Multiple Myeloma Impairs Bone Marrow Localization of Effector Natural Killer Cells by Altering the Chemokine Microenvironment

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Abstract

Natural killer (NK) cells are key innate immune effectors against multiple myeloma, their activity declining in multiple myeloma patients with disease progression. To identify the mechanisms underlying NK cell functional impairment, we characterized the distribution of functionally distinct NK cell subsets in the bone marrow of multiple myeloma-bearing mice. Herein we report that the number of KLRG1\textsuperscript{+} NK cells endowed with potent effector function rapidly and selectively decreases in bone marrow during multiple myeloma growth, this correlating with decreased bone marrow NK cell degranulation in vivo. Altered NK cell subset distribution was dependent on skewed chemokine/chemokine receptor axes in the multiple myeloma microenvironment, with rapid downmodulation of the chemokine receptor CXCR3 on NK cells, increased CXCL9 and CXCL10, and decreased CXCL12 expression in bone marrow. Similar alterations in chemokine receptor/chemokine axes were observed in patients with multiple myeloma. Adoptive transfer experiments demonstrated that KLRG1\textsuperscript{+} NK cell migration to the bone marrow was more efficient in healthy than multiple myeloma--bearing mice. Furthermore, bone marrow localization of transferred CXCR3-deficient NK cells with respect to wild type was enhanced in healthy and multiple myeloma-bearing mice, suggesting that CXCR3 restraints bone marrow NK cell trafficking. Our results indicate that multiple myeloma–promoted CXCR3 ligand upregulation together with CXCL12 downmodulation act as exit signals driving effector NK cells outside the bone marrow, thus weakening the antitumor immune response at the primary site of tumor growth.

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Introduction

Natural killer (NK) cells are innate lymphoid cells that play key roles in the immune surveillance against tumors with a preferential activity toward hematologic malignancies, by exhibiting natural cytotoxic activities and secreting a wide array of cytokine/chemokines (1–5). In C57BL/6 mouse, NK1.1\textsuperscript{+}/CD3\textsuperscript{−} NK cells can be subdivided into functionally and developmentally distinct populations according to CD27 and CD11b expression levels (6). At later stages of differentiation, CD27\textsuperscript{low}/CD11b\textsuperscript{high} NK cells also acquire KLRG1, a receptor that defines an NK cell population with reduced ability to proliferate and mediate effector functions in response to cytokines, but in some instances displaying antitumor activity (7–10).

NK cell recruitment and tumor infiltration have been associated with a less severe disease in human tumors of different histotypes and in a number of mouse tumor models (11–17). A number of G protein–coupled receptors belonging to the chemokine receptor family on NK cell surface and their corresponding tissue counter ligands regulate NK cell mobilization into blood from storage tissues including bone marrow and spleen, and their extravasation and infiltration of tumor-bearing tissues to initiate an antitumor response (1, 18).

Accumulating evidence supports a role for NK cells in the control of development and progression of multiple myeloma, an hematologic neoplasia characterized by abnormal monoclonal plasma cell proliferation in the bone marrow, the main site of NK cell development (19). NK cell number and activity negatively correlate with the clinical stage of the disease, suggesting that multiple myeloma growth interferes with the NK cell ability to counteract tumor expansion (20–22).

Multiple myeloma cells can elude NK cell surveillance through several strategies, including reduction of NKG2D-activating receptor recognition (23), and inhibition of NK cell function (24–27). However, the ability of multiple myeloma microenvironment to affect recruitment/retention of functional NK cell subsets at site of tumor growth has not been investigated so far. To shed light on this matter, we followed the activation state of NK cells and their ability to reach and reside in bone marrow in a well-characterized...
murine model of multiple myeloma (28). Our findings demonstrate a defective migration/retention to the bone marrow of the more functional KLRG1− NK cell subsets in a early multiple myeloma growth phase, which is related to substantial dysregulation of CXCR3 and CXCR4 chemokine receptor/ligand axes. Of note, similar changes in the chemokine system are observable in multiple myeloma patients, suggesting that this might have impact on NK cell response also in the human disease.

**Materials and Methods**

Supplementary Materials and Methods section includes information related to reagents, cell preparation and NK cell enrichment, FACS analysis, ELISA, functional assays, mixed bone marrow chimera, isolation of bone marrow cell populations, and real-time PCR.

**Cell lines and clinical samples**

STGM1 multiple myeloma cells were kindly provided by Dr. Yoneda, (University of Texas, San Antonio, TX) and maintained in RPMI1640 medium supplemented with 10% FBS, 2 mmol/L glutamine, 55 μmol/L β-mercaptoethanol and antibiotics. Cell lines were periodically authenticated by morphologic inspection, verified to be mycoplasma free, and were passaged for no more than 4 to 6 weeks from thawing. Peripheral blood and bone marrow samples were obtained from untreated patients with multiple myeloma to be mycoplasma free, and were passaged for no more than 4 to 6 weeks from thawing. Peripheral blood and bone marrow samples were obtained from untreated patients with multiple myeloma.

**Cell lines and clinical samples**

Materials and Methods was tested, one sample t test was used for each condition with a hypothetical value of 1.

**Results**

Multiple myeloma cells are sensitive to NK cell-mediated antitumor activity in vivo and in vitro.

Our study was aimed at determining the in vivo relevance of NK cells in multiple myeloma clearance and how tumor growth could influence NK cell function in an orthotopic mouse model of multiple myeloma. Tumor growth was evaluated by monitoring the number of IgG2b− cells (produced by STGM1 cells) in bone marrow and spleen at different times after cell injection into syngeneic C57BL/10Scid mice (Fig. 1A). Myeloma cells were already detected at 2 weeks, represented 2% to 3% of total bone marrow cells after 3 weeks and reached 7% to 10% at 4 weeks. Mice began to show macroscopic signs of disease (i.e., paraplegia, weight loss) between the 4th and 5th week, when tumor growth leads to bone marrow architecture disruption (31), and thus we chose 4 weeks as experimental endpoint. Mice depleted of NK1.1+ cells by repeated intraperitoneal injections of anti-NK1.1 monoclonal antibody (mAb) displayed higher rate of tumor growth in bone marrow and spleen as compared with PBS-treated controls (Fig. 1B). Clinical signs of disease were also supported by increased serum paraprotein levels between the 3rd and 4th week that further increased in NK1.1+ cell-depleted mice (Fig. 1D). The in vivo impact of NK cells in the response against multiple myeloma cells was confirmed in vitro with splenic NK cell killing of STGM1 cells using standard 51Cr release assay (data not shown), as previously reported (32, 33). The killing involved granule release as shown by increased NK cell degranulation upon tumor cell interaction (Fig. 1C). The CD11b+KLRG1− subset was the most functional, while the more mature CD11b+KLRG1+ cells displayed the lowest degranulation capacity among NK cell subpopulations.

Multiple myeloma growth results in reduced bone marrow NK cell number

To analyze qualitative and quantitative changes of NK cell populations occurring during tumor growth, total NK cell...
NK cells display anti-multiple myeloma activity in vivo and in vitro. A, bone marrow (BM; two tibiae and femurs) and spleen cells were collected from individual tumor-bearing mice at 2, 3, and 4 weeks after 5TGM1 injection. Tumor cell (IgG2b⁺) frequency (right) and number (left) in the organs are expressed as mean value ± SEM and are representative of at least 12 mice for each group in four separate experiments. Student t test was performed to compare tumor growth in the bone marrow versus spleen; ***, P < 0.001. B, tumor growth in bone marrow and spleen and the concentration of soluble IgG2b in sera were monitored in C57BL/KaLwRij mice depleted of NK1.1⁺ cells. Data are expressed as mean of IgG2b⁺ cell number or of soluble IgG2b concentration ± SEM of at least 9 mice for each group in three separate experiments. Student t test was performed for NK1.1-treated versus the corresponding PBS-treated group; **, P < 0.01; ***, P < 0.001. C, enriched splenic NK cells from healthy mice were incubated with or without 5TGM1 cells and degranulation of NK cell populations was assessed by FACS. Top, representative dot plots showing the frequency of CD107a⁺ on NK cells and NK cell subsets. Bottom, average values ± SD of CD107a⁺ cell frequency upon 5TGM1 stimulation subtracted of degranulation in the absence of target in three independent experiments. Student t test was performed to compare the indicated experimental groups. *, P < 0.05; ***, P < 0.001.
CD3^+ /NK1.1^+ and NK cell subset number in bone marrow and spleen was determined at different times after multiple myeloma cell injection. NK cell number started to decrease at 3 weeks within bone marrow, when tumor burden was low, while it remained unchanged in the spleen (Fig. 2A). No changes of organ cellularity and number of NKT (control: 2.55 \times 10^5 / 1.57 \times 10^4; tumor: 2.49 \times 10^5 / 1.31 \times 10^4) and T cells (control: 8.27 \times 10^5 / 5.9 \times 10^4; tumor: 7.36 \times 10^5 / 6.9 \times 10^4), identified as CD3^+ NK1.1^+ and CD3^-/NK1.1^- lymphocytes, respectively, occurred in the bone marrow and spleen at three weeks after tumor injection. Data are representative of at least 12 individual mice from three independent experiments. C, CXCL10, CXCL9, and CXCL12 concentrations were assessed in the bone marrow extracellular fluids from healthy control (week 0) or multiple myeloma-bearing mice at different weeks by ELISA. Histograms show the mean values ± SEM obtained from at least 5 individual mice per group in at least two independent experiments. Student t test in A, B, and C was performed to compare tumor-bearing versus healthy control mice. *, P < 0.05; **, P < 0.01; †††, P < 0.001.

Multiple myeloma growth results in progressive decrease of NK cell number in bone marrow and in alterations of the bone marrow chemokine milieu. A, CD3^- NK1.1^- NK cell number were quantified in bone marrow and spleen of healthy control (CTRL) and tumor-bearing (TUM) mice during multiple myeloma progression. The graph horizontal bar represents the mean value and each circle represents NK cell number of a single mouse (n < 12). Data were obtained from at least three independent experiments. B, NK cell populations were identified according to their CD11b and KLRG1 expression levels. Histograms show the mean values ± SEM of NK cell numbers in the bone marrow and spleen at three weeks after tumor injection. Data are representative of at least 12 individual mice from three independent experiments.

Multiple myeloma growth results in altered expression levels of CXCR3 and CXCR4 ligands in bone marrow and downmodulation of CXCR3 on bone marrow NK cells

NK cell function is controlled by several chemokines, with a relevant role played by the CXCR4/CXCL12 axis in the regulation of NK cell migration. (CD3^-NK1.1^-) and NK cell subset number in bone marrow and spleen was determined at different times after multiple myeloma cell injection. NK cell number started to decrease at 3 weeks within bone marrow, when tumor burden was low, while it remained unchanged in the spleen (Fig. 2A). No changes of organ cellularity and number of NKT (control: 2.55 \times 10^5 / 1.57 \times 10^4; tumor: 2.49 \times 10^5 / 1.31 \times 10^4) and T cells (control: 8.27 \times 10^5 / 5.9 \times 10^4; tumor: 7.36 \times 10^5 / 6.9 \times 10^4), identified as CD3^- NK1.1^- and CD3^-/ NK1.1^- lymphocytes, respectively, occurred in the bone marrow of tumor-bearing mice at the 3rd week (data not shown), indicating that a low tumor burden selectively affects NK cells among NK1.1^- cells. When NK cell subset distribution was examined, we found that the initial decrease of bone marrow NK cell number was attributable only to the KLRG1^- subsets (Fig. 2B), including both CD11b^low (40% reduction) and CD11b^high cells (35% reduction), while CD11b^high/KLRG1^- NK cell number remained unaffected. A more general dysfunction of the bone marrow compartment was evident at 4 weeks, with higher tumor cell frequency, decreased bone marrow cellularity and NK (all subsets) and T-cell numbers (Fig. 2A and data not shown).

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of NK cell retention in bone marrow (29, 34). Thus, we analyzed the concentration of several chemokines in the bone marrow extracellular fluids by ELISA; those that were found modulated during tumor growth were further tested in spleen and serum. We found that CXCL9 and CXCL10 (CXCR3 ligands) but not CCL3, CCL5 (CCR1 and CCR5 ligands) and CX3CL1 (CX3CR1 ligand, Fig. 2C and data not shown), were upregulated in the bone marrow extracellular fluid of tumor-bearing mice peaking at 3 weeks after tumor cell injection (about 2-fold), when no changes occurred in spleen and only CXCL9 increased in serum (Supplementary Fig. S1A). Conversely, the levels of CXCL11 (CXCR4 ligand) started to decrease in bone marrow from multiple myeloma tumor-bearing mice at the 3rd week (30% reduction), but remained unchanged in spleen and serum (Fig. 2C and Supplementary Fig. S1A). To identify the cellular source of the modulated chemokines, we collected samples from control and 3rd week tumor-bearing mice and performed RT-PCR analysis of freshly isolated and highly purified (>95%) CD11b⁺ bone marrow cell populations, mainly comprising monocyte/macrophages, neutrophils, and dendritic cells. CXCL12 expression was analyzed in CD45⁺CD11b⁺ (>90%) plastic-adherent enriched stromal cell population that represents preferential producers of the chemokine and in the CD45⁺CD11b⁺ (>98%) counterpart. Data shown in Supplementary Table S2 indicate that CXCL9 and CXCL10 mRNA expression levels increased in bone marrow CD11b⁺ cells of tumor-bearing mice, while CXCL12 mRNA expression levels did not change in purified adherent cells as well as in total bone marrow cells. Of note, also STEM1 multiple myeloma cell line produced CXCL10 and may contribute to the increased bone marrow protein levels in tumor-bearing mice (Supplementary Fig. S1B). Thus, multiple myeloma growth is associated with marked changes in the bone marrow chemokine levels being CD11b⁺ cells relevant producers of CXCL9 and CXCL10 during multiple myeloma growth.

To further analyze the relevance of chemokine expression in bone marrow tumor microenvironment, we analyzed the expression levels of their respective receptors on NK cell populations. Among the chemokine receptors tested, only CXCR3 was substantially downmodulated on bone marrow NK cells from tumor-bearing as compared with healthy control mice, with the reduction prevalently confined to the KLRG1⁻ NK cell subsets that display higher levels of this receptor compared with other NK cell subsets (Fig. 3A and B). To investigate the mechanism of CXCR3 regulation, we analyzed CXCR3 downmodulation kinetics on transferred NK cells that migrated to bone marrow of recipient mice (Fig. 3C): similar to endogenous bone marrow NK cells, CXCR3 but not CXCR4 membrane expression on transferred NK cells declined in tumor-bearing as compared with healthy recipient mice; this event occurred at 3 hours after transfer, allowing to exclude transcriptional regulation and/or development alterations, processes that usually do not take place in such a short time.

The mechanisms underlying KLRG1⁻ bone marrow NK cell reduction were further analyzed focusing on the 3rd week of tumor growth, when selective reduction of NK cell populations and higher changes in the bone marrow chemokine milieu occur.

KLRG1⁻ NK cell migration to bone marrow is impaired in multiple myeloma tumor-bearing mice

As no changes were observed in the survival rate of NK cell populations and in the transition of transferred KLRG1⁻ to KLRG1⁺ NK cells in healthy control versus tumor-bearing mice, we excluded the possibility that decreased KLRG1⁻ NK cell number could be due to reduced survival or accelerated KLRG1⁺ NK cell generation promoted by tumor growth (Supplementary Fig. S2). Thus, we characterized the tissue trafficking capacity of NK cell subsets in the multiple myeloma tumor-bearing host as compared with healthy control recipient mice by performing in vivo migration experiments. Like endogenous bone marrow NK cells, adoptively transferred KLRG1⁻ but not KLRG1⁺ NK cells displayed a marked reduction of their localization in bone marrow of tumor-bearing mice, while their number remained higher in the blood and similar in the spleen (Fig. 4A). These data suggest that multiple myeloma cells alter NK cell trafficking in bone marrow by perturbing the chemokine system in an early tumor growth phase occurring between the 2nd and the 3rd week.

CXCR3 acts as a rheostat for NK cell localization in bone marrow in steady state and in multiple myeloma

The results shown above suggest that reduced CXCR3⁺ NK cell number in the bone marrow of multiple myeloma–bearing mice depends on ligand-induced receptor internalization on NK cells recruited to bone marrow and/or on impaired CXCR3⁺ NK cell migration/retention within this compartment. To directly analyze the role of CXCR3 on NK cells, we set up competitive adoptive transfer experiments comparing CD45.1⁺ WT versus CD45.2⁺ CXCR3⁻/⁻ NK cell migration in control and tumor-bearing mice. As we obtained similar results at 3 and 18 hours after transfer, only the data obtained at 18 hours are presented. Similar to WT NK cells, CXCR3-deficient NK cell migration to the bone marrow of multiple myeloma–bearing mice was reduced, but CXCR3 deficiency markedly enhanced NK cell capacity to enter/remain in the bone marrow both in healthy and tumor-bearing recipient mice (Fig. 4B). KLRG1⁻ NK cells were the most affected as evidenced calculating the WT/CXCR3⁻/⁻ ratio of donor NK cell populations in bone marrow that decreased of 1.5-fold for KLRG1⁻ while remained close to 1 for KLRG1⁺ NK cells (Fig. 4C). Regarding peripheral tissues, CXCR3-deficient NK cell trafficking to liver and spleen was significantly impaired under steady-state, supporting a role of CXCR3 ligands in homeostasis (35).

As CXCR4 heterologous desensitization on NK cells has been reported (29), we asked whether one of the effects of CXCR3 engagement was to desensitize CXCR4. Thus, we preincubated WT NK cells with CXCL9 and then measured CXCL9-, CXCL10-, or CXCL12-induced migration. Results in Fig. 4D show that prestimulation with CXCL9 not only inhibits NK cell migration to CXCR3 ligands but also to CXCL12, thus indicating that triggering of CXCR3 can promote both homologous desensitization and heterologous desensitization of CXCR4. The inhibitory effect was confined to KLRG1⁻ NK cells and was not observed in response to CCL3 (data not shown).

Taken together, these data demonstrate that CXCR3 triggering counteracts KLRG1⁻ NK cell localization in the bone marrow both under homeostatic conditions and during multiple myeloma growth, possibly by its ability to influence CXCR4 responsiveness.

Reduced NK cell localization in bone marrow decreases antitumor surveillance

To characterize NK cell functional status during tumor growth, NK cell degranulation capacity was assessed ex vivo by...
Figure 3.

Multiple myeloma growth results in CXCR3 downmodulation on bone marrow NK cells. A, chemokine receptor expression on bone marrow NK cells from healthy control (CTRL) and tumor-bearing (TUM) mice is shown from a representative experiment (left). Filled gray line, isotype control. Right, CXCR3, CXCR4, CCR1, and CCR5 expression levels on CD3^−/C0^− NK1.1^+ NK cells are represented as mean values of median fluorescence intensity (MFI) ± SEM. B, CXCR3 expression on bone marrow KLRG1^− and KLRG1^+ NK cells from healthy control and tumor-bearing mice represented as plot (left) and as average of MFI ± SEM (right). Data from A and B were obtained from at least 10 individual mice in four independent experiments. C, enriched (30%) CFSE^+ splenic NK cells were intravenously transferred into control or tumor-bearing mice at 3 weeks after multiple myeloma injection. CXCR3 and CXCR4 expression levels on transferred NK cells were assessed in bone marrow of independent recipient mice at 3 or 18 hours after injection. Left, the median fluorescence intensity values for each mouse analyzed are displayed and are representative of two independent experiments. The histogram plots (right) show a representative CXCR3 and CXCR4 analysis. The isotype control is pictured as the filled gray line. Numbers in the plots indicate the median fluorescence intensity. Student t test was performed to compare tumor-bearing versus healthy control mice. *, P < 0.05; **, P < 0.01; ns, nonsignificant.
evaluating CD107a expression on freshly collected NK1.1+ CD3−/C0 cells from bone marrow and spleen of healthy control versus tumor-bearing mice as previously described (36). While splenic NK cells from tumor-bearing mice showed increased degranulation at all time points analyzed, NK cell degranulation increased in bone marrow only at 2 weeks, suggesting that at later times NK cells were functionally triggered in vivo by interaction with tumor cells in spleen but not in bone marrow (Fig. 5A). The impaired bone marrow NK cell function was associated with a gradually disadvantageous effector:target ratio for NK cells in bone marrow that became relevant at 3 weeks (NK:multiple myeloma cell ratio: 1/4) and more pronounced at 4 weeks (NK:multiple myeloma cell ratio: 1/18), while remaining always favorable to NK cells in the spleen (NK:multiple myeloma cell ratio: 2.5/1 at 3 weeks and 0.8/1 at 4 weeks; Fig. 5B). These findings suggest that the initial and selective KLRG1− NK cell decrease in bone marrow represents a key phase in multiple myeloma progression contributing to tumor escape from NK cell–mediated immune surveillance. Thus, we analyzed whether NK cell function could be enhanced by increasing the engraftment of KLRG1− NK cells through CXCR3 deficiency. Indeed, transferred CXCR3−/C0 NK cells displayed higher degranulation rate compared with the corresponding WT cells in the bone marrow of

Figure 4.
KLRG1− NK cell trafficking to bone marrow is impaired at 3 weeks after multiple myeloma injection and is regulated by CXCR3. A, PKH26+ splenocytes were intravenously transferred in healthy control (CTRL) or tumor-bearing (TUM) mice at 3 weeks after tumor cell injection. Bone marrow (BM), blood, and spleen cells were stained with anti-CD3, -NK1.1, and KLRG1 mAbs 18 hours after injection and transferred NK cell populations were identified as PKH26+ cells. Histograms represent the mean values ± SEM of the frequency of PKH26+ cell number in each organ among total transferred cells. At least 10 individual recipient mice per group were used in three independent experiments. Student t test was performed to compare TUM versus CTRL mice. *P < 0.05; **P < 0.01. B and C, CFSE− splenocytes from CD45.1 WT and CD45.2 CXCR3−/− mice were intravenously transferred in healthy control (CTRL) or tumor-bearing (TUM) mice at 11 hours. Tissue cells from recipient mice were stained with anti-CD45.1, -CD3, -NK1.1, and -KLRG1 mAbs, and transferred cells were identified as CFSE− cells. WT and CXCR3−/− mice were discriminated on the basis of their CD45 allelic variant. Histograms show the mean values ± SEM of NK cells from WT (CXCR3−/+ ) and CXCR3−/− mice in the analyzed organs, expressed as percentage of CFSE− cell number in the organ among the total input cells (B), or the relative number of KLRG1− and KLRG1+ NK cells from WT and CXCR3−/− mice, expressed as CXCR3−/+ :CXCR3−/− ratio of transferred cells (C). At least 5 individual recipient mice per group in two independent experiments were used. Student t test was performed to compare in vivo migration of CXCR3−/− versus WT NK cell subsets. *P < 0.05; **P < 0.01; ***P < 0.001. D, bone marrow cells from healthy mice were collected and NK cell migration assay in response to medium alone (C), CXCL9 (500 ng/mL), CXCL10 (250 ng/mL), and CXCL12 (200 ng/mL) was performed after cell pretreatment with vehicle (control) or 5 μg/mL CXCL9. Data are expressed as means ± SEM of percentage of input cells in three experiments. *P < 0.05; **P < 0.01.
Multiple Myeloma Perturbs NK Cell Migration to Chemokines

Figure 5.
NK cell function progressively decrease in the bone marrow of multiple myeloma–bearing mice. A, the expression of the degranulation marker CD107a was assessed by FACS analysis on bone marrow and spleen NK cells from healthy and tumor-bearing mice at 2, 3, and 4 weeks after tumor cell injection. Each symbol represents a single mouse (n ≥ 5) from at least three independent experiments. B, mean values ± SEM of the NK:tumor cell ratio obtained by dividing the number of NK cells in bone marrow and spleen from the tumor-bearing mice shown in A by the IgG2b⁺ tumor cell number from the same mouse. Student t test was performed to compare tumor-bearing versus healthy control mice. *P < 0.05; **P < 0.001. C, adoptive transfer was performed as in Fig. 4B and transferred NK cells in the bone marrow of tumor-bearing (TUM) recipient mice were analyzed for CD107a expression. D, one representative experiment out of two showing donor-derived cells in tissues of mixed chimeras from CXCR3⁺/⁺ and CXCR3⁻/⁻ bone marrow, identified with anti-CD45.1 and anti-CD45.2 antibodies. Left, average ratio ± SD of the number of CXCR3⁻/⁻ versus WT NK cells in the bone marrow, spleen, and blood of tumor-bearing recipient mice. Right, mean values ± SD of the percentage of IFN-γ⁺ transferred NK cells from bone marrow of recipient tumor-bearing mice. Student t test was performed to compare CXCR3⁻/⁻ versus WT NK cells in C and D. *, P < 0.05.

To determine whether alterations in the chemokine receptor/ligand axes could be observed also in the human multiple myeloma, we analyzed patients at different disease stages: Monoclonal Gammapathy of Undetermined Significance (MGUS), asymptomatic (Smolde ring), and active multiple myeloma. This indicates that by interfering with KLRG1⁺ NK cell localization in bone marrow, CXCR3 reduces the number of NK cells activated by multiple myeloma cells. Similarly, in mixed bone marrow chimera experiments, CXCR3-deficient donor NK cell number was increased with respect to the WT counterpart in bone marrow (Fig. 5D, left) and displayed higher capacity to produce IFN-γ in response to cytokine stimulation (Fig. 5D, right). Increased CXCL10 expression in bone marrow and blood samples from multiple myeloma patients correlates with downmodulation of CXCR3 on NK cells.

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myeloma (Onset and Relapse). The expression of CXCL10 and CXCL12 was evaluated in bone marrow specimens and serum by ELISA, along with the expression of CXCR3 and CXCR4 on bone marrow and peripheral blood total NK cells and on the functionally distinct CD56\textsuperscript{high} and CD56\textsuperscript{low} NK cell subsets, being the former stronger producers of cytokines and the latter more cytotoxic. CXCL10 was significantly upregulated in bone marrow of patients with active multiple myeloma as compared with MGUS (Fig. 6A, left). Interestingly, not all bone marrow (10 of 26) and serum (not shown) samples from multiple myeloma patients displayed higher levels of CXCL10. CXCR3 expression levels were reduced only in NK cells of patients with asymptomatic disease (all peripheral blood and CD56\textsuperscript{high} bone marrow NK cells; Supplementary Fig. S3). However, CXCR3 expression levels in peripheral blood and bone marrow NK cells were markedly downmodulated on patients with the lower serum levels of CXCL10 as compared with patients with the low serum levels of CXCL10 (Fig. 6B), the latter displaying CXCR3 expression levels on NK cells similar to MGUS in peripheral blood and higher in bone marrow NK cells (dotted line).

We found that CXCL12 expression was significantly downmodulated in bone marrow samples from smoldering and onset patients, while no differences of CXCR4 expression were detected on NK cells from different multiple myeloma stages (Supplementary Fig. S4 and Fig. 6A, right). Thus, a fraction of patients with active multiple myeloma displayed alterations in CXCR3/ligand axes, while CXCL12 levels in bone marrow already decreased in smoldering myeloma and was maintained low in onset, pointing out the importance of evaluating NK cell distribution and function in the bone marrow of multiple myeloma patients.

Discussion

This study was aimed at investigating the influence of multiple myeloma growth on NK cell effector functions and migratory properties. We demonstrated that (i) NK cells play a specific and direct role in the killing of multiple myeloma cells \textit{in vitro} and their clearance \textit{in vivo}; (ii) a skewed CXCR4 and CXCR3 ligand environment impairs NK cell localization in bone marrow when tumor load is low; (iii) The selective reduction of CXCR3\textsuperscript{+} KLRG1\textsuperscript{−} NK cell number leads to reduced effector:target ratio in bone marrow and impairment of NK cell surveillance, paving the way to a vigorous tumor growth phase. The relevance of the multiple myeloma–promoted alteration in the chemokine system is supported by similar observation in patients with active multiple myeloma.

We showed that activated NK cells can kill 5TGM1 multiple myeloma cells, highlighting a major role of the less mature CD11\textsubscript{b}\textsuperscript{low}KLRG1\textsuperscript{−} subset and a decline of the cytotoxic potential in the more mature CD11\textsubscript{b}\textsuperscript{high}KLRG1\textsuperscript{−} and KLRG1\textsuperscript{+} NK cell subsets. We also provided evidence for an \textit{in vivo} role of NK cells in the host immune response against multiple myeloma.

Figure 6.

CXCR3 expression on NK cells and CXCL10 and CXCL12 levels in bone marrow samples of patients with multiple myeloma. A, CXCL10 and CXCL12 concentrations were measured in bone marrow samples from patients with MGUS (n = 16/15), asymptomatic multiple myeloma (Smoldering, n = 9/10), active multiple myeloma (onset, n = 10/12 and relapse, n = 15/16) by ELISA. The horizontal bar represents the mean value and each symbol represents a single sample. Student t test was performed to compare the chemokine concentration in multiple myeloma versus MGUS patients. B, correlation of CXCR3 surface expression by NK cells in bone marrow and peripheral blood of multiple myeloma patients with the serum levels of CXCL10. CXCR3 expression on CD56\textsuperscript{−}CD3\textsuperscript{−} NK cells from MGUS (dotted line) and patients with active multiple myeloma (symbols) was assessed among CD45\textsuperscript{+}CD138\textsuperscript{−} cells. Shown is the median fluorescence intensity of CXCR3 expression on peripheral blood and bone marrow of patients with lower (CXCL10\textsuperscript{low}, <100 pg; n = 9) and higher (CXCL10\textsuperscript{high}, >150 pg; n = 6) CXCL10 serum levels. Student t test was performed to compare CXCR3 expression from CXCL10\textsuperscript{low} versus CXCL10\textsuperscript{high} patients. *, P < 0.05; **, P < 0.01.
confirming recent observations (37). Noteworthy, our results show that multiple myeloma growth determines a decreased trafficking capacity of KLRG1<sup>+</sup> NK cells to bone marrow due to significant changes in the expression levels of several chemokines.

Higher levels of CXCL9 and CXCL10 observed in multiple myeloma–bearing mice may derive from the tumor itself (Supplementary Fig. S1B) and/or from bone marrow environment. In this regard, we found that bone marrow CD11b<sup>+</sup> cells from tumor-bearing mice display increased mRNA expression levels of CXCL9 and CXCL10. Concerning CXCL12, no changes in mRNA expression were detected in total bone marrow cells and in the isolated bone marrow cell populations, suggesting that posttranslational mechanisms are involved in the downmodulation of its bone marrow expression (38, 39). As the highest levels of CXCR3 and CXCR4 expression are detected on KLRG1<sup>−</sup> NK cells (Fig. 3B; refs. 29, 40), we strongly envisage that reduction of CXCL12 protein levels in bone marrow of tumor-bearing mice is involved in the impaired trafficking of KLRG1<sup>−</sup> (CXCR3<sup>+</sup>) NK cells in this organ, consistent with a role of CXCR4 for KLRG1<sup>−</sup> but not KLRG1<sup>+</sup> NK cell retention in bone marrow (34).

Our results also provide new insight regarding the signaling aspects of NK cell egress from bone marrow: the requirement of CXCR4 in bone marrow NK cell retention is partially relieved by genetic ablation of CXCR3 (Fig. 4B). This evidence was unexpected, as other groups reported that CXCR3 is important for NK cell intratumor infiltration (41), and is likely related to the peculiar CXCR4 requirement for bone marrow NK cell retention, together with a prevalent CXCR3 role in NK cell migration to extramedullary compartments (42, 43). Our findings support a model depicted in Supplementary Fig. S5, in which CXCR3 regulates NK cell localization in bone marrow at least in part by overcoming or interfering with CXCR4-mediated retention signals. In a multiple myeloma–conditioned bone marrow environment, NK cells leave this compartment because of impaired CXCR4 signaling. Our observation that NK cells display decreased CXCR4 expression in the bone marrow of multiple myeloma–bearing mice early after adoptive transfer suggests that NK cell localization in bone marrow is limited by local CXCR3 ligand-induced receptor triggering and downmodulation (that may promote CXCR4 desensitization) and/or by impaired CXCR3<sup>−</sup> NK cell migration. In regard to heterologous CXCR4 desensitization, we demonstrated that CXCL9-mediated triggering of CXCR3 inhibits CXCR4 function on NK cells, confirming previous observation on T cells and NK cells (29, 44) and supporting the hypothesis that CXCR3 can directly dampen KLRG1<sup>−</sup> NK cell accumulation in bone marrow at least in part by inhibiting CXCR4 function (Fig. 4D).

The negative impact of the altered CXCR3 and CXCR4 ligand/receptor axes on NK cell localization in bone marrow is associated with selective impairment of in vivo NK cell degranulation capacity in bone marrow but not in spleen (Fig. 5A). This can be attributed to the marked decrease of effector KLRG1<sup>−</sup> NK cell number in the bone marrow of tumor-bearing mice that leads to increased frequency of the poorly functional KLRG1<sup>+</sup> cells and to a lower NK cell/target cell ratio in bone marrow (Fig. 5B).

Similarly to our findings in the mouse model, we evidenced a significant increase of CXCL10 in bone marrow samples from patients with active myeloma as compared with MGUS. Notably, patients with elevated levels of CXCL10 display reduction of CXCR3 plasma membrane expression on peripheral blood and bone marrow NK cells, suggesting that ligand–induced downmodulation of the receptor occurs in multiple myeloma patients and that CXCR3 plays a role in NK cell trafficking to bone marrow also in the human disease. CXCR3 ligand expression by human multiple myeloma cells has been previously reported and CXCL10 has been recently shown to exert a direct antitumor effect (45, 46). Thus, it would be clinically relevant to study serum CXCL10 levels as a prognostic factor for multiple myeloma. CXCL12 expression levels in serum of patients with multiple myeloma were previously shown to positively correlate with the malignant plasma cell burden (47), while we found a different trend of CXCL12 in bone marrow, possibly related to a different regulation of CXCL12 protein in bone marrow versus blood in multiple myeloma.

Overall, we uncovered a critical role for CXCR3 and CXCR4 axes in KLRG1<sup>−</sup> NK cell trafficking to the bone marrow, which affects their localization at the tumor site in an asymptomatic phase of multiple myeloma growth. The exclusion from bone marrow of NK cells endowed with the higher effector capacity represents a novel mechanism of immune evasion applied by multiple myeloma cells and may be one of the underlying causes of several NK cell functional abnormalities observed in multiple myeloma patients during disease progression (20, 22, 48).

The data presented herein bring to the fore the unique properties of bone marrow microenvironment, and first highlight the importance of the altered trafficking of selected bone marrow NK cell effector populations mediated by multiple myeloma–driven dysregulation of bone marrow chemokine/chemokine receptor axes. Targeting of these axes may be exploited in perspective of future NK cell–based adoptive immunotherapies for multiple myeloma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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