small increase was noted in IL-12-activated-NK cells ($35 \pm 4\%$) (Table 1). Following co-culture with BM-MSCs, CD314 expression was reduced in un-activated-, IL-2- and IL-21-activated-NK cells ($15.3 \pm 2.5\%$, $36.6 \pm 2.5\%$ and

Fig. 4 NK ligand expression by BM-MSCs. BM-MSCs were cultivated for 5 days either alone or in the presence of un-activated or IL-2-, IL-12-, IL-15- or IL-21-activated NK cells. The expression levels of CD112, CD155 and ULBP3 in BM-MSCs were analyzed by flow cytometry using monoclonal antibodies as indicated in the Materials and Methods

section. The data are presented as the means $\pm$ SEM of the percentages of MSCs that expressed a given marker from seven independent experiments, each performed in triplicate. $^{***}p < 0.01$ vs. NK cells; Paired Student's *t*-test

Table 1 NK cell expression profiles of NCRs

CD226	NK	NK IL-2	NK IL-12	NK IL-15	NK IL-21
—	48.4 ± 2.4	82 ± 2.5	72.2 ± 3.2	69.8 ± 3.4	79.8 ± 2.5
+ BM-MSCs	53.6 ± 1.52	53.3.3 ± 2.1**	61.6 ± 1.52 *	73 ± 3.6	44.6 ± 4.5 **
CD314	NK	NK IL-2	NK IL-12	NK IL-15	NK IL-21
—	27.4 ± 2.1	55.2 ± 3.5	35 ± 4	51.4 ± 2.7	49.4 ± 2.1
+ BM-MSCs	15.3 ± 2.5	36.6 ± 2.5 **	31.6 ± 2.1	43.3 ± 4.5	29.6 ± 3.5 *
CD335	NK	NK IL-2	NK IL-12	NK IL-15	NK IL-21
—	29.6 ± 1.1	60.2 ± 2.5	47.2 ± 2.9	53.4 ± 3.6	51.6 ± 2.4
+ BM-MSCs	36.3 ± 3.6	59.3 ± 3.7	53.3 ± 5.6	38.3 ± 2.1 *	53 ± 2
CD336	NK	NK IL-2	NK IL-12	NK IL-15	NK IL-21
—	41 ± 1.6	66 ± 3.2	56 ± 4.4	71.6 ± 2.4	58.2 ± 2.6
+ BM-MSCs	32 ± 3 **	32.3 ± 2.5 **	31.3 ± 1.5 **	28 ± 2.6 **	22.3 ± 2.5 **
CD337	NK	NK IL-2	NK IL-12	NK IL-15	NK IL-21
—	25.2 ± 3.2	62.8 ± 3.7	35 ± 3.8	69.6 ± 2.3	35 ± 3.4
+ BM-MSCs	7 ± 1 **	51.3 ± 1.5 *	33 ± 2	50 ± 1 **	12 ± 2.6 *

Un-activated or IL-2-, IL-12-, IL-15- or IL-21-activated NK cells were cultivated for 5 days, in either the absence or presence of BM-MSCs. The expression levels of CD226, CD314, CD335, CD336 and CD337 antigens on NK cells were examined by flow cytometry using the monoclonal antibodies indicated in the Materials and Methods section. The data are presented as the means ± SEM of the percentages of NK cells that expressed a given marker from seven independent experiments, each performed in triplicate. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. NK, NK IL-2/ NK IL-12/ NK IL-15/ NK IL-21-activated cells; Paired Student's *t*-test

29.6 ± 3.5%, respectively) (Table 1). However, co-culture of BM-MSCs with IL-12- or IL-15-activated NK cells only slightly decreased CD314 expression (31.6 ± 2.1% and 43.3 ± 4.5%, respectively) (Table 1).

In the absence of BM-MSCs, CD335 (NKp46) expression levels in un-activated NK cells (29.6 ± 1.1%) were doubled in IL-2-activated-NK cells (60.2 ± 2.5%) but showed a less prominent increase in IL-12-, IL-15- and IL-21-activated-NK cells (47.2 ± 2.9%, 53.4 ± 3.6% and 51.6 ± 2.4%, respectively) (Table 1). Following co-culture with BM-MSCs, CD335 expression partially but significantly decreased in IL-15-activated-NK (38.3 ± 2.1%), while no significant alterations were detected in un-activated, IL-2-, IL-12- or IL-21-activated-NK cells (Table 1).

Prior to co-culture with BM-MSCs, CD336 (NKp44) expression in un-activated NK cells (41 ± 1.6%) substantially increased in IL-15-activated-NK cells (71.6 ± 2.4%) but showed less prominent enhancement in IL-2-, IL-12- and IL-21-activated-NK cells (66 ± 3.2%, 56 ± 4.4% and 58.2 ± 2.6%, respectively) (Table 1). Following co-culture with BM-MSCs, CD336 expression was significantly reduced in un-activated-, IL-2-, IL-12-, IL-15- and IL-21-activated NK cells (32.3 ± 3.1%, 32.3 ± 2.5%, 31.3 ± 1.5%, 28 ± 2.6% and 22.3 ± 2.5%, respectively) (Table 1).

In the absence of BM-MSCs, CD337 (NKp30) expression in un-activated NK cells (25.2 ± 3.2%) strikingly increased in IL-2- and IL-15-activated-NK cells (62.8 ± 3.7% and 69.6 ± 2.3%, respectively), but less prominently in IL-12- and IL-21-

Fig. 5 NK cell expression of LAIR. BM-MSCs were cultivated for 5 days either alone or in the presence of un-activated or IL-2-activated NK cells. The expression levels of CD305 and CD306 in NK cells were analyzed by flow cytometry using monoclonal antibodies as indicated in

the Materials and Methods section. The data are presented as the means ± SEM of the percentages of NK cells that expressed a given marker from seven independent experiments, each performed in triplicate. *** $p < 0.01$ vs. NK cells; Paired Student's *t*-test

activated-NK cells ($35 \pm 3.8\%$ and $35 \pm 3.4\%$, respectively) (Table 1). Following co-culture with BM-MSCs, CD337 expression dramatically decreased in un-activated and IL-21-activated-NK cells ($7 \pm 1\%$ and $12 \pm 2.6\%$, respectively) (Table 1). A significant decrease in CD337 levels was also observed following co-culture of BM-MSCs with IL-2- and IL-15-activated NK cells ($51.3 \pm 1.5\%$ and $50 \pm 1\%$, respectively) (Table 1). No change in CD337 expression was detected in IL-12-activated NK cells following co-culture with BM-MSCs (Table 1).

It is noteworthy that the outcome of BM-MSCs-NK cells interaction was studied only in the context of IL-2 activation in most of subsequent experiments due to the fact that IL-2 was the most powerful activating cytokine for NK cells as well as the most potent cytokine inducing NK cell cytotoxicity and therefore mediating killing of target cells.

NK cell expression of LAIR

Un-activated NK cells displayed constitutively high CD305 expression ($94.3 \pm 4.2\%$) that remained unchanged upon either activation by IL-2 ($99.7 \pm 0.5\%$) or following co-culture with BM-MSCs ($98.2 \pm 2.4\%$) (Fig. 5a). In contrast, CD306 expression exhibited by un-activated NK cells ($59.3 \pm 4.7\%$) was prominently enhanced after activation by IL-2 ($90.7 \pm 4.7\%$) but was substantially reduced following co-culture with BM-MSCs (Fig. 5b).

Effects of BM-MSCs on NK cell proliferation

Depending on their activating cytokines, the proliferation of NK cells was differentially induced and differentially modulated by BM-MSCs (Fig. 6). In the presence of BM-MSCs, the proliferation of IL-12- and IL-21-activated NK cells was modestly but significantly reduced (from $85.6 \pm 0.6\%$ to $73.9 \pm 2.5\%$ and from $82.7 \pm 0.8\%$ to $69.2 \pm 1.7\%$, respectively). For IL-2- and IL-15-activated NK cells, their proliferation showed only a slight decrease (from $61.7 \pm 0.9\%$ to $56.4 \pm 1\%$ and from $77.1 \pm 1.4\%$ to $68.4 \pm 3.8\%$, respectively).

Effects of BM-MSCs on NK cell cytokine secretion

Constitutively, IL-2-activated NK cells secreted substantially more IFN- γ (197.9 ± 13.5 pg/ml) than TNF α (57.6 ± 3.4 pg/ml) (Fig. 7). The level of IFN- γ secreted by IL-2-activated NK cells was considerably increased, by nearly 6 times as much, to reach 1180.2 ± 110.6 pg/ml after co-culturing with BM-MSCs (Fig. 7a). On the other hand, the level of TNF α secreted by IL-2-activated NK cells slightly increased to 80.5 ± 14.5 pg/ml in the presence of BM-MSCs (Fig. 7b). In contrast to IL-2-activated NK cells, BM-MSCs had lightly elevated secretion of these cytokines from un-activated NK cells (Supplementary Table 1).

Fig. 6 Effects of BM-MSCs on NK cell proliferation. IL-2-, IL-12-, IL-15- or IL-21-activated NK cells were cultivated for 5 days either in the absence or presence of BM-MSCs. After labelling NK cells with CFSE, flow cytometry analysis was performed. The values reported represent the means $\pm$ SEM of 7 independent experiments, each performed in triplicate. * $p < 0.05$ vs. NK IL-2/ NK IL-12/ NK IL-15/ NK IL-21-activated cells; Paired Student's *t*-test

Effects of BM-MSCs on NK cell degranulation

As a marker of NK cell degranulation, the expression of CD107a was measured daily during 5 days of culture with BM-MSCs. In the absence of BM-MSCs, the percentage of CD107a expressing-NK cells constantly increased, reaching a maximum of 37.4 ± 1.4 by day 5 (Fig. 8). Interestingly, the presence of BM-MSCs exerted a positive effect on CD107 expression during this time interval. The percentage of CD107a-expressing NK cells was enhanced, from $13.2 \pm 1.5\%$ to $24.8 \pm 1.5\%$ at day 1, from $22.2 \pm 2.1\%$ to $33.4 \pm 2.4\%$ at day 2, from $29 \pm 1.56\%$ to $39.9 \pm 1.6\%$ at day 3, from $31.5 \pm 1.4\%$ to $44.2 \pm 3.7\%$ at day 4 and finally from $37.4 \pm 1.4\%$ to $56.3 \pm 3.1\%$ at day 5, following co-culture with BM-MSCs.

Effects of BM-MSCs on NK cell cytotoxic components

High perforin levels constitutively released by IL-2-activated NK cells (3867.6 ± 551.8 ng/ml) were significantly increased following co-culture with BM-MSCs (5889.6 ± 834.8 ng/ml) (Fig. 9a). Regarding granzymes (Fig. 9b and c), IL-2-activated NK cells displayed distinct secretion profiles, with granzyme A (2247.4 ± 357.6 ng/ml) constitutively produced at higher levels than granzyme B (381.5 ± 54.9 ng/ml). Following co-culture with BM-MSCs, the levels of secreted granzymes were significantly amplified, with granzyme A (6407.59 ± 840.7 ng/ml) at much higher levels than granzyme B (2011.9 ± 191.2 ng/ml). In comparison to IL-2-activated NK cells, BM-MSCs had moderately increased release of these cytotoxic components from un-activated NK cells (Supplementary Table 1).

Fig. 7 Effects of BM-MSCs on NK cell cytokine secretion. IL-2-activated NK cells were cultivated for 5 days either in the absence or presence of BM-MSCs. Cell-free supernatants were collected and then assayed for IFN- γ and TNF α levels via ELISA. The values are expressed

in pg/ml and represent the means $\pm$ SEM of 7 independent experiments, each performed in triplicate. ** $p < 0.01$; *** $p < 0.001$ vs. NK IL-2-activated cells; Paired Student's t -test. The results for un-activated NK cells are presented in Supplementary Table 1

Effects of NK cells on ROS generation by BM-MSCs

We evaluated ROS generation in BM-MSCs after co-culture with IL-2-activated NK cells. As shown in Fig. 10a, we first confirmed ROS generation in BM-MSCs upon exposure to H₂O₂ (for 30 min), which induced a strong oxidative stress response (positive control). Indeed, BM-MSCs showed a statistically significant increase in ROS production after H₂O₂ exposure. Moreover, this ROS generation was blocked by pretreatment with N-acetyl-L-cysteine (NAC), an ROS scavenger. In parallel, IL-2-activated NK cells induced substantial ROS generation in BM-MSCs after 18 h of co-culture (7.1 ± 2.8 -fold induction). Interestingly, the pretreatment with NAC significantly attenuated this ROS production

($p < 0.05$). We also demonstrated that induction of ROS in BM-MSCs was involved in their death after co-culture with IL-2-activated NK cells. Indeed, blocking ROS production via pretreatment of BM-MSCs with NAC rescued them from NK cell-mediated killing and therefore significantly increased their viability following co-culture with IL-2-activated NK cells (Fig. 10b).

Effects of NK cells on serpin expression by BM-MSCs

Constitutively, BM-MSCs exhibited distinct expression patterns of serpins, with B9 being the most highly expressed, followed by moderate C1 and slight A1. Following co-culture with IL-2-activated NK cells, BM-MSCs demonstrated different levels of reduced serpin expression. Serpin A1 and serpin B9 expression significantly decreased (from $13.9 \pm 2.3\%$ to $7.2 \pm 1.6\%$ and from $87.3 \pm 3.1\%$ to $37.5 \pm 4.4\%$, respectively), whereas serpin C1 showed no significant alterations (from $32.1 \pm 3.4\%$ to $27.7 \pm 2.9\%$) (Fig. 11a). As recently suggested by Mangan et al. (Mangan et al. 2016), serpin B9 might be inactivated by ROS generated within cytotoxic lymphocytes. We also observed the down-regulation of serpin B9 expression in BM-MSCs following the induction of ROS. As a positive control, H₂O₂ (known to be a strong inducer of ROS) pretreatment of BM-MSCs caused a dose-dependent decrease in serpin B9 expression. Such a decrease in serpin B9 expression in BM-MSCs was also observed following their co-culture with IL-2-activated NK cells. Pretreatment of BM-MSCs with NAC (a ROS inhibitor) before co-culture with IL-2-activated NK cells prevented the decrease in serpin B9 expression. This finding demonstrates that the reduction of serpin B9 expression within BM-MSCs was associated with ROS generation (Fig. 11b).

Fig. 8 Effects of BM-MSCs on NK cell degranulation. IL-2-activated NK cells were cultivated for 5 days either in the presence or absence of BM-MSCs. NK cell degranulation assays were performed, and the results were determined by flow cytometry. Data correspond to the percentages of NK cells that had externalized CD107a during the 4 h incubation period. The values reported represent the means $\pm$ SEM of 7 independent experiments, each performed in triplicate. ** $p < 0.01$; *** $p < 0.001$ vs. NK IL-2-activated cells. Paired Student's t -test

Fig. 9 Effects of BM-MSCs on NK cell cytotoxic components. IL-2-activated NK cells were cultivated for 5 days in the absence or presence of BM-MSCs. Cell-free supernatants were collected and assayed, using ELISAs, for their levels of perforin (a), granzyme A (b) and granzyme

B (c). Data represent the means $\pm$ SEM of the concentrations from seven independent experiments, each performed in triplicate. * $p < 0.05$; *** $p < 0.001$ vs. NK IL-2-activated cells; Paired Student's t -test. The results for un-activated NK cells are presented in Supplementary Table 1

Discussion

MSCs represent an important therapeutic tool for treating immunological complications, particularly following HSCT

(Bouchlaka et al. 2010). MSCs are known to interact with actors of both innate and adaptive immunity and modulate several of their features accordingly. Moreover, these regulatory roles are sensitive to the environment (Abdelrazik et al. 2011; Ribeiro

Fig. 10 Effects of NK cells on ROS generation by BM-MSCs. The production and role of ROS within BM-MSCs following their co-culture with IL-2-activated NK cells was measured using the 2'-7'-dichlorodihydrofluorescein diacetate (DCF-DA) probe. In some experiments, BM-MSCs were pre-treated or not for 1 h with 10 mM N-acetylcysteine (NAC) before H₂O₂ treatment (100 μ M used as a positive control) or co-culture with IL-2-activated NK cells. The viability of BM-MSCs was determined by flow cytometry after 7-aminoactinomycin D

(7AAD) and CD45-PE-CY7 staining. **a** Representative fluorescence flow cytometry histograms and mean fluorescence intensities (MFIs) of DCFDA, indicating levels of ROS generation in BM-MSCs, are presented. The gray line represents the fluorescence of untreated BM-MSCs. **b** The production of ROS was associated with NK cell-mediated killing of BM-MSCs. The values represent the means $\pm$ SEM of 7 independent experiments, each performed in triplicate. * $p < 0.05$; ** $p < 0.01$ vs. MSCs; Paired Student's t -test

Fig. 11 Effects of NK cells on serpin expression by BM-MSCs. The expression levels of Serpin A1, Serpin C1 and Serpin B9 within BM-MSCs were determined by flow cytometry using the monoclonal antibodies indicated in the Materials and Methods section. In some experiments, BM-MSCs were pre-treated or not 1 h with 10 mM of N-acetylcysteine (NAC, Sigma) before H₂O₂ treatment (50 and 100 μM used as positive controls) or co-culture with IL-2-activated NK cells. **a.** Data showing the percentages of positive BM-MSCs expressing each of

the indicated Serpins. **b.** Representative fluorescence flow cytometry histograms and the percentages of expression, showing the effects of IL-2-activated NK cells and the role of ROS on serpin B9 + BM-MSCs, are presented. The gray line represents a negative control (labeling with the secondary antibody only). The values represent the means ± SEM of 7 independent experiments, each performed in triplicate. * $p < 0.05$; ** $p < 0.01$ vs. MSCs; Paired Student's t-test

et al. 2013). NK cells are major immune components and are actively involved in the Graft-versus Leukemia (GVL) response (Yoon et al. 2015). However, the ability of BM-MSCs to tolerate the presence of activated NK cells and their capacity to modulate NK cell function have not been clearly elucidated. Thus, applying MSC-based approaches to treat problems following HSCT should not alter the beneficial role of NK cells during the GVL response (Wu et al. 1999). In this study, we sought to clarify the functional and phenotypical changes of NK cells, as well as MSCs, following their co-culture. For instance, the impact of MSCs on different immune killer cells showed contrasting results and are likely to be strongly dependent on routes and times of injection, cell-cell contact and cell proportion (Chieregato et al. 2012). Here, we observed altered behaviors during BM-MSC and NK cell interactions that depended greatly on the type of cytokines (IL-2, IL-12, IL-15 and IL-21) used to activate NK cells (Romee et al. 2014).

As previously shown, BM-MSCs are highly sensitive to IL-2-activated NK cell-mediated lysis, regardless of the MSC donor origin (HLA independent) (Spaggiari et al. 2006; Giuliani et al. 2014). In contrast, BM-MSCs were reported to be resistant to allo-specific cytotoxic T lymphocytes (CTLs) and NK cell lysis, which is surprising since MSCs express HLA class I antigens, which are targets for activating lysis (Rasmusson et al. 2003). In agreement with previous data (Poggi et al. 2005; Hoogduijn et al. 2011), the reduced viability and number of BM-MSCs observed following co-culture with activated PBMCs was dependent on the presence of activated NK cells, which are mainly involved in BM-MSC death and cytotoxicity. Nevertheless, a previous study showed that freshly isolated non-activated or IL-2-treated NK cells could both trigger killing of dental pulp stem cells (DPSCs) and BM-MSCs (Jewett et al. 2010). Comparably to lymphocytes (Najar et al. 2009), the immunomodulatory effects of

MSCs towards NK cells greatly depended on the origin of the MSCs and their doses (Ribeiro et al. 2013; Poggi and Zocchi 2014). Such sensitivity to NK cell lysis may explain the rapid disappearance of MSCs following their infusion. The recipient's immune status before and after allograft transplantation is highly associated with the survival and behavior of infused MSCs (Crop et al. 2011).

NK cell activation is thought to be determined by a balance between signals from activating and inhibitory receptors. Results have revealed that such activation step is regulated by distinct receptors and by a combination of co-activating receptors which may also participate but they are not determinant for the strength of activation and therefore for NK cell effector functions (Enk and Mandelboim 2014). Natural cytotoxicity receptors (NCR) including NKp46, NKp30, and NKp44 have been identified as the most prominent NK cell activating receptors and those that play a predominant role in target cell recognition and killing by NK cells (Pazina et al. 2017). In our present study, both ligands and receptors known to mediate the crosstalk between NK cells and BM-MSCs (Wu et al. 1999; Moretta et al. 2001; Moretta and Moretta 2004; Joyce and Sun 2011) showed differential expression and modulation following co-culture. Interestingly, the origin of MSCs, as well as inter-donor variations, may substantially influence the expression of ligands for DNAM-1 (CD112, CD155), for NKG2D (MICA/B and ULBPs), for CD244 (CD48) and for NCR (NKp46-Fc, NKp44-Fc and NKp30-Fc) receptors (Spaggiari et al. 2006; Götherström et al. 2011; DelaRosa et al. 2012) and consequently alter NK cell interactions and functions. We showed that the constitutively high expression of NK ligands, e.g., CD112 and CD155, was modulated following co-culture with activated NK cells in a manner dependent on the cytokine used. NK cell-mediated lysis could be triggered upon ligation of the NKG2D (CD314) receptor by its ligands, MIC-A and ULBP3, which are expressed by BM-MSCs (Poggi et al. 2005). This finding is in contrast with our observation reporting the absence of ULBP3 expression within BM-MSCs. Additional surface molecules such as the leukocyte adhesion molecule DNAX accessory molecule-1 (DNAM-1; CD226) appear to function as a co-receptor and might be implicated in the activation of NK cells following the binding to Nectin-2 or PVR (Bottino et al. 2003). Remarkably, DNAM-1 function is dependent on the surface expression of lymphocytes function-associated antigen 1 (LFA-1; CD18/CD11a) (Bryceson et al. 2006). Of importance, the expression of CD226 is reported to be associated to but not required for NK cell education (licensing) that allows NK cells to become functionally competent (He and Tian 2016). It is likely that DNAM-1 expression may contribute, but not be essential, to promote cellular interactions required for NK cell education (Wagner et al. 2017). The altered CD226 expression may account for the reduced NK cell activity by MSCs. Interestingly, MSCs may stimulate

NK cell functions despite the downregulation of CD226. The neutralization of CD226 has not rescued BM-MSCs from NK cell mediated cytotoxicity. In agreement, the interaction between bone marrow stromal cells and NK cells has been shown to result in NK cell triggering and activation independently of CD226 (Poggi et al. 2005; DelaRosa et al. 2012). CD226 as an accessory adhesion molecule seems to be non-relevant during NK-MSC co-cultures and that other signaling pathways are involved. Context-dependent modulation of NCRs and other NK cell receptors has shown contrasting results and may highlight differential capacities of BM-MSCs to alter the activation status of NK cells, as well as the involvement of different regulatory pathways (Spaggiari et al. 2006; Spaggiari et al. 2008; Pradier et al. 2011; Li et al. 2011; DelaRosa et al. 2012; Zhao et al. 2012; Ribeiro et al. 2013; Chatterjee et al. 2014a; Giuliani et al. 2014). Accordingly, the reduced NCR expression following co-culture with BM-MSCs might negatively affect the lysis effect achieved by NK cells. On the other hand, down-regulated expression of CD314, CD244 CD336 and CD337 on NK cells might significantly contribute to BM-MSC-mediated impairment of NK-cell function (Poggi et al. 2005; Sotiropoulou et al. 2006; Giuliani and Oudrhiri 2011). These contrasting outcomes suggest that the functions of both cell types are sensitive to their surrounding environment. Since these receptors have been found to interact with distinct specificities and activate distinct signaling pathways in response to diverse ligands (Pazina et al. 2017), their differential alteration may reflect distinct pathways by which MSCs can modulate NK-cell function. In particular, pro-inflammatory cytokines from ongoing immune responses, such as those used for NK cell activation, may critically modulate their pattern of receptors and therefore their features (de Rham et al. 2007).

In addition to NCRs, NK cells are endowed with a wide array of molecules, such as Leukocyte-Associated Immunoglobulin-like Receptor (LAIR), that mediate their interaction with extracellular matrix components and are thus potentially involved in their regulation. In addition to LAIR-1 (CD305), a collagen-receptor that impairs immune cell function, LAIR-2 (CD306) is likely a competitor for LAIR-1, thereby controlling its inhibitory potential (Lebbink et al. 2008). Within connective tissues, MSC–NK interactions occur in the presence of several extracellular-matrix proteins whose receptors are expressed on NK cells. In our study, BM-MSCs had no significant effect on the expression levels of both LAIR types, thus excluding a possible regulatory role of these pathways during BM-MSCs and NK cell crosstalk. Several different effects of NK cell interaction with several matrix proteins, such as collagen, have been reported in the literature. However, no direct evidence for the interaction of LAIR1 expressed by NK cells and collagen has been reported to date, despite the fact that several other immune cells are inhibited following such interactions (Poggi and Zocchi

2014). Recently, decidual MSCs (DMSCs) were shown to modulate the function of decidual NK (dNK) cells via an interaction between collagen and LAIR-1 (Fu et al. 2017). It has been demonstrated that collagen produced by DMSCs increased the expression of KIR2DL1 and IL-4, decreasing the expression of NKp30 and TNF- α . Because these receptors may trigger different signaling pathways, their modulation may reflect multiple regulatory ways of NK-cell function. The selective expression and modulation of several receptors and/or ligands can significantly influence the outcome of MSC and NK cell interactions. Thus, a better understanding of these diverse expression profiles and interactions are needed.

To display their biological effects, NK cells proliferate in response to immunological cytokines as part of productive or pathologic consequences of immunity (Warren 1996). BM-MSCs differentially modulated the proliferation of cytokine-activated NK cells with a partial effect exerted on IL-12- and IL-21-activated NK cells. To date, controversial results regarding the effect of MSCs on NK cell proliferation have been reported, with several studies describing a suppressive effect and others reporting a stimulatory one (Sotiropoulou et al. 2006; Spaggiari et al. 2006; Spaggiari et al. 2008; Pradier et al. 2011; Blanco et al. 2016). Although BM-MSCs significantly inhibited IL-2-triggered proliferation of resting NK cells, they had only a partial inhibitory effect on proliferating activated NK cells (Spaggiari et al. 2006). It is likely that the influence of MSCs on NK cells depends not only on the cell ratio but also on the activation status of NK cells. Such restricted ability of BM-MSCs to impair NK cell proliferation might be correlated with their altered viability and their differential modulation of NCR expression.

The ability of NK cells to secrete cytokines may be more important than their cytolytic function. NK cells have the potential to guide the developing immune response towards a Th1 response and to facilitate the inflammatory response (Warren 1996). Our data demonstrated that BM-MSCs promoted the secretion of IFN- γ and TNF- α by IL-2-activated NK cells. These cytokines, among others, are produced following NK cell activation and may participate in regulating inflammatory and immune responses independent, at least in part, of their cytotoxic functions (Perussia 1996). The striking difference in all results regarding MSCs and NK cells appears, to an extent, dependent on the experimental conditions, such as the cell ratio of the co-culture and, in particular, the type of cytokine used to activate NK cells (Thomas et al. 2014). Several studies have reported the ability of MSCs, regardless of their tissue origin, to decrease IFN γ and TNF α secretion from activated NK cells, thereby contributing to the inhibition of the immune response (Aggarwal and Pittenger 2005; Sotiropoulou et al. 2006; Pradier et al. 2011; Ribeiro et al. 2013; Chatterjee et al. 2014a; Chatterjee et al. 2014b). In contrast, our study highlighted a positive effect of BM-

MSCs on the cytokine secretion ability of activated NK cells. This enhanced cytokine secretion highlights a stimulatory effect of MSCs on NK cell activity, thus empowering their immunological effects (Poggi et al. 2005; Li et al. 2011; Almeida et al. 2012; DelaRosa et al. 2012; Noone et al. 2013; Thomas et al. 2014; Chatterjee et al. 2014a). Indeed, increased IFN- γ secretion from NK cells after contact with MSCs might improve the defense against infections at the site of injury but might additionally affect tissue regeneration (Thomas et al. 2014). Recently, a novel positive feedback loop involving the release of CCL2/MCP-1 by MSCs and IFN- γ by NK cells was reported. As they are immunostimulatory, MSCs reinforce NK cell activation and increase their inflammatory immune response (Cui et al. 2016). Targeting MSCs or their soluble factors to the patients' NK cells might improve the immune function after severe injury and thereby extend the indications for MSC therapy.

Killing of target cells is a complex, multi-stage process that concludes with degranulation of lytic granules, releasing perforin and granzymes at the immunological synapse. Upon delivery to a target cell, perforin mediates the generation of pores in membranes, allowing granzymes to access target cell cytoplasm and induce cell death. Therefore, lytic granules of NK cells are indispensable for normal NK cell cytolytic function (Krzewski and Coligan 2012). Increasing the expression of lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) on NK cells has been recognized as a marker of degranulation (Alter et al. 2004). Here, we showed that IL-2-activated NK cell expression of CD107a was constantly and time-dependently increased following co-culture with BM-MSCs. The percentage of CD107a-expressing IL-2 and IL-15-activated NK cells increased considerably in the presence of MSCs derived from human umbilical cords (ucMSCs) (de Witte et al. 2017). In line with our findings, by enhancing NK cell degranulation activity, MSCs of different types exerted a positive influence on NK cell activity despite using distinct pathways (Poggi et al. 2005; Götherström et al. 2011; DelaRosa et al. 2012; Cui et al. 2016). Indeed, enhanced CD107a expression has been strongly associated with increased NK cell cytokine secretion and lysis of target cells (Alter et al. 2004). Such intense degranulation of the cytotoxic granules will therefore be followed by a consequent discharge of their contents (e.g., perforin, granzymes A and B), strengthening the cytolytic activity of NK cells.

In the presence of BM-MSCs, the secretion of perforin was markedly enhanced, illustrating the increased level of NK cell degranulation. This high level of perforin, probably by creating several pores in the target-cell membrane, will thus mediate the rapid entrance of granzyme (Voskoboinik et al. 2006; Krzewski and Coligan 2012). Accordingly, the release of granzymes A and B by activated NK cells was notably enhanced by BM-MSCs, demonstrating an enhanced cytolytic capacity of NK cells. Indeed, granzymes (granule-secreted

enzymes), by triggering different apoptotic pathways, induce target cell death. While granzyme B activates an intracellular cascade of caspases leading to cell apoptosis, granzyme A additionally induces IL-1, TNF α and IL-6 expression from other immune cells. This finding correlates with our previous observations, suggesting that increased MSC secretion of IFN- γ and TNF- α by activated NK cells may favor an inflammatory immune response. Several studies have reported that the perforin and granzyme pools of NK cells are differentially altered by MSCs according to their origins, suggesting that modulation of the NK cell cytolytic machinery is not universal (Giuliani and Oudrhiri 2011; Pradier et al. 2011; Ribeiro et al. 2013; Chatterjee et al. 2014a). In parallel, IL-2-activated NK cells by inducing substantial generation of reactive oxygen species (ROS) in BM-MSCs, which is likely involved in their cell death. Interestingly, inhibition of these ROS rescued BM-MSCs from NK cell-mediated killing and therefore significantly increased their viability. Following co-culture, the increased release of granzymes from NK cells may trigger the production of ROS in MSCs, which therefore participate in cell death. Indeed, granzyme B (GB) may induce cell death in ROS-dependent manner. GB-induced ROS were found to be necessary for optimal apoptogenic factor release, rapid DNA fragmentation and lysosomal rupture. Consequently, GB-induced ROS significantly promoted cell death by favoring apoptosis (Jacquemin et al. 2015). In parallel, the results suggested that granzyme A could induce cell death via increasing the production of ROS by a caspase-independent death pathway (Williams and Henkart 2005). As ROS are essential second messengers for immune cells, modulating the levels of ROS may also be critical for the direction of the inflammatory response (Chen et al. 2016). In agreement with Padgett et al. (2013), by stimulating the secretion of cytokines, such as IFN- γ , TNF- α and IL-1 β , low levels of ROS may activate innate and adaptive immune responses, thus exacerbating immunity.

Although they have important immunological and cytotoxic roles, unrestrained granzyme activity may be deleterious by causing excessive tissue destruction and cellular apoptosis. Serpins, a family of protease inhibitors, can be used by MSCs to regulate granzyme activity and thus modulate NK cell-mediated killing (Kaiserman and Bird 2010). Here, we showed that BM-MSCs are characterized by differential expression of serpins, with serpin B9 being the most expressed, followed by serpin C1 and finally serpin 1A. To date, only serpin B9 (Serine protease inhibitor 9) has been ascribed a cytoprotective function in MSCs (El Haddad et al. 2011). After co-culture with activated NK cells, the expression of A1 and B9 serpins by BM-MSCs was considerably reduced and may have a negative impact on their defense strategy against granzymes. Of note, we have demonstrated that ROS increases within BM-MSCs after co-culture with activated NK cells are responsible for the reduced expression of serpin

B9. Consistently, Mangan et al. have concluded that serpin B9 might be inactivated by ROS as part of a mechanism regulating lymphocyte numbers, activity and viability (Mangan et al. 2016). An increased number and activity of granzymes will undoubtedly exacerbate NK cell-cytotoxicity and consequently intensify the death of MSCs. Indeed, both granzyme A- and B-mediated cytotoxicity is responsible for inducing cell death and loss of defense within MSCs in an ROS-dependent manner.

Conclusion

Collectively, we have demonstrated that several biological and immunological attributes of BM-MSCs and NK cells are substantially modulated following their co-culture. Due to NK cell activation, BM-MSCs showed reduced viability, altered ligand expression and impaired serpin defense in an ROS generation-dependent manner. In turn, activated NK cells are likely immunostimulated by BM-MSCs, as they demonstrated elevated cytokine secretion as well as cytotoxicity activity but only limited inhibition of their proliferation. Understanding such interactions between MSCs and NK cell is necessary to improve their therapeutic potential for specific cell-based applications.

Acknowledgements This project was supported by “Le Fonds National de la Recherche Scientifique, F.R.S.-FNRS” and the “Télévie”.

Compliance with ethical standards

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the ethics committee of the “Institut Jules Bordet” (Belgium) and with the 1964 Helsinki declaration and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

Conflict of interest All authors declare that they have no conflict of interest

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