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Interaction between Human NK Cells and Bone Marrow Stromal Cells Induces NK Cell Triggering: Role of NKp30 and NKG2D Receptors[1]

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In this study we have analyzed the interaction between in vitro cultured bone marrow stromal cells (BMSC) and NK cells. Ex vivo-isolated NK cells neoexpressed the activation Ag CD69 and released IFN-γ and TNF-α upon binding with BMSC. Production of these proinflammatory cytokines was dependent on ligation of ICAM1 expressed on BMSC and its receptor LFA1 on NK cells. Furthermore, the NKp30, among natural cytotoxicity receptors, appeared to be primarily involved in triggering NK cells upon interaction with BMSC. Unexpectedly, autologous IL-2-activated NK cells killed BMSC. Again, LFA1/ICAM1 interaction plays a key role in NK/BMSC interaction; this interaction is followed by a strong intracellular calcium increase in NK cells. More importantly, NKG2D/MHC-I-related stress-inducible molecule A and/or NKG2D/UL-16 binding protein 3 engagement is responsible for the delivery of a lethal hit. It appears that HLA-I molecules do not protect BMSC from NK cell-mediated injury. Thus, NK cells, activated upon binding with BMSC, may regulate BMSC survival. *The Journal of Immunology,* **2005, 175: 6352–6360.**

It is generally accepted that human NK cells play a role in eliminating virus-infected cells as well as in controlling tumor cell growth (1–4). This lymphocyte population mainly resides in the peripheral blood and bone marrow, although they have also been detected in primary and secondary lymphoid tissues (1–7). NK cells, beside exerting cytolytic activity, produce and secrete cytokines, including TNF-α and GM-CSF, that are possibly involved in regulating myeloid and lymphoid precursors maturation/differentiation (1). NK cells are characterized by the surface expression of receptors for self-HLA class I molecules, including some members of the inhibitory receptor superfamily (IRS),[3] whose engagement leads to the inhibition of cytolysis and cytokine production (2–4). Thus, according to the missing self-hypothesis (8), NK cells can kill target cells that do not express (or express low levels) of HLA class I Ags, such as tumor and virus-infected cells. However, it has been reported that NK cells can also interact and kill autologous APCs, despite the expression of normal levels of HLA class I molecules (9–13). Furthermore, surface receptors involved in this process have been identified (12); indeed, the natural cytotoxicity receptors (NCRs) (14), NKp30 and NKp46, have been shown to have a key role in inducing the killing of autologous dendritic cells (DC) (12). Apparently, only activated NK cells lyse self-DC and, in some instances (13), only immature DC, suggesting that physiologically, this killing occurs during viral infections when NK cells are activated and that this phenomenon may regulate the adaptive immune response. In addition, NK cells can be triggered by the engagement of an NKG2D surface molecule (15–18) by its counterligands, represented by MHC-I-related stress-inducible molecule A (MIC-A) or MIC-B, molecules up-regulated on normal autologous cells stressed by several stimuli, including temperature and retinoic acid (15, 19, 20), or constitutively expressed by tumor cells (21, 22). Additional ligands for NKG2D represented by UL-16 binding protein (ULBP) 1–4 receptors have been discovered during a search for ligands of the UL16 glycoprotein of human CMV (16). NKG2D is present on NK cells or on cytolytic effector $CD8^+$ $\alpha\beta$T cells and T lymphocytes bearing $\gamma\delta$ TCR (15, 16, 20, 24), and recently, it has been claimed that NKG2D-mediated triggering may have a critical role in eliminating tumor cells (16, 18, 20, 22, 23, 24). Because NK cells reside in the bone marrow (1), it is conceivable that they may interact with stromal cells. BMSC are thought to have a role in the regulation of hemopoietic stem cell proliferation and differentiation as well in the growth of some hemopoietic malignancies (25–27). More recently, it has been reported that MSC present within BMSC are able to differentiate into mesodermic tissues or even into epithelial cells and neurons (28–34). In addition, BMSC can exert a tolerogenic effect on T lymphocytes, supporting their use in bone marrow transplantation to favor bone marrow engraftment and to avoid graft-vs-host disease (GVHD) (35–43). In this context, it is important to determine whether the innate arm of the immune system may interact with BMSC and the consequence of this interaction.

In this study we show that ex vivo-isolated NK cells neoexpress the activation Ag CD69 and secrete IFN-γ and TNF-α, via NKp30 triggering, upon binding with BMSC. Unexpectedly, BMSC can be killed by autologous IL-2-activated NK cells that bind to BMSC

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[3] Abbreviations used in this paper: IRS, inhibitory receptor superfamily; a.u., arbitrary unit; BMSC, bone marrow stromal cell; $[Ca^{2+}]_i$, intracellular free calcium concentration; CMA, concanamycin; DC, dendritic cell; GAM, goat anti-mouse; GVHD, graft-vs-host disease; LAK, lymphokine-activated killer; MSC, mesenchymal stem cell; NCR, natural cytotoxicity receptor; MIC, MHC-I-related stress-inducible molecule A; ULBP, UL-16 binding protein.

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through LFA1/ICAM1 interaction. Lysis of BMSC is initiated by ligation on NK cells of the NKG2D receptor by its natural ligands, MIC-A and ULBP3, expressed by BMSC.

Materials and Methods

mAbs and reagents

The anti-CD69 mAb (clone 31C4, IgG2a), the anti-CD45 mAbs (TA218/12, IgM; T205/23, IgM), the anti-CD31 mAb (89D3), the anti-CD16 mAbs (NK1, IgG1; NK54, IgG2a), the anti-CD18 mAb (70H12, IgG2a), the anti-CD54 mAb (ICAM1, clone SM89, IgM), and the anti-CD44 mAbs (T61/12, IgG1; TA153/G8, IgG2a) were obtained in our laboratory as previously described (44, 45). Affinity-purified, azide-free mAbs recognizing NKp30, NKp44, and the NKp46 were purchased from Immunotech. The anti-CD3 mAb (UCHT-1, IgG1) was obtained from Ancell. The anti-NKG2D azide free was purchased from R&D Systems Europe. The anti-HLA class-I W6/32 (IgG2a), the anti-SH2 (CD105, IgG1), the anti-SH3 (CD73, IgG2b), the anti-SH4 (IgG1), the anti-CD34 (clone IgG1), the anti-CD11a (LFA1a, TS1.22, IgG1), and the anti-CD18 (LFA1b, TS1.18, IgG1)-producing hybridomas were purchased from American Type Culture Collection. Anti-HLA class I mAb (clone 3A3, IgM) and the anti-CD14 mAb (63D3, IgG1) were gifts from E. Ciccone (Institute of Anatomy, University of Genoa, Genoa, Italy) and D. Vercelli (Scientific Institute San Raffaele, Milan, Italy), respectively. The anti-β_1 integrin (CD29) mAb (3E1, IgG1) was a gift from Dr. L. Zardi (IST-Genoa, Genoa, Italy), the anti-MIC-A mAbs AMO1 was obtained from Immatics Biotechnologies, and the anti-ULPBs mAbs (anti-ULBP1, M291, IgG1; anti-ULBP2, M311, IgG1; anti-ULBP3, M551, IgG1; and anti-ULBP4, M478, IgG1) were provided by Amgen. The anti-ICAM2 and anti-ICAM3 mAbs were purchased from Bender MedSystem. The anti-prolyl-4-hydroxylase mAb (clone 5B5, IgG1) was purchased from DakoCytomation. PHA, the calcium chelator EGTA, the vacuolar H^+ATPase inhibitor concanamycin (CMA), and PI3K inhibitors wortmannin or LY294002 were obtained from Sigma-Aldrich, and rIL-2 was purchased from PeproTech EC. Complete medium was composed of RPMI 1640 (Biochrom) with 10% FCS (Sigma-Aldrich) supplemented with antibiotics (penicillin and streptomycin) and L-glutamine (Biochrom).

Isolation of bone marrow and peripheral blood T and NK lymphocytes and generation of T and NK polyclonal cell populations

Three healthy subjects (HD1–3) (from Bone Marrow Transplantation Unit, Ospedale Careggi, Florence, Italy), eight patients (Pt1–8) with acute myeloid leukemia, and one with acute lymphoblastic leukemia (Pt9) in postchemotherapy complete remission (from Clinical Hematology, University of Genoa) were subjected to heparinized samples of bone marrow aspirates or peripheral blood during conventional diagnostic procedures. Mononuclear cells were obtained by Ficoll-Hypaque (Biochrom) gradient centrifugation. $CD8^+$ T or NK cell samples were isolated from bone marrow or peripheral blood with the specific RosetteSep kit (StemCell Biotechnologies). The resulting $CD8^+$ T cell populations were >98% $CD3^+CD8^+$, whereas purified NK cells were $CD56^+$ (80–90%; $n = 10$) and $CD16^+$ (90–98%; $n = 10$). NK cell populations were immediately used in functional assays (ex vivo-isolated NK cells) or cultured in complete RPMI 1640 medium (RPMI 1640 supplemented with 10% FCS, antibiotics, and L-glutamine) with 10^5 irradiated PBMC in the presence of 1 mg/ml PHA and 100 IU/ml IL-2 for 7 days (46, 47). Usually strong proliferation was observed at this time by microscopic analysis, and then cell cultures were split and expanded for an additional 10 days. Under these culture conditions, NK cell populations homogeneously expressed CD56, CD16, and NKG2D Ags. No remarkable differences in the expression of these molecules or of the IRS members (2–4) as CD158 family or of the CD94/NKG2 complex was found between NK cells derived from bone marrow or peripheral blood (not shown).

Generation of BMSC

BMSC were obtained by culturing the bone marrow cell suspensions from healthy donors ($n = 3$) or from patients affected by acute myeloid leukemia ($n = 8$) or acute lymphoblastic leukemia ($n = 1$) in complete remission in six-well plates (5×10^6 cells/plates) in RPMI 1640 (comparable results were obtained using DMEM) complete medium for 3 days. After this period, nonadherent cells were washed away, and adherent cells were cultured for an additional 7 days. On day 10 small groups of adherent cells (100–200 cells/group) were detectable under microscopic examination, medium was changed, and cells were cultured for an additional 7 days. Then confluent adherent cells were harvested and expanded in 25-cm^2 flasks. At this time the surface phenotype of adherent cells was the following: 99% $SH3/CD73^+SH4^+SH2/CD105^+$, 100% $CD44^+\beta_1$ integrin$(CD29)^+$ $ICAM1(CD54)^+$, 100% $HLA\text{-}I^+$, and 98% $CD45^-CD31^-CD34^-CD33^-CD3^-CD2^-CD16^-CD14^-ICAM2^-ICAM3^-$. This phenotype was maintained during the culture, and BMSC were used in a functional assay within the fourth passage. Two types of BMSC were obtained: spindle-shaped cells resembling morphology of fibroblasts and polygon-shaped cells similar to epithelial cells. If not otherwise indicated, the results obtained in functional experiments were superimposable by using BMSC of either one type or another. It is of note that polygonal cells were positive for the intracytoplasmic expression of prolyl-4-hydroxylase, whereas the spindle cells were negative. Both spindle and polygonal stromal cells strongly inhibited (range, 50–90%; mean, 70%; $n = 6$) the proliferation of T lymphocytes in MLR when added at a BMSC:T responder ratio of 1:5. Furthermore, the amounts of TGF-β spontaneously released in culture by both types of BMSC from the same donor were similar. Thus, both phenotypic and functional features of BMSC obtained under our culture conditions were in agreement with those observed previously by other authors (35–40). The expression of MIC-A and the different ULBPs was also analyzed at the first passage and when BMSC were used in functional assays. The expression of MIC-A and ULBPBs remained constant during the culture period. Indeed, the expression of these receptors was not modified when cells were cultured with either human sera (from two healthy donors) or FCS (eight batches from different preparations).

Immunofluorescence and cytofluorometric analysis

Expression of surface markers was performed on both trypsin/EDTA-treated BMSC and BMSC adherent to glass coverslips. Immunofluorescence on either BMSC or T or NK cell populations was performed with the various mAbs, followed by the addition of anti-isotype-specific goat anti-mouse (GAM-Ig) antisera (Southern Biotechnology Associates) conjugated with PE or FITC as indicated. Control aliquots were stained with isotype-matched irrelevant mAb (BD Biosciences), followed by GAM-PE or GAM-FITC. Samples of BMSC detached from plastic culture flasks were run on a flow cytometer (FACScan; BD Biosciences) equipped with an argon ion laser exciting PE at 488 nm. Data were analyzed using the CellQuest computer program and are expressed as the log red fluorescence intensity (arbitrary units (a.u.)) vs the log green fluorescence intensity or vs the number of cells, or as the mean fluorescence intensity (a.u.). Calibration was assessed with CALIBRITE particles (BD Biosciences) using the AutoCOMP computer program. Samples of BMSC cultured on glass coverslips and stained as described above were analyzed on an IX81 microscopy with the confocal equipment FV500 (Olympus Byosystem).

Cytotoxicity assay

The cytolytic activity of $CD8^+$ T or NK cell lines from healthy donors or leukemia patients was analyzed in a 4-h ^{51}Cr release assay with autologous or allogenic BMSC, activated PHA blasts, or the K562 erithroleukemia cell line labeled with ^{51}Cr at an E:T cell ratio of 20:1 to 1:1 in a final volume of 200 ml of culture medium in V-bottom microwells (46, 47). One hundred microliters of supernatant was counted in a gamma counter, and the percent ^{51}Cr specific release was calculated as described previously (46, 47). Some experiments were performed by adding to the cytolytic assay saturating amounts (3 μg/ml) of anti-NKG2D, anti-MIC-A, anti-ULBP1, anti-ULBP2, anti-ULBP3, anti-HLA class I, anti-LFA1, or anti-ICAM1 mAbs or EGTA (0.5 mM), CMA (3 μM), or the PI3K inhibitors, wortmannin (100 nM) or LY294002 (5 μM). Cytolytic assays were performed with ^{51}Cr-labeled trypsin/EDTA-treated BMSC as well as with BMSC labeled with ^{51}Cr still adherent in flat-bottom microwells. Results obtained under these conditions were superimposable. In some experiments lymphokine-activated killer (LAK) cells were obtained from PBMC (10^5 cells/well in 96-well, U-bottom microwells) cultured for 5 days with 100 IU/ml IL-2 in the absence or the presence of BMSC at the indicated ratios (PBMC/BMSC ratio of 20:1, 200:1, 400:1, or 800:1). The numbers of cells obtained from PBMC expanded in IL-2 with or without BMSC were comparable. In the presence of IL-2 and BMSC, the percentage of $CD3^-CD16^+$ NK cells was similar to that found in culture of PBMC with IL-2.

IFN-γ, TNF-α, and TGF-β production assay

Ex vivo highly purified NK cells (10^5 cells) were cultured for different periods of time (24, 36, and 48 h) with BMSC (5×10^3 or 10^4 or 2×10^4 cells/well) at a 20:1, 10:1, or 5:1 NK:BMSC ratio in RPMI 1640 complete medium at 200 μl/well in flat-bottom, 96-well plates at 37°C. In some experiments to determine whether the contact between NK cells and BMSC was necessary to evoke NK cell triggering, NK cells were seeded at the same ratios in Millicell (Millipore) wells provided with a permeable

membrane with 0.4-μm diameter pores put into 24-well plates on which were cultured BMSC.

Saturating amounts of anti-LFA1 mAb (5 μg/ml) or affinity-purified azide-free mAbs recognizing NKp30, NKp44, and NKp46, either alone or in combination (5 μg/ml), were added to NK/BMSC cocultures to evaluate the roles of these receptors in the NK/BMSC interaction. Culture supernatants were harvested and analyzed for TNF-α and IFN-γ using an ELISA kit (PeproTech). The presence of TGF-β in culture supernatants from BMSC was determined using an ELISA kit from R&D Systems, following the manufacturer's instructions.

Evaluation of mRNA for MIC-A and ULBPs

Total RNA was prepared from BMSC or from cell lines (H9, T cell line surface ULBP3$^+$MIC-A$^-$; CIR-neo, lymphoblastoid cell line ULPB3$^-$; CIR-MIC-A, CIR transfected with MIC-A (gift from Dr. A. Steinle, Eberhard-Karls University, Tubingen, Germany) using TRIpure (Sigma-Aldrich) and was reverse transcribed using the RT kit from Amplimedical. The resulting cDNA was amplified by PCR with specific primer pairs in 32 cycles at 95°C for 1 min and 60 and 72°C for 1 min. Oligonucleotide sequences (forward and reverse) were: β-actin, 5′-CATACTCCTGCTTGCTGATCC-3′ and 5′-ACTCCATCATGAAGTGTGACG-3′; MIC-A, 5′-CCTTGGCCATCAACGTCAGG-3′ and 5′-CCTCTGAGGCCTCRCTGCG-3′; ULBP1, 5′-GTACTGGGAACAAATGCTGGAT-3′ and 5′-AACTCTCCTCATCTGCCAGCT-3′; ULBP2, 5′-TTACTTCTCAATGGGAGACTGT-3′ and 5′-TGTGCCTGAGGACATGGCGA-3′; and ULBP3, 5′-CCTGATGCACAGGAAGAAGAG-3′ and 5′-TATGGCTTTGGGTTGAGCTAAG-3′. Amplicons were examined on 2% agarose gel for correct size (20). Images were acquired by Chemi 550 (AlphaInnotech) and analyzed by Gel Pro Analyzer 3.1 (Media Cybernetics).

Microscopic analysis and evaluation of intracellular calcium concentration in NK cells during interaction with BMSC

BMSC morphology was analyzed at different culture passages in plastic petri dishes using the inverted microscope Olympus IX70 equipped with Normaski and the Cell^R/Cell^M imaging video system (Olympus Biosystem). NK cell-mediated killing of BMSC was analyzed along time by taking video images every 20 s of BMSC adherent on glass coverslips in chamber slides kept at 37°C using the microincubator TM102 and the temperature controller (Medical System) in 2 ml of buffer (48). NK cells were added to BMSC at a ratio of 5:1, and interaction between NK and BMSC was recorded for 4 h. In some experiments NK and BMSC were labeled with fura 2-AM (1 μM; Sigma-Aldrich) for 1 h at 37°C. BMSC were placed on a Leiden coverslip dish, NK cells were added to this chamber, and the whole apparatus was maintained at 37°C by a thermostatically controlled water bath (48). Fura 2-AM was excited at 334 and 380 nm, emitted light was filtered at 510 nm, and fluorescence was monitored with the Cell^R/Cell^M system. The intracellular free calcium concentration ($[Ca^{2+}]_i$) was calculated as previously described (48) during interaction between NK and BMSC by gating the analysis on NK cells.

Results

Interaction between ex vivo-isolated NK cells and BMSC leads to NK cell activation and cytokine production

To determine the outcome of NK/BMSC interaction, we isolated highly purified NK cells (>98% CD16$^+$CD3$^-$) from peripheral blood or bone marrow of healthy donors. Thus, we investigated whether the BMSC/NK interaction could lead to activation of NK cells and/or production of cytokines by NK cells. First, expression of the activation surface marker CD69 (44) on NK cells after coculture with BMSC was analyzed. CD69 surface expression was strongly up-regulated in 24 h upon NK cell incubation with BMSC, and this effect was dependent on the NK:BMSC ratio. Indeed, the highest CD69 up-regulation was detected at the 20:1 NK/BMSC ratio (Fig. 1*A*, *lower left quadrant*; range, 25–57%; $n = 6$), whereas CD69 was expressed on only ~10% of NK cells (Fig. 1*A*, *lower right quadrant*; range, 5–15%; $n = 6$) at an NK/BMSC ratio of 5:1. Accordingly, FACS and microscopic analysis showed that NK cells became blasts (>65% of the whole NK cell population) when cocultured with BMSC at an NK:BMSC ratio of 20:1, whereas at a 5:1 ratio, no evident differences between NK cells in medium alone and coculture with BMSC were observed (not shown). In an additional series of experiments, among the cytokines that NK cells are able to produce (1), we focused on IFN-γ and TNF-α. Interestingly, we consistently found that ex vivo-isolated NK cells released high amounts of IFN-γ and TNF-α upon binding with BMSC. Indeed, this effect was maximal at a 20:1 NK/BMSC ratio, but it was still detectable at a 5:1 ratio (Fig. 1*B*). In contrast, BMSC did not produce either IFN-γ or TNF-α (not shown).

LFA1/ICAM1 and NKp30 are involved in the production of proinflammatory cytokines

The binding between NK and BMSC was needed to induce NK cell triggering; indeed, when NK cells and BMSC were separated from each other by a Transwell porous membrane, neither CD69 (not shown) expression on NK cells nor IFN-γ and TNF-α release in culture supernatant was detectable (Fig. 1*C*). We also found that anti-LFA1 mAb strongly inhibited (45–70% inhibition; $n = 4$) the production of both IFN-γ and TNF-α evoked by NK/BMSC binding (Fig. 1*C*), suggesting that the LFA1/ICAM1 adhesion system is involved in NK/BMSC interaction. The addition to NK/BMSC cocultures of anti-LFA3 mAb did not affect the production of the above-mentioned cytokines, indicating that CD2/LFA3 interaction does not play a major role in NK/BMSC binding (Fig. 1*C*). It is of note that anti-NKp30 mAbs inhibited (Fig. 1*C*; range of inhibition, 65–85%; $n = 4$) both IFN-γ and TNF-α production, suggesting that this NK cell-activating receptor may recognize on BMSC its counterligand. In contrast, NKp46 and NKp44 did not appear to be involved in the triggering of NK cells, because specific mAbs did not affect IFN-γ and TNF-α production (Fig. 1*C*).

Resting NK cells do not lyse BMSC, which, in turn, impair LAK generation from PBMC

We analyzed whether ex vivo-isolated NK cells could affect BMSC survival (Fig. 1*D*) and whether this effect was comparable to that exerted by NK cells on the NK-sensitive target cell K562. In a 4-h ^{51}Cr release assay at a high NK/BMSC ratio (20:1), we found that allogeneic BMSC were lysed by ex vivo-isolated NK cells at a low level (Fig. 1*D*). Indeed, using NK cells from 10 different healthy donors, lysis of allogeneic BMSC at an NK/BMSC ratio of 20:1 ranged from 5 to 30% (range of 10 independent experiments). Also, the degree of K562 lysis varied from donor to donor; however, the range of lysis at an NK/K562 ratio of 20:1 was consistently higher (range, 10–55%; $n = 10$) than that observed using BMSC as target cells (Fig. 1*D*). Because BMSC express HLA class I Ags, which can deliver an inhibitory signal on NK cell-mediated cytolysis by interacting with IRS present on NK cells, we performed some experiments in the presence of anti-HLA-I mAb. Although not shown, the addition of anti-HLA-I mAb did not increase the lysis of BMSC exerted by ex vivo-isolated NK cells.

Furthermore, we determined whether BMSC can affect the generation of LAK cells from PBMC cultured in the presence of high amounts of IL-2. Indeed, the large majority of LAK activity present in IL-2-activated PBMC is due to activated NK cells (1–4). To this aim, PBMC was seeded with BMSC (at different PBMC/BMSC ratios) with 100 U/ml IL-2 for 5 days. As shown in Fig. 1*E*, PBMC harvested from cocultures with BMSC, even at very high PBMC/BMSC ratios, was not able to lyse LAK-sensitive target cells such as JA3 (Fig. 1*E*). Although not shown, we found that JA3 lysis, in agreement with a previous report (18), was strongly dependent on the triggering on PBMC of NKG2D receptors, because its covering with anti-NKG2D mAbs down-regulated LAK cell-mediated lysis of JA3 (70–90% inhibition; $n = 3$). Furthermore, coculture of PBMC with BMSC reduced the expression of

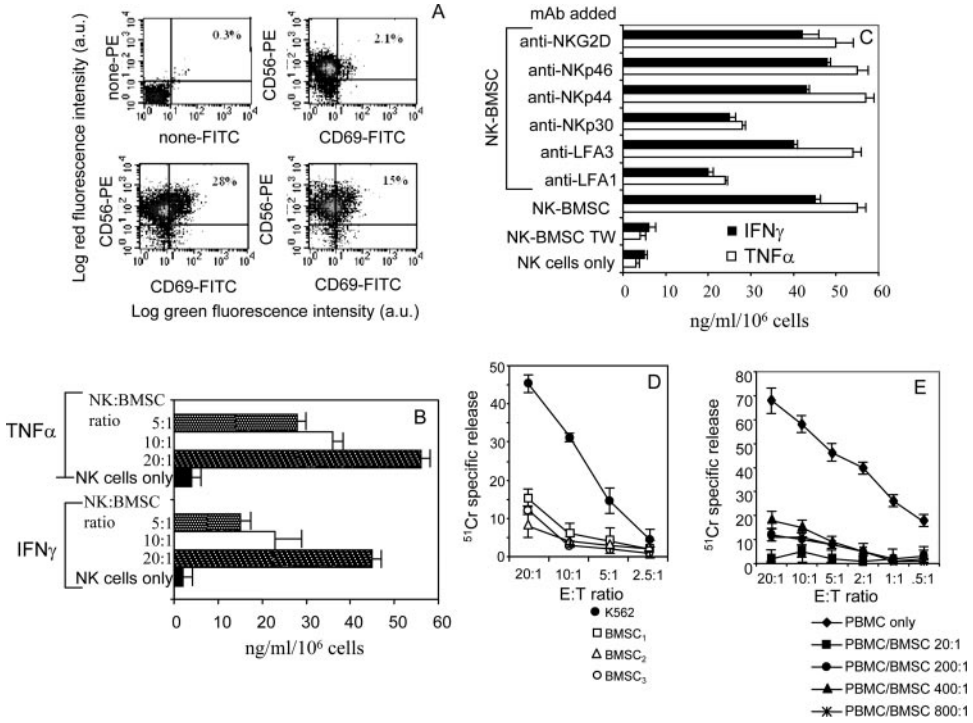

FIGURE 1. BMSC activate NK cells and down-regulate LAK cell generation. *A*, Double-immunofluorescence analysis for expression of the NK-related marker CD56 and activation of Ag CD69 on ex vivo-isolated NK cells cocultured with BMSC at different NK:BMSC ratios. *Upper left quadrant*, Staining of ex vivo-isolated NK cells with unrelated mAb (none-PE and none-FITC). *Upper right quadrant*, Expression of CD56 and CD69 on ex vivo-isolated NK cells; *lower left quadrant*, coculture of NK cells with BMSC (ratio 10:1); *lower right quadrant*, coculture of NK cells with BMSC (ratio 5:1). *B*, IFN-γ and TNF-α production by ex vivo-isolated NK cells upon coculture with BMSC at the indicated NK:BMSC ratios. NK cells only, cytokines released by ex vivo-isolated NK cells. After 24 h of incubation, culture supernatants were harvested and analyzed by ELISA. Results are expressed as nanograms per milliliter per 10^6 cells and are the mean $\pm$ SD of triplicate samples. Similar results were obtained with NK isolated from six different healthy donors cocultured with one of three samples of BMSC (BMSC from HD1, Pt3, and Pt9). *C*, IFN-γ and TNF-α production by ex vivo-isolated NK cells upon coculture with BMSC at a 20:1 ratio in the absence (NK-BMSC) or the presence of saturating amounts of mAbs (5 μg/ml) directed to the indicated molecules (NKG2D, NKp46, NKp44, NKp30, LFA3, and LFA1). Some experiments were performed by seeding NK cells in MilliCell wells separated from BMSC by a filter (NK-BMSC TW). Results are shown as the mean $\pm$ SD of triplicate samples. Similar results were obtained with NK isolated from six different healthy donors cocultured with one of three samples of BMSC (BMSC from HD1, Pt3, and Pt9). *D*, Ex vivo-isolated NK cells from one healthy donor were challenged with allogeneic BMSC from three different donors ($BMSC_1$, HD-1; $BMSC_2$, Pt5; $BMSC_3$, Pt9) in a 4-h ^{51}Cr release assay at the indicated E:T cell ratios. Lysis of the NK-sensitive cell line K562 is shown for comparison. *E*, Cytolytic activity of LAK cells generated from PBMC (10^5 cells) after 5 days of culture with 100 IU/ml IL-2 in the absence (PBMC only) or the presence of BMSC at the indicated PBMC/BMSC ratios was analyzed in a ^{51}Cr release assay against the LAK-sensitive target cell JA3 at the indicated E:T cell ratio. Results are expressed as ^{51}Cr specific release and are the mean $\pm$ SD of triplicate samples. Superimposable results were obtained with LAK cells derived from peripheral blood of three healthy donors (HD1, HD2, and HD3).

NKG2D receptor on both $CD3^-CD16^+$ NK cells (mean fluorescence intensity of NKG2D, 161 $\pm$ 15 a.u. (PBMC alone) vs 120 $\pm$ 10 a.u. (PBMC with BMSC)) and $CD3^+$ T cells (123 $\pm$ 13 vs 85 $\pm$ 6 a.u.; $n = 3$). Finally, PBMC cultured with IL-2 supplemented with the supernatant derived from BMSC containing TGF-β (0.5–08 ng/ml) did not affect the generation of LAK activity (not shown).

Autologous IL-2-activated NK cells kill BMSC

It is generally accepted that BMSC may favor cell proliferation and produce soluble factors involved in myeloid precursor differentiation (25–27). Thus, we addressed the question of whether IL-2-activated NK lymphocytes can affect BMSC survival, as it may occur during a viral infection (1–4). As shown in Fig. 2, IL-2-activated NK cells efficiently lysed both autologous and allogeneic BMSC, and this killing was evident at very low E:T cell ratios (2:1 or 1:1). This would imply that during the interaction with one BMSC, a single activated NK cell can deliver an efficient lethal hit. Lysis of autologous BMSC was similar using either NK cells from peripheral blood or bone marrow of healthy donors (Fig. 2, *A* and *B*) or from leukemic patients (not shown). In contrast, IL-2-activated $CD8^+$ T lymphocytes, representative of a cytolytic T cell population, did not kill BMSC (Fig. 2, *A* and *B*). To determine the surface structures involved in NK-BMSC interaction, cytolytic assays were performed in the presence of anti-ICAM1 and anti-LFA1 mAbs, because the ICAM1/LFA1 adhesion system plays a key role in NK cell adhesion to target cells (1, 49, 50). As shown in Fig. 2*C*, the addition of anti-LFA1 and/or anti-ICAM1 mAb strongly reduced (by 70%) the killing of BMSC mediated by NK cells, indicating that LFA1/ICAM1 binding is needed to deliver the lethal hit. Killing of BMSC by NK cells was inhibited by the calcium chelator EGTA (Fig. 2*D*) or by the vacuolar H^+-ATPase inhibitor CMA (51) (Fig. 2*E*), indicating that both the calcium increase and the release of perforins are involved. The involvement of perforins (52) in NK cell-mediated lysis of BMSC was also supported by the inhibition of lysis exerted by PI3K (53–55) inhibitors, wortmannin or LY294002 (Fig. 2*F*). Indeed, it is well known that the secretion of lytic enzymes by cytolytic effector lymphocytes is PI3K dependent (53–55).

We also analyzed whether increases in $[Ca^{2+}]_i$ occurred during NK/BMSC interaction. Both NK and BMSC were labeled with fura 2 (48), and NK cells were added to a coverslip with adherent

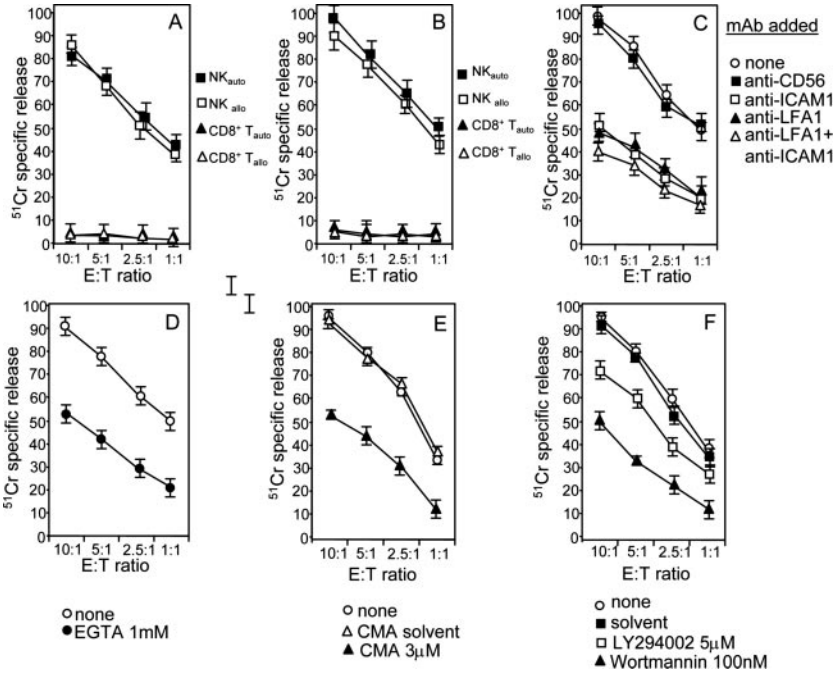

FIGURE 2. Killing of BMSC by IL-2-activated NK cells. Cytolytic activity of IL-2-activated NK cells exerted against BMSC was evaluated in a 4-h ^{51}Cr release assay at the indicated E:T cell ratios. *A* and *B*, Autologous or allogeneic NK cells (NK_{auto} and NK_{allo}) derived from peripheral blood (*A*) or bone marrow (*B*) were challenged with BMSC. Results obtained using autologous or allogeneic $CD8^+$ T cells ($CD8^+$ T_{auto} and $CD8^+$ T_{allo}) are shown for comparison. *C*, Effect of anti-LFA1 and/or anti-ICAM1 mAb (added at the onset of the cytolytic assay in saturating amounts, 5 μg/ml) on NK cell killing of autologous BMSC. Anti-CD56 mAb was used as an isotype-matched negative control. Results are shown as the mean ± SD of triplicate samples. Killing of BMSC by IL-2-activated NK cells (Pt5; in the presence of the calcium chelator EGTA (1 mM; *D*), the H^+-ATPase inhibitor CMA (3 μM; *E*), or the PI3K inhibitors LY294002 (5 μM) and wortmannin (100 nM; *F*). Results obtained with solvent of the above-mentioned drugs is shown for comparison. None, lysis in medium alone. Results are expressed as ^{51}Cr specific release. Results are shown as the mean ± SD of triplicate samples. Comparable results were obtained with six bulk NK cell populations derived from peripheral blood of Pt3, Pt4, Pt8, HD1, HD2, and HD3.

BMSC. Upon interaction with BMSC, $[Ca^{2+}]_i$ strongly increased in NK cells, and this increase lasted for 60–90 min (Fig. 3*A*). The addition of EGTA inhibited the $[Ca^{2+}]_i$ increase observed during the NK/BMSC interaction, suggesting that calcium influx from the extracellular milieu was involved (Fig. 3*A*). Shrinking of BMSC after ligation with NK cells was evident after 120–180 min, and alteration of the BMSC cell membrane occurred later, as shown in Fig. 3*B*.

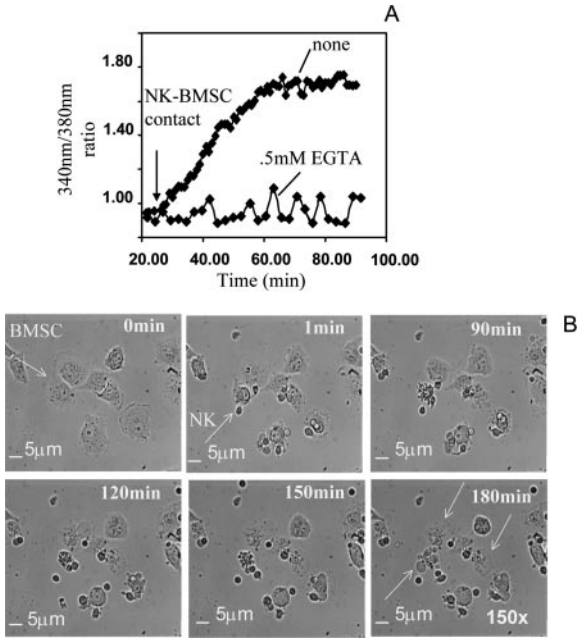

FIGURE 3. Interaction between NK and BMSC evokes increases in $[Ca^{2+}]_i$. *A*, Analysis of $[Ca^{2+}]_i$ during NK-BMSC interaction. NK cells and BMSC (from Pt5) were labeled with fura 2-AM and $[Ca^{2+}]_i$ was monitored during NK-BMSC interaction. The 340 nm/380 nm ratio of fura 2-AM emission was analyzed at 510 nm during the NK-BMSC interaction in the absence (none; basal level of $[Ca^{2+}]_i$, 75 nM; peak level at 80 min, 575 nM) or the presence of 0.5 mM EGTA as indicated. *B*, Brightfield images showing NK-BMSC binding (arrows) were taken at the indicated time points. Results shown are representative of six independent experiments performed with NK cells from Pt3, Pt4, Pt7, HD1, HD2, and HD3 incubated with autologous BMSC. Magnification, ×150.

Killing of BMSC is mediated by NK cell activation through NKG2D engagement by MIC-A or ULBP3

To define the surface activating receptors expressed on NK cells involved in the lysis of BMSC, cytolytic assays were performed by masking with specific mAbs either NKG2D or NCR represented by NKp30, NKp44, and NKp46 molecules, because these triggering receptors have been reported to play a role in delivering the lethal hit to target cells by human NK cells (14, 18, 56). A sharp inhibitory effect (range, 50–75%; mean, 60%; $n = 8$) was found by adding the anti-NKG2D mAb (Fig. 4*A*). This prompted us to analyze BMSC for the surface expression of the known counter ligands of the NKG2D receptor, represented by MIC-A and ULBP1–4 molecules (15, 16). Indeed, we found that BMSC (both spindle-shaped and polygonal cells) bear at the cell surface MIC-A, ULPB1, and ULBP3, whereas ULPB2 was expressed at lower levels, and ULBP4 was absent (Fig. 4*B*). The expression of MIC-A and ULBP3 was also confirmed by confocal microscopic analysis of BMSC cultured on glass coverslips (not shown) and by RT-PCR analysis of mRNA coding for ULBP3 or MICA (Fig. 4, *C* and *D*) from cultured BMSC. That MIC-A and ULBP3 were the surface structures recognized by NK cells on BMSC was indicated by the inhibition of lysis of autologous BMSC obtained by adding anti-MIC-A- and/or anti-ULBP3-specific mAbs to the cytolytic assay (Fig. 4*A*).

In contrast, covering of a given NCR by the addition to the cytolytic assay of specific anti-NCR mAb, either alone or in combination, slightly reduced (range of inhibition, 10–30%; mean, 25%; $n = 10$) NK cell-mediated lysis of autologous (Fig. 5*A*) or allogeneic BMSC (not shown).

Role of surface HLA-I in NK cell-mediated killing of autologous BMSC

The lysis of BMSC by autologous activated NK cells contrasts with the generally accepted concept that NK cells do not kill

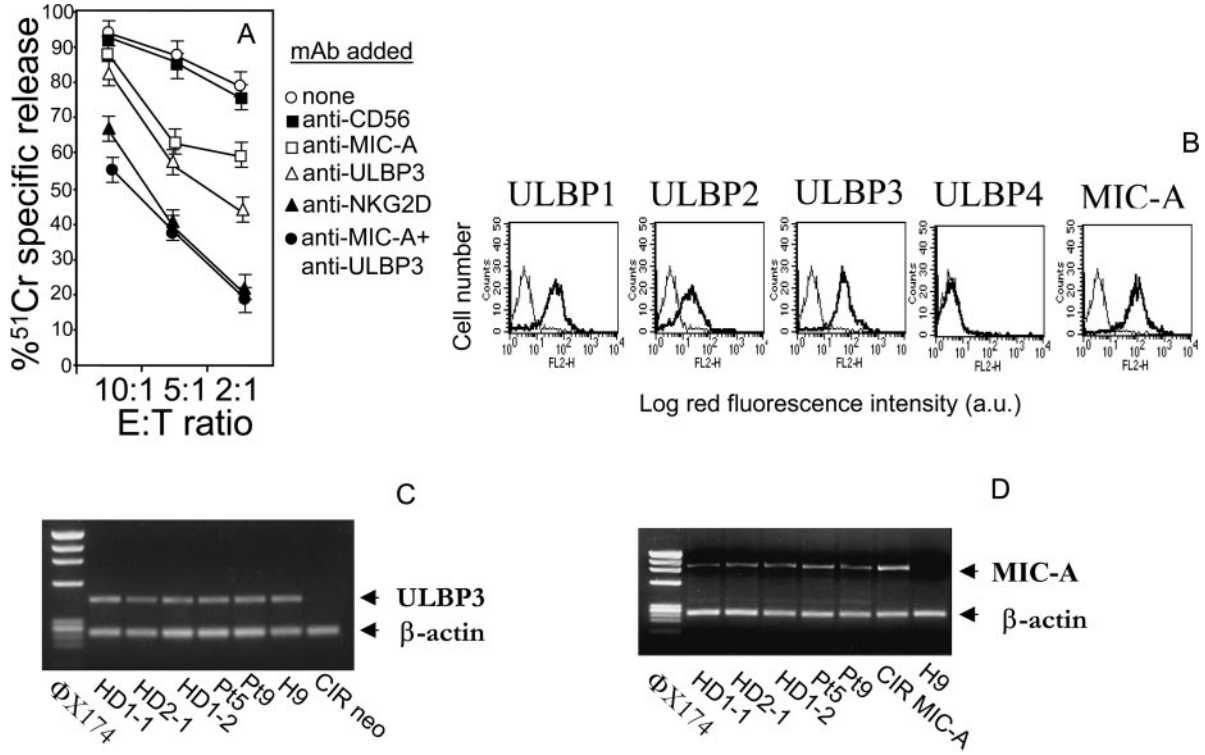

FIGURE 4. NKG2D is responsible for triggering of BMSC lysis by activated NK cells. *A*, Cytolysis of NK cells against autologous BMSC (from Pt6) at the indicated E:T cell ratios was evaluated in a 4-h ^{51}Cr release assay in the absence (none) or the presence of the indicated mAbs. Anti-CD56 mAb was used as an isotype-matched negative control. Results are expressed as ^{51}Cr specific release and are the mean ± SD of triplicate samples. Comparable results were obtained with NK cells from Pt5, Pt7, HD1, and HD2 using as target cells autologous or allogeneic BMSC from three different donors (Pt2, Pt9, and Pt11). *B*, Analysis of surface expression on BMSC of the indicated NKG2D counterligands. BMSC (from HD1) were stained with mAbs recognizing the indicated surface NKG2D counterligand, followed by PE-conjugated GAM-Ig. Thin gray histograms, BMSC stained with an unrelated mAb matched for the isotype; bold black line histograms, BMSC stained with mAbs recognizing the indicated molecules. *C*, RT-PCR analysis of mRNA coding for ULBP3 in BMSC from HD1, HD2, Pt5, Pt9, Pt11 (HD1-1 and HD2-1, spindle-shaped BMSC; HD1-2, Pt5, and Pt9, polygon-shaped BMSC). H9, mRNA for ULBP3 in the ULBP3$^+$ T cell line H9. CIR-neo mRNA for ULBP3 in the ULBP3$^-$ lymphoblastoid cell line CIR. *D*, RT-PCR analysis of mRNA coding for MIC-A in BMSC from HD1, HD2, Pt5, Pt9 (HD1-1 and HD2-1, spindle-shaped BMSC; HD1-2, Pt5, and Pt9, polygon-shaped BMSC). CIR-MIC-A, mRNA for MIC-A in the MIC-A-transfected lymphoblastoid cell line CIR. H9, mRNA for MIC-A in the MIC-A$^-$ T cell line H9. The mRNA for β-actin in each sample is shown. The mRNA for β-actin in each sample is shown. Fx174, control markers.

autologous cells (1–4). Indeed, it has been shown that HLA-I molecules expressed on autologous target cells down-regulate NK cell-mediated cytolysis (1–4), interacting with members of IRS that are constitutively expressed on NK cells (2–4). To demonstrate this protective effect, anti-HLA-I mAb were added to a cytolytic assay using NK cells as effectors and autologous PHA blasts as target cells to avoid the interaction between HLA-I and IRS (2–4). In this assay, the addition of anti-HLA-I mAb should result in an enhancement of PHA blast lysis. As BMSC expressed at the cell surface amount of HLA-I similar to that displayed by autologous PHA blasts (n = 8; data not shown) it is conceivable that the lysis of autologous BMSC is not due to a deficiency in HLA-I Ag expression. Furthermore, the addition of anti-HLA-I mAb to the cytolytic assay did not significantly enhance the lysis of autologous BMSC (range of enhancement, 5–10% in 10 experiments; Fig. 5*B*). Nevertheless, the addition of anti-HLA-I mAb strongly enhanced (60–100% increase in basal lysis; n = 9) the lysis of autologous PHA blasts by NK cells (Fig. 5*C*). These findings indicate that NK cells may also kill self-BMSC in the presence of a negative signal initiated by IRS.

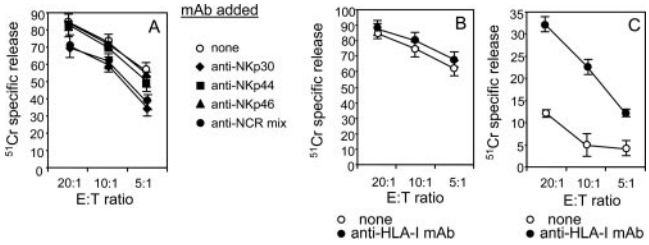

FIGURE 5. Roles of HLA-I Ags and natural cytotoxicity receptors in NK cell-mediated lysis of BMSC. *A*, Effect of anti-NKp30, anti-NKp44, or anti-NKp46 mAbs (each at 5 μg/ml) or a combination of these mAbs (anti-NCR mix) on NK cell-mediated lysis of autologous BMSC. None, lysis in the absence of any mAb. *B*, NK cell-mediated lysis of autologous BMSC was analyzed in a 4-h ^{51}Cr release assay in the absence (none) or the presence of anti-HLA-I mAb (5 μg/ml) at the indicated E:T cell ratios. *C*, Lysis of PHA blasts exerted by autologous NK cells in the absence (none) or the presence of anti-HLA-I mAb (5 μg/ml). Results are expressed as ^{51}Cr-specific release and are shown as the mean ± SD of triplicate samples. Results similar to those depicted in *A–C* were obtained with NK cells from five different donors (HD1, HD2, Pt3, Pt4, and Pt9).

Discussion

In this study we provide evidence that ex vivo-isolated human NK cells become activated upon interaction with BMSC. Indeed, NK cells de novo expressed the activation Ag CD69 and released IFN-γ and TNF-α after binding to BMSC. These effects depend on

LFA1/ICAM1 interaction, and the NKp30 receptor appears to play a key role NK cell activation. Recently, it has been reported that BMSC can down-regulate NK cell-mediated IFN-γ production induced by IL-2 (43); this is in contrast to the strong release of IFN-γ found in our cocultures of ex vivo NK cells with BMSC. A possible explanation for this discrepancy would be that BMSC exert different effects on NK cells depending on whether these cells are triggered (43) or not (this work) by IL-2. Secondly, it is possible that the ratio and the number of NK cells and BMSC used in different experimental settings have a role in triggering an activation signal or an inhibiting effect. Actually, it is difficult to determine whether in vivo just one or, alternatively, many NK cells can interact at the same time with a single stromal cell or vice versa. Probably this depends on the different portion of bone marrow analyzed. Indeed, next to or within bone, NK cells are in contact with more BMSC (fibroblasts, adipocytes, or osteoblasts) than in portions that represent bone marrow aspirates.

In our experiments a wide range of ratios between NK cells and BMSC has been used; furthermore, in calcium mobilization experiments, we have shown that during the interaction of a single NK cell with a single BMSC, NK cells can be activated. Thus, we are confident that the NK:BMSC ratios used conceivably represent the range of ratios that may occur in vivo.

On the one hand, BMSC down-regulate the generation of LAK activity from PBMC possibly by down-regulating NKG2D expression, although the binding of IL-2-cultured NK cells to autologous or allogeneic BMSC is followed by the activation of lytic function through the ligation of NKG2D receptor, expressed on NK cells, by its counterligands MIC-A and/or ULBP3 expressed on BMSC. Indeed, MIC-A and ULPB3 and, to a lesser extent, ULBP1 and ULBP2, all natural ligands of NKG2D molecule (15–17), are expressed on BMSC, and the inhibition of BMSC lysis observed in the presence of anti-MIC-A and/or anti-ULBP3 mAbs strongly suggests that these two ligands are mainly involved in the interaction with NKG2D.

Our present findings do not clarify whether MIC-A and/or ULBP3 are actually expressed in vivo by marrow stromal cells. Attempts to stain BMSC in bone marrow aspirates and subsequent analysis by flow cytometry have been hampered by the very low number of BMSC in these specimens (one BMSC in 10,000–100,000 cells). However, we should stress that MIC-A and ULBP3 molecules were present on our BMSC bulk populations or subclones from early culture passages, and they did not vary during the culture. Furthermore, MIC-A is present not only on tumors (15–24), but also on normal cells, such as some thymic epithelial cells and patches of gastrointestinal epithelial cells (57). These findings suggest that MIC-A expression is not induced on BMSC upon in vitro culture, and likewise thymic epithelium, which displays a nursing function, BMSC may express MIC-A in vivo. However, we cannot exclude that up-regulation of MIC-A and/or ULBPs may occur in vivo as a consequence of stress signals, such as during chronic inflammation or viral infections (15–17).

Our results indicate that NK cells, depending on their activation status, use different triggering receptors, such as NKp30 or NKG2D, to interact with BMSC. It does not appear exceptional that NK cells can interact with autologous cells through the NKp30-activating receptor, because we and others reported that this molecule is involved in the cross-talk between autologous cells and effector NK cells (12, 13, 58). In contrast, the triggering NK cell receptor mainly involved in the delivery of the lethal hit is apparently NKG2D (15–24).

In our experiments, the lysis of BMSC by activated NK cells is not down-regulated by the expression of HLA-I on BMSC, as should conceivably happen in an autologous context (8). Moreover, the addition of anti-HLA-I mAb did not induce an enhancement of BMSC lysis, because it appears for autologous PHA blasts (2–4, 8). It is also evident that the lytic effect of activated NK cells on BMSC is not inhibited by the ligation of IRS at the NK cell surface and HLA class I Ags present on BMSC.

Together, these findings suggest that upon NK-BMSC binding, NK cells produce and release proinflammatory cytokines, and after activation, NK cells can affect BMSC survival. Direct elimination of BMSC may happen after physiological activation of NK cells, which occurs during viral infection (1), or upon activation with bacterial products through the engagement of TLRs, recently described at the NK cell surface (59).

Several recent reports pointed out the tolerogenic effect exerted by MSC, which are present within bone marrow stroma (34–43). Indeed, MSC can strongly inhibit in vitro the lymphocyte proliferation observed in MLR when added as a third party. This effect suggested that MSC may play a regulatory role during bone marrow transplantation by reducing the GVHD reaction and facilitating engraftment (34–43). In our experimental conditions, among BMSC derived from either leukemic or healthy donors, on the basis of morphology, two types of stromal cells have been identified: 1) spindle-shaped cells similar to fibroblasts, and 2) polygon-shaped, epithelial-like adherent cells. Both types of BMSC expressed several markers of those usually present, and considered typical, on MSC, whereas they were negative for surface receptors expressed on leukocytes. Importantly, the polygon-shaped BMSC, differently from the spindle-shaped BMSC, were intracytoplasmic positive for expression of the enzyme 4-prolyl-hydroxylase, which is involved in the hydroxylation of collagen and other protein with collagen-like amino acid sequences (60). Although both these BMSC types were sensitive to NK cell-mediated lysis, only the spindle-shaped BMSC were able to differentiate into adipocytes or osteoblasts under appropriate culture conditions (not shown), suggesting that within this cell population, some cells with a differentiating potential similar to that of MSC are present. In contrast, polygonal BMSC as well as spindle BMSC were able to inhibit MLR proliferation, suggesting that this functional feature is not actually confined to BMSC populations containing MSC. This may imply that different types of BMSC can be used in vivo to different purposes: MSC to repair tissues, and BMSC without differentiating potential to counteract GVHD. Whatever the usage, we suggest that if BMSC is planned for in vivo therapy, one should take into account that NK cells might affect their survival, possibly impairing the BMSC-dependent therapeutic effect.

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Disclosures

The authors have no financial conflict of interest.

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