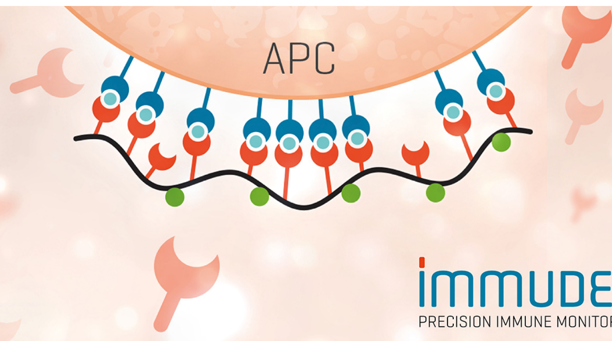

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Neuroblastoma-Derived TGF-β1 Modulates the Chemokine Receptor Repertoire of Human Resting NK Cells

Roberta Castriconi,*,†,1 Alessandra Dondero,*,1 Francesca Bellora,* Lorenzo Moretta,†,‡ Aurora Castellano,§ Franco Locatelli,¶ Maria Valeria Corrias,‡ Alessandro Moretta,*,†,1 and Cristina Bottino*,‡,1

In this study, we show that neuroblastoma (NB) cell conditioning affects the chemokine receptor repertoire of human resting NK cells. In particular, NB cells upregulated the expression of CXCR4 and CXCR3 in all NK cells and downregulated CX_3CR1 in the $CD56^{dim}$ subset. On the contrary, the expression of CXCR1 and CCR7 remained unaltered. The phenomenon was dependent on the release by NB cells of TGF-β1, and rTGF-β1 induced a chemokine receptor repertoire identical to that of NB-conditioned NK cells. The immune modulatory role of TGF-β1 appears to be dose dependent because low amounts of the cytokine were sufficient to modulate CXCR4 and CX_3CR1 expression, intermediate amounts modified that of CXCR3, and high amounts were necessary to downregulate the expression of the NKp30 activating receptor. Notably, a similar receptor modulation was observed in rTGF-β2–conditioned NK cells. Finally, the analysis of NK cells from patients with stage 4 NB suggests that NB conditioning could exert in vivo an immune modulatory effect resembling that emerged from in vitro experiments. Altogether our data propose a novel tumor escape-mechanism based on the modulation of chemokine receptors that play pivotal roles in NK cells bone marrow homing, egress, or recruitment into peripheral tissues. ***The Journal of Immunology*, 2013, 190: 5321–5328.**

Human NK cells represent important effectors against hematological and nonhematological malignances (1–4). Cytokine-conditioned NK cells kill ex vivo–derived tumor cells of different histotypes (5–7) including those with stem cell properties, which now are considered the ultimate target of both conventional and innovative therapies (8). The molecular mechanisms responsible for killing have been clarified and are represented by the engagement of triggering NK receptors such as NKp46, NKp30, DNAM-1, and NKG2D with specific ligands expressed at the tumor cell surface (9). The NK-mediated lysis occurs when, together with upregulation or de novo expression of ligands for triggering NK receptors, transformed cells present an altered expression of HLA class I molecules, which results in the defective engagement of HLA class I–specific inhibitory killer Ig–like receptors and the CD94/NKG2A heterodimer. Downregulation of HLA class I molecules is frequent in various types of malignancies and represents an important mechanism set up by tumor cells to escape T cell–mediated recognition (10).

Besides the antitumor cytotoxicity, NK cells are endowed with several other effector functions and regulate both innate and adaptive immune responses. NK cells release β-chemokines (i.e., CCL3 and CCL5), cytokines such as IFN-γ and interact with other effectors such as dendritic cells (DC), T cells, and macrophages (11, 12).

On a per cell basis, NK cells might represent optimal candidates for the cure of neoplastic patients and adoptive immunotherapy with NK cells is a novel promising treatment strategy (1–4). In vivo, however, the tumor microenvironment could affect NK cell recruitment and activation, steps that are essential for the development of effective antitumor responses. Tumor cells and/or immune cell types present at the tumor site can release soluble MICA/B and ULBPs, ligands of NKG2D (13), and/or immunosuppressive mediators such as MIF (14), PGE_2 (15), the tryptophan catabolite *l*-kynurenine (16) and TGF-β1. In NK cells TGF-β1 has been shown to downregulate the expression of NKp30, and NKG2D, thus impairing the cytotoxicity against different tumor targets (17–20). TGF-β1 belongs to a large family of dimeric polypeptide growth factors counting more than 40 members. The three isoforms of TGF-β (β1, β2, and β3) are encoded by distinct genes and are highly conserved in mammals, suggesting critical biological functions (21). Because of its role in multiple immune suppressive pathways, TGF-β1 might represent an attractive target for innovative therapeutic approaches. In preclinical models, TGF-β receptor I kinase inhibitors extended the survival of glioma-bearing animals, and the effect correlated with an increased tumor infiltration by macrophages, CD8 T cells, and NK cells (22). In this context, several studies have shown a correlation between the degree of NK cell infiltration at the tumor site and increased patient survival (23, 24). NK cells, however, are often scarce within tumors, despite high local levels of chemokines that could allow their recruitment (25, 26).

In humans, two maturative NK cell subsets exist in peripheral blood that are characterized by high or low expression of CD56

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; LAP, latency-associated peptide; MFI, median fluorescence intensity; NB, neuroblastoma; VEGF, vascular endothelial growth factor.

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($CD56^{bright}$ and $CD56^{dim}$) and by a different chemokine receptor repertoire. $CD56^{bright}$ and $CD56^{dim}$ cells preferentially home to secondary lymphoid organs and inflamed peripheral tissues, respectively. $CD56^{bright}$ cells are guided to secondary lymphoid organs via CCR7, the CCL19 and CCL21 receptor. The homing property of human $CD56^{dim}$ cells is linked to the expression of CXCR1 (CXCL8 or IL-8R), ChemR23 (chemerin receptor), and CX_3CR1 (fractalkine receptor). $CD56^{bright}$ express levels of CXCR3 (CXCL4, 9, 10, 11 receptor) higher than $CD56^{dim}$ cells, whereas both subsets express similar amounts of CXCR4, the unique receptor for the CXCL12 (11, 27, 28). The CXCR4/CXCL12 axis is crucial in homing and maintenance of hematopoietic stem cells in the stromal cell niches within the marrow (29–31). In mice it has been shown that CXCR4 is lost during NK cell differentiation and blockade of CXCR4 signaling by plerixafor (AMD3100) led to increased NK cells bone marrow (BM) egress (30–33).

Neuroblastoma (NB) is a poorly differentiated extracranial neuroectodermal tumor that accounts for 15% of all childhood cancer deaths. Stage 4 patients present a metastatic disease characterized often by BM infiltrates and by a very poor prognosis because of drug resistance and relapse (34). We have shown previously that, similar to NB cell lines, BM-derived neuroblasts were susceptible to lysis mediated by cytokine-conditioned NK cells and that killing involved NKp30 and DNAM-1 triggering receptors (5, 35).

In the current study, we focused on the mechanisms that could be involved in the escape of NB from NK-mediated immune surveillance. In particular, we analyzed whether NB cells could affect the chemokine receptor repertoire of NK cells.

Materials and Methods

Cells used in the study

NK cells were purified from PBMC of healthy donors using Human NK Cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). NB cell line HTLA-230, donated by Dr. E. Bogenmann (Children's Hospital Los Angeles, Los Angeles, CA) (36), and GI-LI-N, established at the laboratory of oncology at the Giannina Gaslini Institute (37), were periodically checked for *MYCN* amplification by fluorescence in situ hybridization analysis. The NB cell lines SH-SY5Y, SK-N-F1, IMR32, and SK-N-SH NB tumor cell lines were purchased from Banca Biologica and Cell Factory (IRCCS Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la ricerca sul cancro, Genova, Italy), which use multiplex short tandem repeat profiling for cell line identification. NB cell lines were cultured in the presence of RPMI 1640 medium supplemented with 2 mM glutamine, 50 mg/ml penicillin, 50 mg/ml streptomycin, and 10% heat-inactivated FCS (Sigma-Aldrich). All NB cell lines were tested for mycoplasma contamination, morphology, and proliferation rate after thawing and within four passages in culture. For coculture experiments, 2×10^5 resting NK cells were cultured for 2 d with 3×10^5 NB cell lines. For Transwell experiments, NK cells and NB cell lines were placed in 24-well Transwell (0.3-μm pore size; Corning Costar), upper and bottom chamber, respectively.

PBMC and BM aspirates were collected from children diagnosed with stage 4 NB. Diagnosis and staging were performed according to the International Neuroblastoma Staging System. Control PBMC were obtained from residual blood drawn from healthy age-matched children admitted at Emergency Department for accidental injuries. Samples were used after they were made anonymous, and legal guardians of healthy children and patients signed a written consent allowing the use of samples for research purposes. The study has been approved by the Istituto G. Gaslini Ethics Committees, and the procedures were in accordance with the Helsinki Declaration.

PBMC from patients or healthy children were frozen first and later thawed to perform comparative cytofluorimetric analyses.

Monoclonal Abs

The following mAbs were produced in our laboratories: BAB281 (IgG1, anti-NKp46), AZ20 (IgG1, anti-NKp30), ON72 (IgG1, anti-NKG2D), JT3A (IgG2A, anti-CD3), and 5B14 (IgM, anti–4IgB7-H3) (5, 17, 38).

Anti–CD56-PC7 and a mixture of anti–CD56-PC5 and anti–CD3-FITC (IgG1) mAbs were purchased from Beckman Coulter, Immunotech (Marseille, France); anti-GD2 (IgG2A) mAb was purchased from BD Biosciences Pharmingen (San Diego, CA). Anti-CXCR3 (IgG1), anti-CXCR4 (IgG2b), and anti-CCR7 (IgG2a) mAbs were purchased from R&D Systems (Minneapolis, MN); anti-CXCR1 (IgG1) mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-CX_3CR1-PE (rat IgG2b) and the isotype control (rat IgG2b-PE) were purchased from MBL (Woburn, MA). The Abs listed above are of mouse origin unless otherwise specified. The neutralizing anti–TGF-β1 (chicken), anti–TGF-β2, anti–IL-8 (CXCL8), anti-MIF, anti-vascular endothelial growth factor (VEGF), anti–TNF-α, anti-SDF1α (CXCL12), anti–IP-10 (CXCL10), anti-MCP1 (CCL2), and anti-MDC (chicken, CCL22) were purchased from R&D Systems. Ab-mediated neutralization experiments were performed adding Abs at various concentrations (from 100 to 0.1 μg/ml) at the onset of the cell cultures. Results show data obtained with 12 μg/ml (anti–TGF-β1), 3 μg/ml (anti–TGF-β2), 10 μg/ml (anti-VEGF), and 12 μg/ml (anti-MDC) Abs. Neutralizing Abs are of goat origin unless otherwise specified.

Recombinant proteins

Human rTGF-β1, TGF-β2, and VEGF were purchased from PeproTech (Rock Hill, NJ).

Flow cytofluorimetric analysis

For cytofluorimetric analysis (FACSCalibur; BD Biosciences, Mountain View, CA), cells were stained with PE- or FITC-conjugated mAbs or with unconjugated mAbs, followed by PE-, FITC-, allophycocyanin-, or PE-Cy7–conjugated isotype-specific goat anti-mouse second reagent (Southern Biotechnology Associates, Birmingham, AL). Viability of NK cells was assessed by staining with Annexin V-FITC (eBioscience Diagnostic, Vienna, Austria) and TO-PRO-3 iodide solution in DMSO (Life Technologies) (10 min at room temperature). The percent viability of NK cells was >98%. On every experimental session, the flow cytometer performances were controlled, and the reproducibility of the fluorescence intensity was aligned using calibrite microsfere (BD Biosciences). Samples of patients and healthy donors were analyzed in the same daily session of cytometric analysis.

Analysis of soluble factors release

NB cells were cultured for 24 h in RPMI 1640 medium supplemented with 10% FCS in 96-well plates (3×10^5 cells/well). After centrifugation, supernatants were filtered and analyzed using Bio-Plex Human Cytokine 27-Plex Panel (IL-1β, IL-1 receptor antagonist, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 [p70], IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1 [monocyte chemotactic and activating factor], MIP-1α, MIP-1β, platelet-derived growth factor-BB, RANTES, TNF-α, and VEGF), Bio-Plex Human Group II Cytokine 23-Plex Panel (IFN-α2, IL-1α, IL-2Rα, IL-3, IL-12 [p40], IL-16, IL-18, CTACK, growth-related oncogene-α, HGF, ICAM-1, LIF, MCP-3, M-CSF, MIF, MIG, β-nerve growth factor, stem cell factor, SCGF-β, SDF-1α, TNF-β, TRAIL, and VCAM-1), and Bio-Plex TGF-β1,TGF-β2, and TGF-β3 Plex Panel (Bio-Plex System and Suspension Array Technology; Bio-Rad, Milan, Italy). RPMI 1640 medium supplemented with 10% FCS was used as negative control.

Statistical analysis

Wilcoxon-Mann-Whiteny p value test (nonparametric significance test) was used for assessing whether two independent samples of observations have equally large value. The test is a statistical technique that is used to analyze the rank sum of two independent groups. The statistical level of significance (p) is indicated. Graphic representation and statistical analyses were performed using the PASW Statistics, version 20.0 software (formerly SPSS Statistics) (IBM, Milan, Italy) and GraphPad Prism 6 (GraphPad Software La Jolla, CA).

Results

The SH-SY5Y NB cell line modulates CXCR4 and CX_3CR1 expression in freshly purified NK cells

Human NK cells were purified from peripheral blood of healthy donors and cultured under Transwell conditions in the presence of two representative NB cell lines, HTLA-230 characterized by MYCN amplification and 1p deletion and SH-SY5Y that lack MYCN amplification and 1p deletion. After cocultures, NK cells were analyzed for

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the surface expression of different chemokine receptors. Although coculture with HTLA-230 cells did not modify the chemokine receptors expression (Fig. 1A, 1B), coculture with SH-SY5Y cells induced in NK cells a significant upregulation of CXCR4 that was appreciable in both $CD56^{bright}$ and $CD56^{dim}$ NK cells (Fig. 1A, 1C). Moreover, SH-SY5Y conditioning resulted in downregulation of CX_3CR1 by $CD56^{dim}$ NK cells ($p < 0.05$) (Fig. 1A, 1C). The expression of CXCR1, CXCR3, and CCR7 remained unaltered (Fig. 1B).

To characterize the soluble factor(s) potentially involved in the SH-SY5Y–mediated CXCR4 and CX_3CR1 modulation, supernatants of NB cell cultures were analyzed for the presence of up to 50 soluble mediators using a Multiplex Assay (see *Materials and Methods*). SH-SY5Y and HTLA-230 cells secreted comparable amounts of the proangiogenic factors VEGF and MIF (data not shown). On the contrary, SH-SY5Y released significant higher amounts of MCP-1 (CCL2), HGF and M-CSF growth factors, IP-10 (CXCL10) proinflammatory chemokine, SDF1-α (CXCL12) and CTACK (CCL27) homeostatic chemokines, TNF-α, IL12p40, IL-16, and IFN-2α cytokines and IL-2 receptor antagonist (Fig. 2). Regarding TGF-β, SH-SY5Y produced amounts of TGF-β1 six times higher than HTLA-230 cells, whereas the two NB cell lines released comparable low amounts of TGF-β2 (Fig. 2). All the other soluble mediators analyzed (see *Materials and Methods*), including TGF-β3 (Fig. 2) were undetectable in the supernatants of both SH-SY5Y and HTLA-230 cell lines.

Overall, the analysis showed a great heterogeneity in the secretory profile of the NB cells analyzed and proposed a number of SH-SY5Y-derived soluble factors being possibly involved in the modulation of CXCR4 and CX_3CR1 in NK cells.

TGF-β1 is responsible for chemokine receptor modulation in NB-conditioned NK cells

In order to clarify which soluble factor(s) played a role in CXCR4 and CX_3CR1 modulation, SH-SY5Y/NK cocultures were performed in the presence of neutralizing Abs specific for the soluble mediators secreted by the SH-SY5Y cell line. Ab-mediated blocking of TGF-β1 alone interfered with the effect of SH-SY5Y conditioning. Indeed, NK cells cocultured in the presence of SH-SY5Y and anti–TGF-β1 Ab showed a diminished upregulation of CXCR4 ($p < 0.05$) and downregulation of CX_3CR1 ($p < 0.05$) (Fig. 3A). Thus, in the presence of the neutralizing Ab, SH-SY5Y–conditioned NK cells expressed levels of CXCR4 and CX_3CR1 comparable to those detected in unconditioned NK cells (p = ns). A representative experiment is shown in Fig. 3B. Ab-mediated neutralization of other factors such as MCP-1, IP-10, SDF1α, TNF-α, IL-8, and VEGF, as well as of TGF-β2, did not interfere with SH-SY5Y-conditioning. Moreover, the anti–TGF-β2 Ab did not show a significant additive or synergistic effect when used in combination with the anti–TGF-β1 Ab (Fig. 3).

To establish whether SH-SY5Y shared with other NB cell lines the capability of modulating the chemokine receptor expression in NK cells, NK cells were cocultured with additional NB cell lines. GI-LI-N, IMR-32, and SK-N-SH cell lines did not modify the expression of the chemokine receptors analyzed (Supplemental Fig. 1A, 1B). In contrast, NK cells cocultured with the SK-N-F1 cell line displayed a significant CXCR4 upregulation ($p < 0.001$ for both median fluorescence intensity [MFI] and percentage of positive cells) and CX_3CR1 downregulation ($p < 0.05$ and $p < 0.01$ for MFI and percentage of positive cells, respectively) (Supplemental Fig. 1A, 1B). Interestingly, the effect of SK-N-F1 conditioning was even higher than that mediated by SH-SY5Y cells. Moreover, different from SH-SY5Y, SK-N-F1 conditioning also induced significant upregulation of CXCR3 ($p < 0.05$ for both MFI and percentage of positive cells) (Supplemental Fig. 1A, 1B). The Multiplex Assay showed that SK-N-F1 cells secrete amounts of TGF-β1 approximately two times higher than those released by SH-SY5Y cells (Supplemental Fig. 1C). Importantly, in SK-N-F1/NK cocultures, Ab-mediated blocking of TGF-β1 interfered not only with the modulation of CXCR4 and CX_3CR1 but also with that of CXCR3 (Fig. 4A). A representative experiment is shown in Fig. 4B.

Treatment of NK cells with rTGF-β1 and rTGF-β2 results in modulation of the chemokine receptor repertoire similar to that of NB-conditioned NK cells

To confirm the role of TGF-β1 in CXCR4, CX_3CR1, and CXCR3 modulation, resting NK cells were cultured in the presence of

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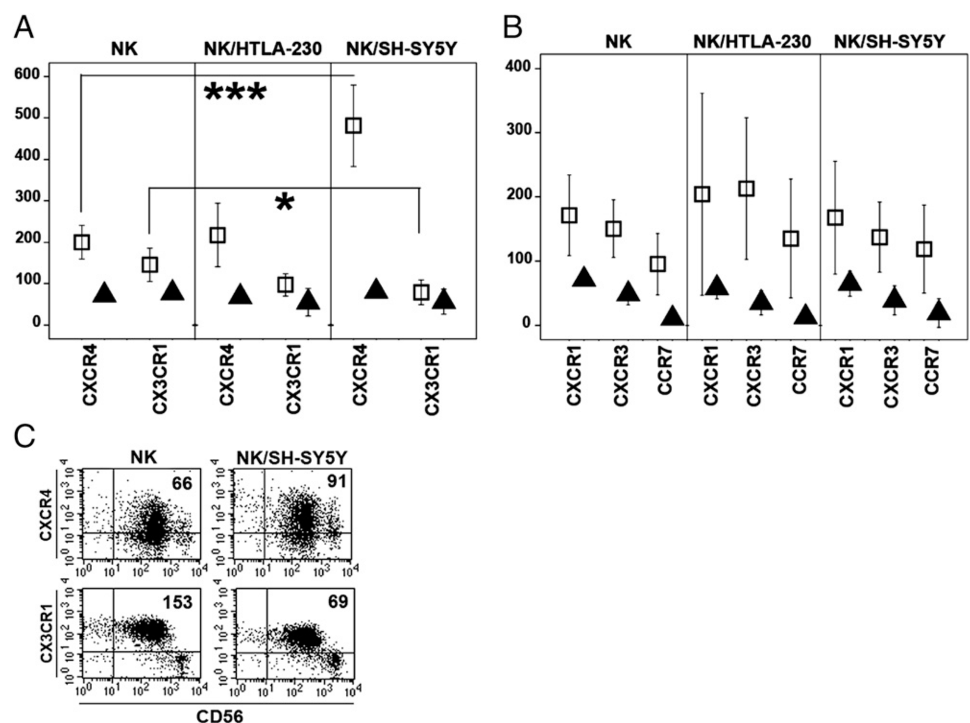

FIGURE 1. SH-SY5Y NB cell line modulates the expression of CXCR4 and CX3CR1 in NK cells. (**A** and **B**) Peripheral blood NK cells were cocultured with HTLA-230 or SH-SY5Y NB cell lines in Transwell conditions. After 2 d, NK cells were analyzed by flow cytometric analysis for the expression of the indicated chemokine receptors. Squares and triangles indicate the MFI and the percentage of positive cells (%), respectively. Data pooled from six independent experiments. Mean of the values, 95% confidence intervals, and significance are shown. *$p < 0.05$, ***$p < 0.001$. (**C**) Representative two-color flow cytometric analysis of CXCR4 and CX3CR1 expression in SH-SY5Y–conditioned NK cells. MFI is indicated.

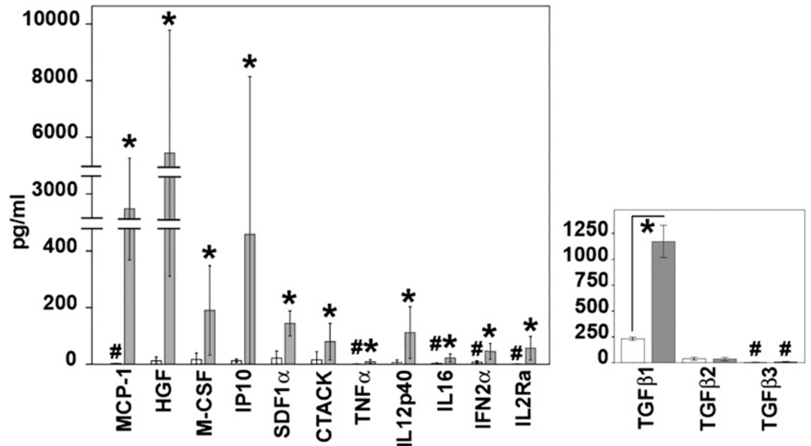

FIGURE 2. HTLA-230 and SH-SY5Y NB cell lines display different secretory profiles. Supernatants from HTLA-230 (white bar) and SH-SY5Y (gray bar) cell lines were analyzed by Multiplex Assays for the presence of the indicated cytokines. Data pooled from three independent experiments. Mean, 95% confidence intervals, and significance are shown. $^*p < 0.05$; #, undetectable.

different amounts of rTGF-β1 (or rTGF-β2). Recombinant VEGF was used as negative control. As shown in Fig. 5, rTGF-β1 induced CXCR4 and CXCR3 upregulation as well as CX_3CR1 downregulation. Thus, rTGF-β1 mimicked the chemokine receptor repertoire modifications detected in NK cells upon conditioning by NB cell lines. Interestingly, a similar pattern of chemokine receptor modulation was induced by rTGF-β2. It is of note that the concentrations of rTGFβ2 that were effective were 20 times higher than those released by NB cell lines (Fig. 5). In this context, not only TGFβ1, as previously described (17), but also TGF-β2 caused downregulation of the NKp30 activating NK receptor. The modulation of the NKp30 expression required high amounts of either rTGF-β1 or rTGF-β2 (>10 ng/ml), whereas its expression was unaffected at cytokines concentrations (<10 ng/ml) that were sufficient to induce modulation of the chemokine receptors (Fig. 5).

Altogether, these data suggest that the immune modulatory role played by tumor cells such as NB via TGF-β1 might be extremely dose dependent. Thus, low amounts of the cytokine are sufficient to modulate CXCR4 and CX_3CR1 expression, modification of CXCR3 expression requires intermediate amounts of TGF-β1, whereas very high amounts are needed to downregulate the expression of the NKp30 activating receptor. Our data also show that TGF-β2 might exert a redundant or alternative role in the modification of the NK cell triggering and chemokine receptor repertoire.

Analysis of the chemokine receptor repertoire in NK cells from NB patients

Peripheral blood samples were derived from six NB patients with stage 4 disease and, as control, from six age-matched healthy children. NK cells were analyzed for the expression of different chemokine receptors by cytofluorimetric analysis. Results are depicted in Fig. 6A, and a representative experiment is shown in Fig. 6B. In NB patients, $CD56^{dim}$ NK cells showed significant downregulation of CX_3CR1 expression both in term of MFI and percentage of positive cells ($p < 0.05$ for both parameters). Regarding CXCR4 and CXCR3, no significant differences in both $CD56^{dim}$ and $CD56^{bright}$ subpopulations were observed within all the samples analyzed. However, CXCR3 expression was clearly

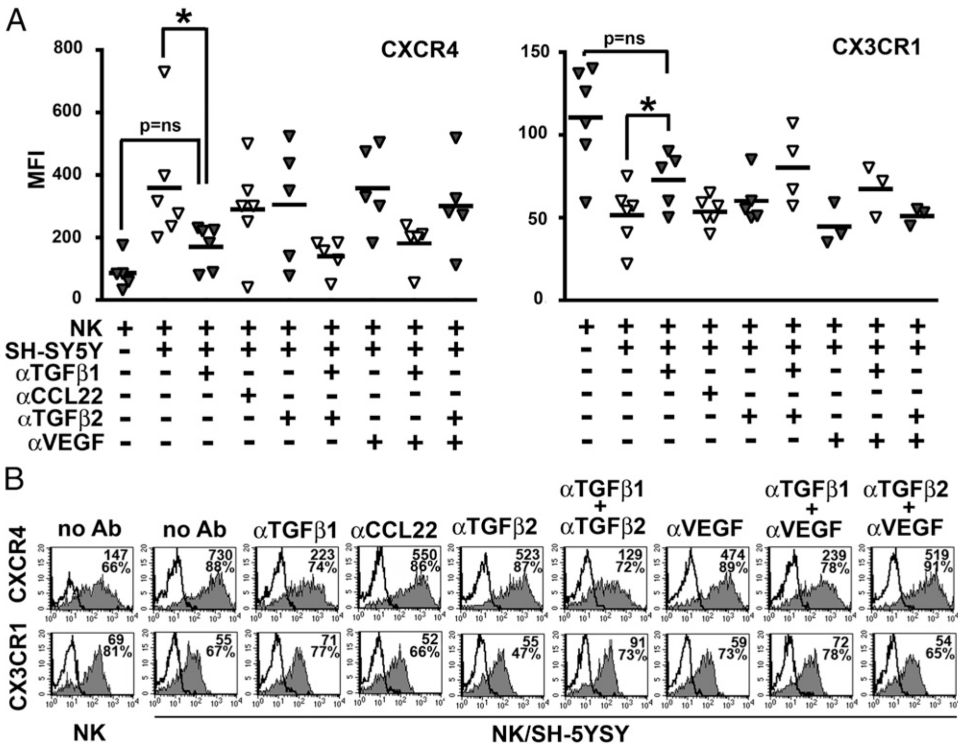

FIGURE 3. TGF-β1 is responsible for CXCR4 and CX3CR1 modulation in SH-SY5Y–conditioned NK cells. (**A**) Peripheral blood NK cells were cocultured with SH-SY5Y NB cell line either in absence or in the presence of neutralizing Abs to the indicated soluble factors. Cells were analyzed by flow cytometric analysis for the expression of CXCR4 and CX3CR1. MFI is indicated. Data pooled from six independent experiments. Mean of the values and significance are shown. $^*p < 0.05$. (**B**) A representative experiment is shown. MFI and percentage of positive cells are indicated.

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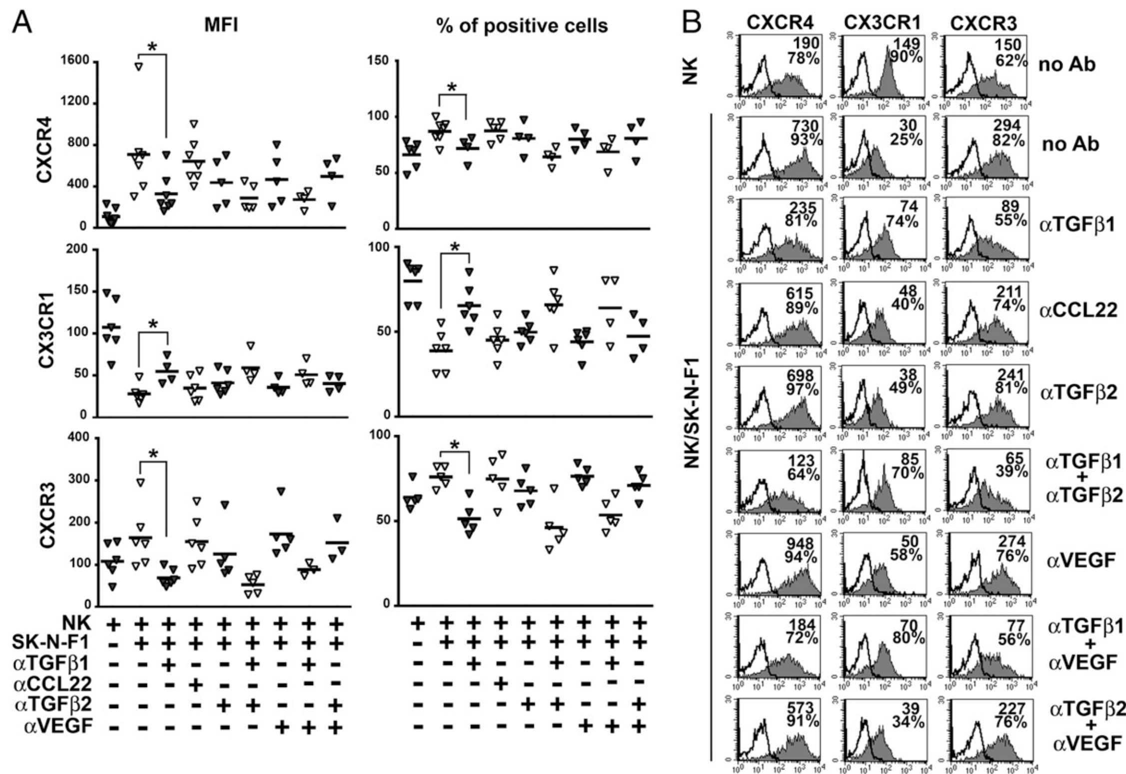

FIGURE 4. TGF-β1 released by SK-N-F1 is responsible for CXCR4, CX3CR1, and CXCR3 modulation in NK cells. (**A**) Peripheral blood NK cells were cocultured with SK-N-F1 NB cell line either in absence or in the presence of neutralizing Abs to the indicated soluble factors. NK cells were analyzed by flow cytometric analysis for the expression of CXCR4, CX3CR1, and CXCR3. MFI and percentage of positive cells are shown in *left* and *right panels*, respectively. Data pooled from six independent experiments. Mean of the values and significance are shown; $*p < 0.05$. (**B**) A representative experiment is shown. MFI and percentage of positive cells are indicated.

upregulated in CD56^{brigh} NK cells from some patients as compared with healthy children (Fig. 6A). This might reflect a tendency toward increase in the CXCR3 expression by NK cells derived from NB patients. Finally, no statistically significant differences were observed in the expression of NKp30 and NKG2D triggering NK receptors (Supplemental Fig. 2), which can be downregulated in vitro by high concentrations of TGF-β1 (17).

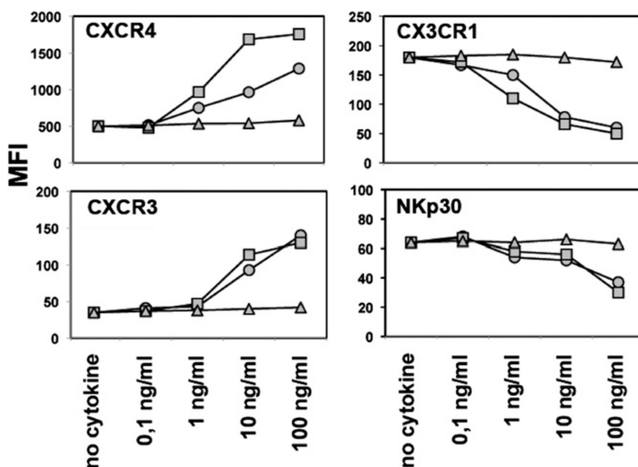

FIGURE 5. rTGF-β1 and rTGF-β2 modulate CXCR4, CX3CR1, CXCR3, and NKp30 receptors in NK cells. Peripheral blood NK cells were cultured for 2 d in the presence of increasing concentrations of rTGF-β1 (circle), rTGF-β2 (square), and rVEGF (triangle) and analyzed by flow cytometric analysis for the expression of the indicated molecules. MFI is shown.

Next, we analyzed the expression of the various chemokine receptors in NK cells present in BM aspirates of NB patients with stage 4 disease, with and without marrow infiltration by NB cells (Fig. 6B). According to histopathological examination, flow cytometric analysis showed high numbers of CD56$^+$4Ig-B7-H3$^+$GD2$^+$ NB cells in infiltrated BM samples (5, 38), while these cells were absent in noninfiltrated BM samples (Fig. 6B, Supplemental Fig. 3). The (CD3$^-$CD56$^+$4IgB7-H3$^-$NKp46$^+$) BM NK cell population displayed both CX_3CR1 downregulation and CXCR3 upregulation. Interestingly, the chemokine receptor modulation was higher in NK cells from the patient with BM metastasis as compared with that found in NK cells from patients without NB cell infiltration (Fig. 6B).

Altogether these data suggest that NB conditioning could exert in vivo an immune modulatory effect resembling that emerged from in vitro experiments. It is of note that NK cells from NB patients also displayed a reduced expression of CXCR1 as compared with healthy donors. This was detected not only in peripheral blood NK cells but also in BM NK cells (Fig. 6). CXCR1 modulation was not detected in NK cells conditioned in vitro with either NB cell lines or rTGF-β1 and rTGF-β2.

Discussion

In the current study, we show that NB cells modulate the chemokine receptor repertoire of human NK cells. The phenomenon was observed with some but not all NB cells studied in our laboratory, this finding confirming the heterogeneity of NB cells. Searching for NB-derived soluble mediators endowed with immunomodulatory properties, we identified NB-derived TGF-β1 as a pivotal factor involved in the CXCR4, CXCR3, and CX_3CR1 modulation in resting NK cells. This is in line with previously published results

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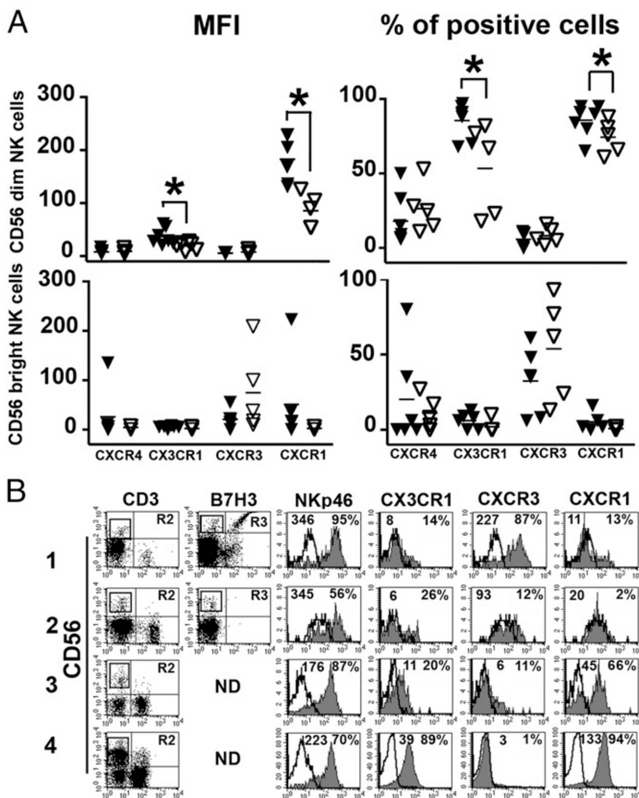

FIGURE 6. Altered expression of chemokine receptors in NK cells of NB patients. (**A**) $CD3^{-}CD56^{dim}$ (up) or $CD3^{-}CD56^{bright}$ (down) NK cells from healthy donors (▼) or neuroblastoma patients (▽) were analyzed by three-color flow cytometric analysis for the expression of CXCR4, CX3CR1, CXCR3, and CXCR1. MFI (*left*) and percentage of positive cells (*right*) are shown. Mean of the values and significance are shown; $*p < 0.05$. (**B**) NKp46, CX3CR1, CXCR3, and CXCR1 expression was analyzed by four-color flow cytometric analysis in NK cells from 1) NB-infiltrated or 2) NB-free BM aspirates, 3) peripheral blood of NB patients, or 4) healthy donors gating on $CD3^{-}CD56^{+}$ (4Ig-B7-$H3^{-}$) NK cells. MFI and percentage of positive cells are shown.

showing that rTGF-β1 is capable of upregulating CXCR4 and CXCR3 in human NK cells (39). Interestingly, although the low concentrations of TGF-β2 secreted by NB cells had no effects, high concentrations of rTGF-β2 modulated the NK cell chemokine receptor repertoire in a manner similar to that observed with TGF-β1. This suggests that TGF-β2 could provide an additional/alternative immunomodulatory mechanism used by given tumor types to escape/modulate immune surveillance.

NB-derived TGF-β1, rTGF-β1, as well as rTGF-β2, increased the expression of CXCR4 and CXCR3 while downregulating that of CX_3CR1. The opposite modulation of CXCR4 and CX_3CR1 might be extremely interesting in light of the data described in human and mice. CXCR4 is essential for development, homing, and maintenance of NK cells in stromal cell niches within BM (29, 30). In contrast, CX_3CR1 is a crucial receptor that regulates the egress of NK cells from BM in homeostatic conditions (32, 33). Indeed, in BM, $CXCR4^{+}CX_3CR1^{-}$ ($KLRG1^{+}$) NK cells were mainly positioned into parenchyma, whereas $CXCR4^{-}CX_3CR1^{+}$ ($KLRG1^{-}$) NK cells preferentially resided within the sinusoids (33). Thus, TGF-β1 and TGF-β2 by inducing in human NK cells a $CXCR4^{bright}CX_3CR1^{dull/neg}$ phenotype might favor their retention in BM, hindering their recruitment in peripheral tissues including the tumor sites.

Our study also shows that high levels of NB-derived TGF-β1 and rTGF-β2 induced a significant upregulation of CXCR3. During the late phase of tumor progression, tumor cells (that are resistant to growth inhibition by TGF-β) and infiltrating myeloid suppressor cells increase the production of TGF-β (40). Moreover, it has been shown that NK cells preferentially driven toward tumors belong to the $CD56^{bright}$ immature, poor cytolytic NK cell subset (25). In this context, it is of note that $CD56^{bright}$ but not $CD56^{dim}$ express CXCR3 in steady-state conditions (11, 28). Our data show that NK cells within BM of NB patients upregulate CXCR3, the effect reaching higher levels in highly infiltrated BM aspirates. Moreover, peripheral blood NK cells from NB patients showed levels of CXCR3 higher than healthy (age-matched) donors, and this phenomenon was restricted to the $CD56^{bright}$ NK cell subset. These data suggest that in NB patients upon TGF-β1 conditioning, $CD56^{bright}$ NK cells might reach CXCR3 levels sufficient to overcome CXCR4-mediated anchoring in BM, thus driving this NK cell subset into the tumor. On the contrary, NB-derived TGF-β1 could mediate the preferential retention in BM of $CD56^{dim}$ NK cells by increasing CXCR4 expression and reducing that of CX_3CR1. It is well established that CX_3CR1 drives NK cell migration in response to the membrane-bound CX_3CL1 (fractalkine) ligand. In addition, the CX_3CR1/CX3CL1 interaction can increase NK cell responsiveness to CCL4 (MIP1b) and CXCL8 (IL-8) (41). CX_3CR1 was also reported to promote IFN-γ production and cytotoxicity by NK cells (42). Thus, the NB-mediated downregulation of CX_3CR1 might represent a "Janus escape mechanism" impairing both NK cell migration toward tumors and the function of tumor-infiltrating NK cells.

In vitro experiments performed under transwell culture conditions showed that TGF-β1 (and rTGF-β2) upregulate CXCR4 in both $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets. In this context, a significant upregulation of CXCR4 was also detected in the presence of cell-to-cell contacts between NB and NK cells (Supplemental Fig. 4A), although differences in the modulation of other chemokine receptors could be appreciated. In contrast, CXCR4 upregulation was not measurable in NK cells from BM aspirates of NB patients. A possible explanation is that in NB patients (and healthy donors), CXCR4 might be masked by the high concentrations of the CXCL12 (SDF1) ligand present in BM microenvironment (43).

Our data also show that very high concentrations ($>$10 ng/ml) of rTGF-β1 and rTGF-β2 are able to downregulate the NKp30 activating receptor. Thus, the effect of these cytokines on NK cells might be extremely dose dependent, ranging from systemic modulation of the NK cell chemokine receptor repertoire to reach, possibly via a paracrine in situ effect, the downregulation of crucial NK cell triggering receptors. In this context, NKp30 is involved in the recognition and killing of most tumors including ex vivo–derived NB cells (5).

Quantification of blood levels of immunostimulatory or immunomodulatory cytokines could help to monitor various diseases including cancer. This method, however, cannot be applied to TGF-β. Indeed, unlike other cytokines that are secreted in an active form, TGF-β is released as "small latent complex," that is, noncovalently associated with the latency-associated peptide (LAP), which renders TGF-β inactive. Moreover, TGF-β/LAP heterodimers can associate with the TGF-β–binding protein to form "large latent complexes." Finally, as a further complication, LAP homodimers have been detected (44). To date, the available assays are unable to discriminate between the free/active TGF-β and the latent complexes. Thus, quantification of TGF-β is poorly informative, and high levels of TGF-β with unknown activation status can be detected in plasma from both healthy donors and neoplastic patients (Supplemental Fig. 4B). An interesting hypothesis is that in neoplastic patients tumor cells might activate locally the latent complex (via integrins and/or matrix metalloproteinase), thus re-

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leasing amounts of free/active TGF-β sufficient to exert a systemic immunomodulatory effect (44).

Finally, it is of note that NK cells from NB patients downregulated CXCR1. This was detected in both peripheral blood and BM-derived NK cells, particularly in the presence of BM metastases. The factor(s) responsible for this phenomenon remains unclear. Indeed, the modulation of CXCR1 is clearly TGF-β independent because it was not observed in rTGF-β1– and rTGF-β2–conditioned NK cells. In addition, it appears to be independent also from other soluble mediators including MIF, which was released in very high amounts by all NB cell lines analyzed.

In summary, our results shed new light on NB-mediated modulation of NK cell function and homing that could have relevance in terms of clinical translation for treating children affected by this aggressive neoplasm.

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Disclosures

A.M. is founder and shareholder of Innate Pharma (Marseille, France). The other authors have no financial conflicts of interest.

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