

# Natural Killer Cell Accumulation in Tumors Is Dependent on IFN- $\gamma$ and CXCR3 Ligands

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## Abstract

Several studies have correlated high numbers of tumor-infiltrating natural killer (NK) cells with a good prognosis for cancer patients. Our study aimed at identifying factors controlling intratumoral NK cell accumulation in s.c. injected NK cell sensitive tumor models and at studying their effect on survival time of recipient mice. We observed that fewer NK cells infiltrated the tumors in IFN- $\gamma$  receptor knockout (IFN- $\gamma$ R<sup>-/-</sup>) mice compared with wild-type controls that correlated with decreased survival rate. Exogenous application of IFN- $\gamma$  in the tumor augmented levels of ligands of the chemokine receptor CXCR3, increased NK cell accumulation, and prolonged survival. Furthermore, our data show that CD27<sup>high</sup> NK cells, which under steady-state conditions express CXCR3, preferentially accumulated in the tumor tissue. Accordingly, significantly lower numbers of tumor-infiltrating NK cells were detected in CXCR3<sup>-/-</sup> mice, and the capacity of adoptively transferred CXCR3<sup>-/-</sup> NK cells to accumulate in the tumor was severely impaired. Finally, exogenous application of the CXCR3 ligand CXCL10 in the tumor or ectopic expression of CXCL10 by tumor cells increased the numbers of NK cells in the tumors and prolonged NK cell-dependent survival. Our results identify IFN- $\gamma$  and the expression of CXCR3 on NK cells as prerequisites for NK cell infiltration into tumors. Exploiting strategies to augment NK cell accumulation in the tumor might lead to the development of effective antitumor therapies. [Cancer Res 2008;68(20):8437–45]

## Introduction

In a variety of different cancer types, several studies have provided evidence that high amounts of tumor-infiltrating natural killer (NK) cells correlate with a good prognosis and increased patient survival (1–4). For this reason, it is important to identify and exploit factors regulating NK cell accumulation in tumors to improve the success of antitumor therapies.

NK cells are cytolytic and cytokine-producing effector cells of the innate immune system and represent the first line of defense against virally infected and transformed cells (5). Their effector functions include release of cytotoxic granules containing perforin and granzymes (6) and induction of death receptor-mediated apoptosis (7) resulting in direct killing of target cells. In addition, NK cells produce the pleiotropic cytokine IFN- $\gamma$  that is important

for activation of antigen-presenting cells (8) and the induction of T<sub>H</sub>1 responses (9). Furthermore, IFN- $\gamma$  was described to exert antiangiogenic function (10). NK cell activation is regulated by a delicate balance of activating and inhibitory signals with most inhibitory receptors being specific for self MHC class I molecules (11). One prominent activating receptor is NKG2D that recognizes multiple ligands, including RAE-1 family members (12, 13). According to the “missing self” hypothesis of NK cell function, cytotoxicity is inhibited when appropriate MHC class I molecules are expressed by target cells and enhanced when target cells are deficient in MHC class I expression (14). Therefore, NK cells are particularly important for the elimination of tumors with reduced or absent MHC class I expression that evade CD8<sup>+</sup> T cell-mediated killing (15).

To exert antimicrobial or antitumor effects, NK cells are mobilized from the bone marrow and subsequently recruited from peripheral blood to infected target organs or tumor tissues, respectively. NK cell migration is mediated by adhesion molecules, chemokine receptors, and chemokines (16). The trafficking of mouse bone marrow NK cell subsets was reported to be regulated by the chemokines CCL3 and CXCL12 (17). Certain chemokine receptors, including CCR2 (18), CCR5 (19), CXCR3 (9), and CX3CR1 (20), were described to direct NK cells to sites of inflammation. Very recently, the sphingosine 1-phosphate (S1P) receptor S1P<sub>5</sub> was reported to control NK cell trafficking in steady-state and inflammatory conditions (21).

Thus far, only little is known about chemokines or other factors governing accumulation of NK cells in tumors and tumor metastases. These factors are largely dependent on the tumor type and tumor microenvironment. It was reported that the lack of MHC class I expression or ectopic expression of NKG2D ligands on tumor cells enhanced the accumulation of NK cells at the site of tumor cell injection (13, 22). In addition, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was shown to be critical for the recruitment of NK cells into the peritoneum after i.p. application of RMA-S tumor cells (23). Moreover, certain chemokine receptors were implicated in the accumulation of NK cells in tumors. NK cell recruitment to a CX3CL1-transduced EL4 lymphoma (24) or to s.c. B16 tumors after intratumor injection of an adenoviral vector expressing CX3CL1 (25) was attributed to the CX3CR1 receptor. After injection of B16 melanoma cells, lower amounts of NK cells were recruited to the lungs of CX3CR1<sup>-/-</sup> mice compared with wild-type (WT) mice (26). The importance of CCR5 in NK cell recruitment to B16 tumors was shown after injection of CpG-activated plasmacytoid dendritic cells (27). Furthermore, there is evidence that NK cells accumulate in CXCR3 ligand-transduced tumors (28, 29), after intratumor injection of the CXCR3 ligand CXCL9 (30), Sendai virus particles, inducing CXCL10 (31), or CXCL10-encoding plasmid DNA (32). Thus far, it is not known whether CXCR3 directly mediates NK cell accumulation in tumors. The chemokine receptor CXCR3 binds the structurally and functionally related chemokines CXCL9 (Mig, monokine induced by IFN- $\gamma$ ), CXCL10 (IP-10, IFN-inducible

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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protein-10), and CXCL11 (I-TAC, IFN-inducible T-cell  $\alpha$  chemoattractant; refs. 33, 34). Expression of these three ligands is induced by IFN- $\gamma$  in a wide variety of cell types (34–36), and CXCR3 expression on mouse NK cells was recently shown to be confined to the NK cell subset expressing high levels of CD27 (37).

In the present study, we describe IFN- $\gamma$  as an essential prerequisite for NK cell accumulation in the tumor tissue of s.c. growing MHC class I-deficient RMA-S lymphoma and B16 melanoma cells transduced with the NKG2D ligand RAE-1 $\epsilon$ . Importantly, the CD27<sup>high</sup> NK cell subset predominantly accumulated in the tumors. Furthermore, our data reveal the importance of CXCR3 expression on NK cells for their tumor infiltration. Ectopic CXCL10 expression by tumor cells or application of exogenous CXCL10 augmented accumulation of NK cells in the tumors, which correlated with improved host survival.

## Materials and Methods

**Mice.** C57BL/6 (WT) and congenic C57BL/6-Ly5.1<sup>+</sup> mice were purchased from Charles River Laboratories (Sulzfeld and Erembodegem). IFN- $\gamma$  receptor knockout (IFN- $\gamma$ R<sup>-/-</sup>) mice were purchased from Jackson Laboratory. CXCR3<sup>-/-</sup> mice were kindly provided by Prof. C. Gerard (Children's Hospital Boston, Boston, MA; ref. 38). Mice were housed under specific pathogen-free conditions and used in experiments at 6 to 8 wk of age. All experiments were performed according to local animal experimental ethics committee guidelines and permission.

**Tumor cell lines and tumor cell injection.** MHC class I-deficient RMA-S or MHC class I-expressing RMA lymphoma cells (14) were cultured in RPMI 1640 supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin/streptomycin (Life Technologies Invitrogen). B16BL6 melanoma cells that were transduced with RAE-1 $\epsilon$  were cultured in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. If not indicated otherwise, mice were injected s.c. in the left flank with 10<sup>6</sup> tumor cells in PBS. For infiltration experiments, mice were injected with 10<sup>6</sup> tumor cells suspended in 300  $\mu$ L growth factor-reduced Matrigel (BD Biosciences). This experimental set-up (39) enabled us to investigate early accumulation events at the site of tumor cell injection, before a solid tumor became palpable.

**Generation of the tumor cell line RMA-S/CXCL10.** The retroviral vector pMXs-IG containing an IRES-GFP was kindly provided by Prof. T. Kitamura (University of Tokyo, Japan; ref. 40). Mouse CXCL10 cDNA was prepared and amplified from the IFN- $\gamma$  stimulated macrophage cell line J774A.1. The following primers were used: 5'-CGGAATTCATGAACC-CAAGTGTGCCGTCATT-3' (forward) and 5'-ATAGTTTAGCGCCGCT-TAAGGAGCCCTTTAGACCTTTTGG-3' (reverse). Stable RMA-S-derived cell lines were obtained by retroviral infection, as described elsewhere (41). RMA-S cells transduced with empty pMX-IG vector are called RMA-S/vc. Transduced cells were enriched by flow cytometry sorting for GFP using a FACSVantage (BD Biosciences). Single-cell clones were selected according to their CXCL10 production as assessed by ELISA (R&D Systems).

**In vivo depletion experiments.** Mice were depleted of NK1.1<sup>+</sup> cells by i.p. injection of 200  $\mu$ g anti-NK1.1 monoclonal antibody (mAb; clone PK136, Bioexpress) on days -2, +2, +9, and +16 of tumor cell inoculation. At day 10 and the end point, the efficiency of NK cell depletion was >90% in blood, spleens, and tumor tissues monitored by flow cytometry analysis using mAb directed against CD3 and NK1.1. For *in vivo* neutralization of IFN- $\gamma$ , 300  $\mu$ g anti-IFN- $\gamma$  mAb (clone XMG1.2, Bioexpress) were injected i.p. thrice weekly, beginning at the day of tumor cell inoculation.

**In vivo application of cytokines and chemokines.** For infiltration experiments, 1  $\mu$ g recombinant mouse CXCL10 (Peprotech) or 10,000 units recombinant mouse IFN- $\gamma$  (Peprotech) were mixed with Matrigel and coinjected with the tumor cells. CXCL10 was reinjected into the Matrigel plug after 2 d. For the monitoring of survival time, 1  $\mu$ g CXCL10 or 10,000 units IFN- $\gamma$  in 50  $\mu$ L PBS were injected intratumorally thrice weekly beginning on day 4 after tumor cell inoculation. 100,000 units/d of human

interleukin-2 (IL-2; Chiron) were given i.p. for 5 d starting on the day of tumor cell inoculation (42).

**Cell preparation, cell quantification, and flow cytometry.** Tumors or Matrigel plugs were removed, cut into small pieces, and digested with 5 or 1.5 mg/mL collagenase type 4 (Cell Systems GmbH), respectively, supplemented with 0.5 mg/mL DNase I (Sigma-Aldrich) at 37°C for 25 min. Erythrocytes were lysed with buffered ammonium chloride solution. A list of the antibodies used for flow cytometry is provided in the supplementary data. For quantification of absolute NK cell numbers, percentages of NK cells among viable cells in the tumor (defined as 7-AAD-negative cells) were multiplied by live cell numbers counted in the target organ using a Neubauer chamber.

**Adoptive NK cell transfer.** Splenocytes from WT, IFN- $\gamma$ R<sup>-/-</sup>, or CXCR3<sup>-/-</sup> mice were depleted of CD3<sup>+</sup> cells by staining with APC-conjugated anti-CD3 mAb and subsequent negative magnetic selection using anti-APC microbeads (Miltenyi Biotec). NK cells were further enriched by positive magnetic selection using CD49b (DX5) microbeads (Miltenyi Biotec). Purity of CD3<sup>-</sup>NK1.1<sup>+</sup> cells was >90%, as assessed by flow cytometric analysis. 4  $\times$  10<sup>6</sup> CD3<sup>-</sup>NK1.1<sup>+</sup> cells were injected i.v. into C57BL/6-Ly5.1<sup>+</sup> hosts. Two days later, tumor cells suspended in Matrigel were injected. On day 5 after injection, Matrigel plugs were removed and ratios of Ly5.2<sup>+</sup> donor to Ly5.1<sup>+</sup> host NK cells were determined in blood, spleens, and tumors. All ratios were normalized to the donor-to-host ratios determined in the blood.

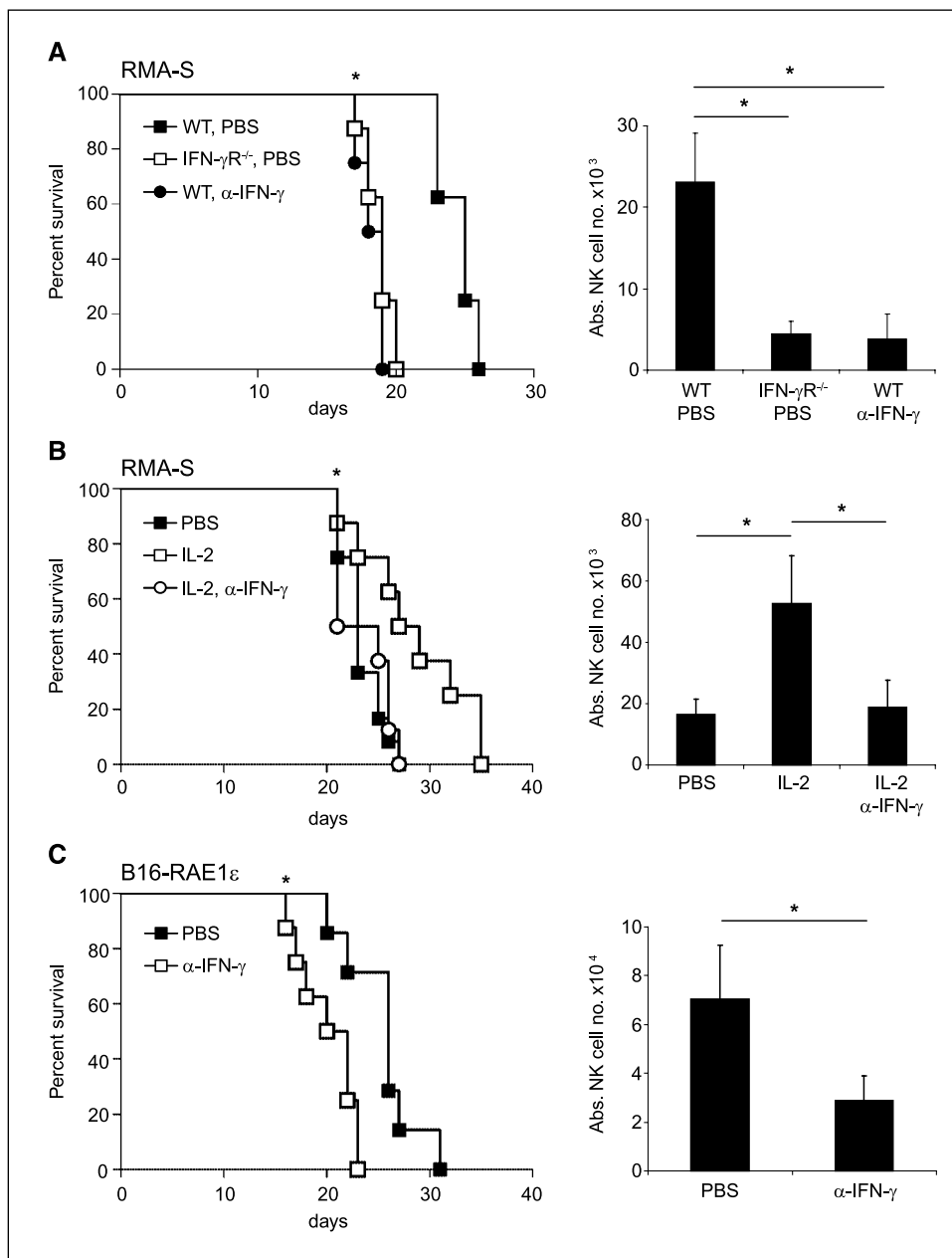
**Real-time PCR.** Total RNA was extracted from cells isolated from Matrigel plugs 5 d after tumor cell inoculation using the RNeasy Mini Kit (Qiagen). RNA was reverse-transcribed to cDNA by SuperScript II RT (Invitrogen) using oligo (dT) primers. Real-time PCR was performed on a Lightcycler 480 using the Probe Master system (Roche). Primer sequences are provided in the supplementary data.

**Statistical analysis.** *P* values were calculated using Student's *t* test with two-tailed distribution and two-sample equal variance variables. For comparison of survival curves, the log-rank test was used. *P* values for log-rank test were calculated using the GraphPad Prism software. Asterisks in figures indicate *P* values <0.05.

## Results

**IFN- $\gamma$  regulates NK cell accumulation in tumors.** RMA-S, a MHC class I-deficient variant of the T-cell lymphoma RMA, is rejected by NK cells in recipient mice, when injected at relatively low cell numbers (14). Injection of 10<sup>6</sup> RMA-S cells into WT mice resulted in progressive tumor growth with a median survival time of 25 days (Fig. 1A). On day 5 after tumor cell inoculation, 2.3  $\pm$  0.6  $\times$  10<sup>4</sup> (*n* = 8) NK cells were detectable in the tumor tissue. When RMA-S cells were injected into IFN- $\gamma$ R<sup>-/-</sup> or WT mice treated with a neutralizing anti-IFN- $\gamma$  mAb, survival was significantly reduced compared with the WT control group (Fig. 1A). Decreased survival correlated with a significant reduction in the amounts of NK cells in the tumors (Fig. 1A). Treatment of tumor-bearing mice with IL-2 significantly prolonged survival that correlated with a massive accumulation of NK cells in the tumors (Fig. 1B). Simultaneous neutralization of IFN- $\gamma$  in IL-2-treated mice reduced survival time to control group levels (Fig. 1B) that correlated with decreased numbers of NK cells in the tumor. Also in the s.c. B16-RAE-1 $\epsilon$  melanoma model, application of anti-IFN- $\gamma$  mAb decreased the survival time that correlated with lower amounts of NK cells in the tumors (Fig. 1C). Of note, similar numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells accumulated in RMA-S tumors in the absence of IFN- $\gamma$  and upon treatment with IL-2 compared with control-treated mice (data not shown).

To determine whether exogenous IFN- $\gamma$  affected NK cell infiltration into tumors, we injected recombinant IFN- $\gamma$  together with RMA-S cells. Quantification of absolute cell numbers revealed a significant increase of NK cell numbers after IFN- $\gamma$  treatment



**Figure 1.** IFN- $\gamma$ -dependent NK cell accumulation in tumors correlates with survival. **A**, WT or IFN- $\gamma$ R<sup>-/-</sup> mice ( $n = 8$ ) were injected with RMA-S cells (*left*) or RMA-S cells in Matrigel (*right*) and treated with anti-IFN- $\gamma$  mAb or PBS, and survival was monitored for 30 d (*left*; \*,  $P < 0.05$ , for IFN- $\gamma$ R<sup>-/-</sup>, PBS or WT, anti-IFN- $\gamma$  compared with WT, PBS, respectively). Matrigel plugs were removed 5 d after inoculation, and absolute numbers of infiltrated NK cells were determined (*right*). Data are representative of four experiments. **B**, C57BL/6 mice ( $n = 8$ ) were injected with RMA-S cells (*left*) or RMA-S cells in Matrigel (*right*) and treated with IL-2 or IL-2 and anti-IFN- $\gamma$  mAb or PBS, and survival was monitored for 40 d (*left*; \*,  $P < 0.05$ , for IL-2 compared with PBS). Matrigel plugs were removed 5 d after inoculation and absolute numbers of infiltrated NK cells were determined (*right*). **C**, C57BL/6 mice ( $n = 8$ ) were injected with B16-RAE-1 $\epsilon$  cells (*left*) or B16-RAE-1 $\epsilon$  cells in Matrigel (*right*) and treated with anti-IFN- $\gamma$  mAb or with PBS, and survival was monitored for 40 d (*left*). Matrigel plugs were removed 5 d after inoculation, and absolute numbers of infiltrated NK cells were determined (*right*). Values at the right represent mean  $\pm$  SD ( $n = 8$ ).

compared with the PBS-treated control group (Fig. 2A). Upon injection of MHC class I-expressing RMA cells, lower amounts of NK cells were detected in the tumor tissues compared with injection of MHC class I-deficient RMA-S tumor cells. Importantly, IFN- $\gamma$  treatment effectively increased NK cell numbers in RMA tumors compared with the control group (Fig. 2A). Similar results were obtained in the s.c. B16-RAE-1 $\epsilon$  melanoma model (Supplementary Fig. S1A). To test whether this enhanced NK cell accumulation correlated with increased survival, IFN- $\gamma$  was injected intratumorally starting on day 4 after tumor cell

inoculation. Median survival time of mice was significantly prolonged by IFN- $\gamma$  treatment (Fig. 2B). Exogenous application of IFN- $\gamma$  also significantly increased CD4<sup>+</sup> T-cell numbers, whereas the amounts of CD8<sup>+</sup> T cells remained unaffected compared with control-treated mice (data not shown).

To dissect the mechanisms by which IFN- $\gamma$  injection enhanced NK cell accumulation, we first investigated whether it affected host cells or RMA-S tumor cells, which also express the IFN- $\gamma$ R (data not shown). Injection of IFN- $\gamma$  into IFN- $\gamma$ R<sup>-/-</sup> mice did not significantly increase NK cell numbers in the tumors compared

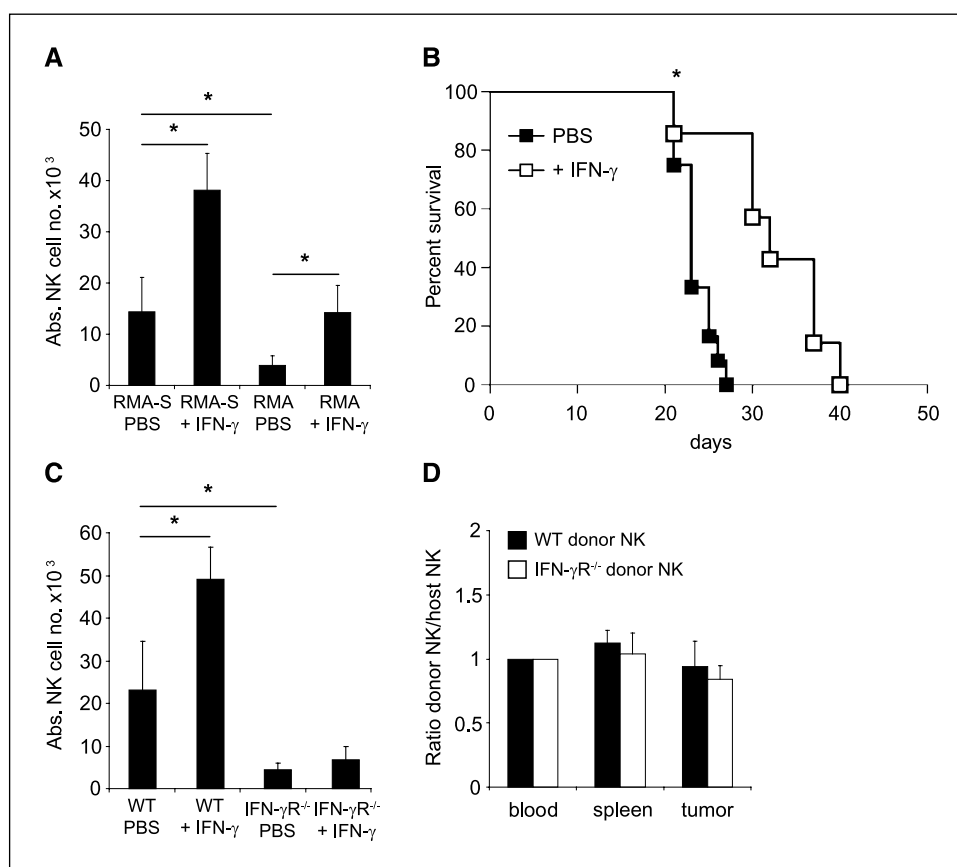
with PBS-treated control mice (Fig. 2C), suggesting that the application of exogenous IFN- $\gamma$  had a direct effect on the host cells. Upon adoptive transfer of Ly5.2<sup>+</sup> IFN- $\gamma$ R<sup>-/-</sup> or WT NK cells into congenic Ly5.1<sup>+</sup> recipients, we detected similar ratios of donor to host NK cells for IFN- $\gamma$ R<sup>-/-</sup> and WT donors in peripheral blood, spleens, and tumors of recipient mice (Fig. 2D), indicating that IFN- $\gamma$ R expression on NK cells was not a prerequisite for their accumulation in tumors.

Taken together, our data show that endogenous IFN- $\gamma$  is an essential factor for accumulation of NK cell at the tumor site. This accumulation can be further enhanced by exogenous application of IFN- $\gamma$ . However, IFN- $\gamma$  does not directly modulate NK cell tumor infiltration.

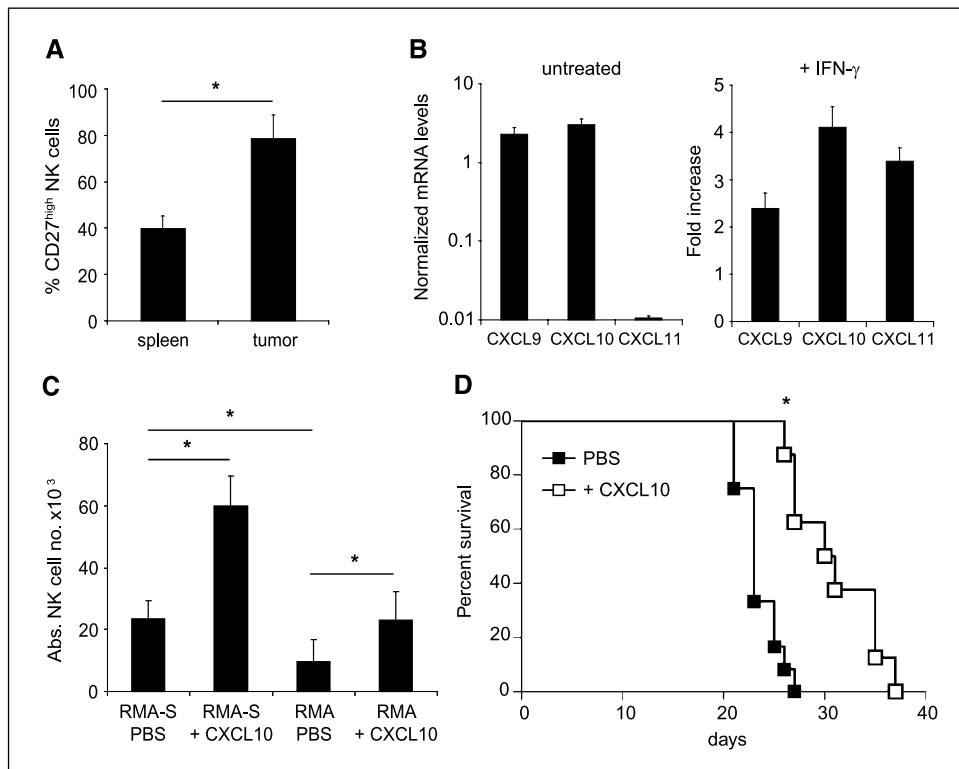
**CD27<sup>high</sup> NK cells predominantly accumulate in the tumor.** We analyzed the phenotype of NK cells isolated from RMA-S tumors and observed that the subset of NK cells expressing high levels of CD27 was significantly enriched within RMA-S (Fig. 3A) and B16-RAE1 $\epsilon$  (Supplementary Fig. S1B) tumors compared with spleens of tumor-bearing mice. Similar percentages of CD27<sup>high</sup> NK cells were detected in spleens of naive mice compared with spleens isolated from tumor-bearing mice (data not shown). In addition, the markers CD11b, B220, MHC class II, and CD11c were up-regulated on tumor-infiltrating NK cells compared with NK cells from spleens (data not shown). The chemokine receptor CXCR3

was recently described to be expressed on CD27<sup>high</sup>, but not on CD27<sup>low</sup> NK cells (37). Interestingly, almost all CD27<sup>high</sup> NK cells in the tumors were CXCR3-negative, whereas NKT and T cells isolated from tumors expressed CXCR3 (data not shown). Because CXCR3 engagement was reported to result in receptor internalization (43), we assumed that NK cells might have used the CXCR3 receptor to accumulate in the tumors. Thus, we examined mRNA expression levels of the three CXCR3 ligands, CXCL9, CXCL10, and CXCL11, in RMA-S tumors. These experiments revealed that CXCL9 and CXCL10 were much more abundant than CXCL11 (Fig. 3B, left). After separation of tumors into leukocytes (Ly5.1<sup>+</sup>) and nonleukocytes (Ly5.1<sup>-</sup>), we observed that these chemokines were mainly produced by the leukocyte fraction, which did not include Ly5.1<sup>-</sup> RMA-S cells (data not shown). In concordance with previous reports (34–36), mRNA levels of all ligands were increased after application of recombinant IFN- $\gamma$  (Fig. 3B, right). When we analyzed the overall expression profile of chemokines and chemokine receptors in RMA-S tumors on day 10 after tumor cell inoculation, CXCL9 and CXCL10 were among the most abundant chemokines expressed by tumor-infiltrating leukocytes (data not shown).

To investigate whether CXCR3 ligands mediate intratumoral NK cell accumulation *in vivo*, we injected recombinant CXCL10 together with RMA-S or RMA cells. CXCL10 injection resulted in a



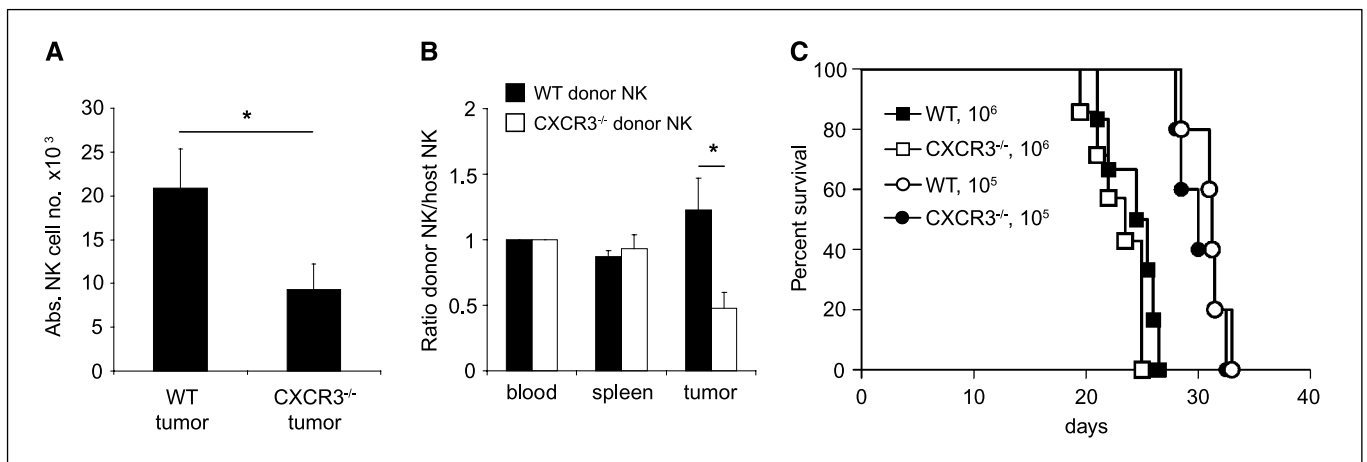
**Figure 2.** IFN- $\gamma$  regulates the numbers of tumor-infiltrating NK cells. **A**, C57BL/6 mice were injected with RMA-S or RMA cells in Matrigel with or without recombinant IFN- $\gamma$ . Matrigel plugs were removed 5 d after inoculation, and absolute numbers of infiltrated NK cells were determined. **B**, C57BL/6 mice ( $n = 8$ ) were injected with RMA-S cells. Starting on day 4 after tumor inoculation, mice were injected with IFN- $\gamma$  or PBS intratumorally and survival was monitored for 40 d. **C**, WT and IFN- $\gamma$ R<sup>-/-</sup> mice were injected with RMA-S cells in Matrigel with or without recombinant IFN- $\gamma$ . Matrigel plugs were removed 5 d after inoculation, and absolute numbers of infiltrated NK cells were determined. **D**, WT (black columns) or IFN- $\gamma$ R<sup>-/-</sup> (white columns) donor NK cells were transferred into congenic hosts 2 d before injection of RMA-S cells in Matrigel. On day 5 after tumor cell inoculation, ratios of donor to host NK cells were determined in blood, spleens, and Matrigel plugs, as described in Materials and Methods. Data are representative of four (A) or two (C and D) experiments. Values in A, C, and D represent the mean  $\pm$  SD ( $n = 5$ ). \*,  $P < 0.05$ .



**Figure 3.** CD27<sup>high</sup> NK cells predominantly accumulate in the tumor. *A*, C57BL/6 mice were injected with RMA-S cells, and CD27 expression on splenic and tumor-infiltrating NK cells was analyzed on day 10 after tumor inoculation. *B*, C57BL/6 mice were injected with RMA-S cells in Matrigel (*left*) or with RMA-S cells in Matrigel and recombinant IFN- $\gamma$  (*right*). Matrigel plugs were removed 5 d after inoculation. mRNA levels of CXCL9, CXCL10, and CXCL11 were determined by real-time PCR. *Right*, fold increase in mRNA levels between IFN- $\gamma$ -treated and untreated groups. *C*, C57BL/6 mice were injected with RMA-S or RMA cells in Matrigel with or without recombinant CXCL10. Matrigel plugs were removed 5 d after inoculation, and absolute numbers of infiltrated NK cells were determined. Data are representative of three (*A–C*) experiments. *D*, C57BL/6 mice ( $n = 8$ ) were injected with RMA-S cells. Starting on day 4 after tumor inoculation, mice were injected with CXCL10 or PBS intratumorally and survival was monitored for 40 d. Values in *A–C* represent the mean  $\pm$  SD ( $n = 5$ ). \*,  $P < 0.05$ .

significant increase in the numbers of NK cells infiltrating RMA-S or RMA tumors (Fig. 3C). In addition, injection of CXCL10 significantly increased NK cell numbers in the tumor when RAE-I $\epsilon$ -expressing B16 melanoma cells were inoculated (Supplementary

Fig. S1C). CXCL9 and CXCL11 were not as potent as CXCL10 in inducing NK cell accumulation in the tumors when injected in similar concentrations (data not shown). Intratumoral application of CXCL10 significantly prolonged median survival time of



**Figure 4.** CXCR3 expression on NK cells is required for their tumor infiltration. *A*, WT or CXCR3<sup>-/-</sup> mice were injected with RMA-S cells in Matrigel. Matrigel plugs were removed 5 d after inoculation, and absolute numbers of infiltrated NK cells were determined. *B*, WT (black columns) or CXCR3<sup>-/-</sup> (white columns) donor NK cells were transferred into congenic host 2 d before injection of RMA-S cells in Matrigel. After 5 d, ratios of donor to host NK cells were determined in blood, spleen, and Matrigel plugs. *C*, WT or CXCR3<sup>-/-</sup> mice ( $n = 8$ ) were injected with 10<sup>5</sup> or 10<sup>6</sup> RMA-S cells, and survival was monitored for 35 d. Data are representative of four (*A* and *C*) or two (*B*) experiments. Values in *A* and *B* represent the mean  $\pm$  SD ( $n = 5$ ). \*,  $P < 0.05$ .

tumor-bearing mice (Fig. 3D). After exogenous application of CXCL10, also CD4<sup>+</sup> and CD8<sup>+</sup> T-cell numbers significantly increased in RMA-S and B16-RAE1 $\epsilon$  tumors, although to a lesser extent than NK cells numbers (data not shown).

In summary, our data indicate that IFN- $\gamma$  treatment induced expression of CXCR3 ligands in RMA-S tumor tissues and that CXCR3 ligands chemoattracted CD27<sup>high</sup> NK cells *in vivo*.

**Intratumoral NK cell accumulation is dependent on CXCR3 expression.** To investigate the importance of the CXCR3 receptor in tumor infiltration by NK cells, RMA-S or B16-RAE1 $\epsilon$  cells were injected into CXCR3<sup>-/-</sup> recipient mice and tumor infiltrates were analyzed. Significantly decreased NK cell numbers were detected in tumors isolated from CXCR3<sup>-/-</sup> mice compared with tumors of WT mice (Fig. 4A and Supplementary Fig. S1D), whereas CD4<sup>+</sup> and CD8<sup>+</sup> T-cell infiltration was not significantly affected. Similar results were obtained when MHC class I-expressing RMA cells transduced with RAE-1 $\gamma$ , a ligand of the activating NK cell receptor NKG2D (12, 13), were injected (data not shown). Because it was reported that CXCR3 mediates NK cell entry into inflamed lymph nodes (LN; ref. 9), we also determined the cellular composition of tumor-draining LN. No difference in NK cell numbers between draining LN of WT and CXCR3<sup>-/-</sup> mice was detected on day 5 after tumor cell inoculation (data not shown). Of note, tissue distribution of the NK cell subsets CD11b<sup>low</sup>/CD27<sup>high</sup>, CD11b<sup>high</sup>/CD27<sup>high</sup>, and CD11b<sup>high</sup>/CD27<sup>low</sup>, which were described to differ in CXCR3 expression (37), was not significantly altered in blood, spleens,

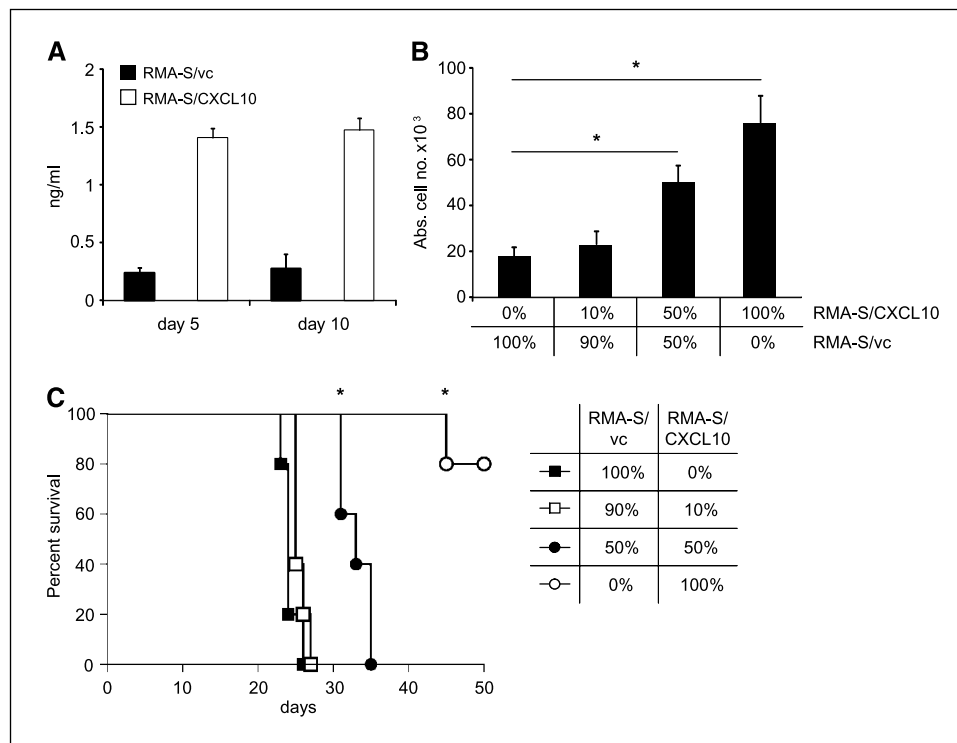
LN, and livers of naive CXCR3<sup>-/-</sup> mice compared with WT controls (data not shown).

To determine whether the expression of CXCR3 on NK cells was required for their efficient accumulation in the tumor tissue, we adoptively transferred NK cells from either CXCR3<sup>-/-</sup> or WT donors into congenic WT hosts. We observed similar ratios of CXCR3<sup>-/-</sup> and WT donor to host cells accumulating in spleens and peripheral blood of tumor-bearing hosts. In contrast, a significantly lower donor to host ratio was detected in the tumor infiltrates for CXCR3<sup>-/-</sup> donor NK cells compared with WT donor cells (Fig. 4B).

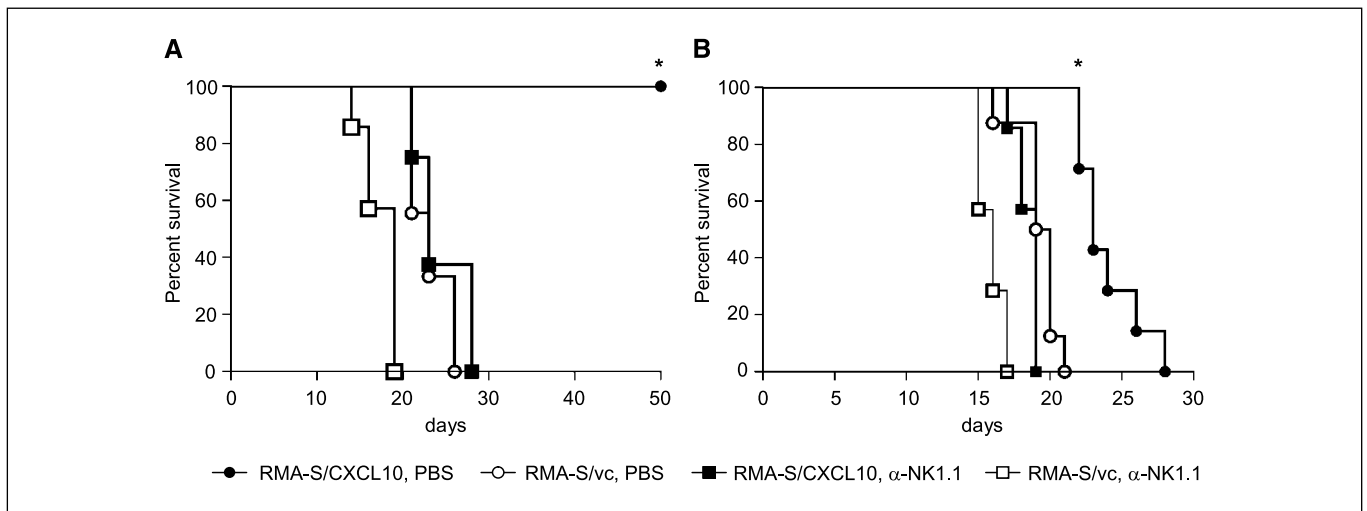
Remarkably, despite the reduction of NK cells infiltrating tumors in CXCR3<sup>-/-</sup> mice, no difference in survival was observed between CXCR3<sup>-/-</sup> and WT RMA-S recipients, regardless of whether high (10<sup>6</sup>) or comparably low (10<sup>5</sup>) tumor cell numbers were inoculated (Fig. 4C).

In summary, our results show the importance of CXCR3 expression on NK cells for their capacity to accumulate in RMA-S tumors. However, the reduction of NK cells in tumors of CXCR3<sup>-/-</sup> mice did not result in reduced survival.

**CXCL10-induced intratumoral NK cell accumulation correlates with improved survival.** To determine the role of CXCR3 ligands in RMA-S lymphoma, RMA-S cells were transduced with a CXCL10-encoding plasmid and clones were selected for their ability to produce high amounts of CXCL10 *in vitro* (data not shown). We selected to ectopically express CXCL10, but not CXCL9 or CXCL11, because in our experiments CXCL10



**Figure 5.** CXCL10 mediates dosage-dependent NK cell accumulation in the tumor. **A**, C57BL/6 mice were injected with RMA-S/vc or RMA-S/CXCL10 cells and amounts of CXCL10 in whole-tumor lysates were determined by specific ELISA on days 5 and 10 after inoculation. **B**, C57BL/6 mice were injected with mixtures of RMA-S/vc and RMA-S/CXCL10 cells at the indicated ratios in Matrigel. Depicted values represent the percentages of RMA-S/CXCL10 and RMA-S/vc, respectively, among 10<sup>6</sup> cells in total. Matrigel plugs were removed 5 d after inoculation, and absolute numbers of infiltrated NK cells were determined. **C**, C57BL/6 mice ( $n = 5$ ) were injected with mixtures of RMA-S/vc and RMA-S/CXCL10 cells. Depicted values represent the percentages of RMA-S/CXCL10 and RMA-S/vc, respectively, among 10<sup>6</sup> cells in total. Survival was monitored for 50 d (\*,  $P < 0.05$ , compared with 100% RMA-S/vc). **A-C**, data are representative of three experiments. Values in **A** and **B** represent the mean  $\pm$  SD ( $n = 5$ ).



**Figure 6.** Ectopic expression of CXCL10 on RMA-S leads to prolonged NK cell-dependent survival. *A*, C57BL/6 mice ( $n = 8$ ) were injected with RMA-S/vc or RMA-S/CXCL10 cells and treated with depleting anti-NK1.1 mAb or PBS. Survival was monitored for 50 d. *B*, RAG2<sup>-/-</sup> mice ( $n = 8$ ) were injected with RMA-S/vc or RMA-S/CXCL10 cells and treated with anti-NK1.1 mAb or PBS. Survival was monitored for 30 d. *A* and *B*, data are representative of four experiments. \*,  $P < 0.05$ , compared with RMA-S/vc, PBS.

exerted the strongest chemotactic activity on NK cells *in vitro* (data not shown) and *in vivo* (Fig. 3*D* and data not shown). The *in vitro* proliferation of RMA-S/CXCL10 cells was not significantly altered by CXCL10 overexpression compared with RMA-S/vc and RMA-S cells (data not shown). Furthermore, NK cells cocultured with RMA-S/CXCL10 or RMA-S/vc cells *in vitro* showed similar degranulation and IFN- $\gamma$  production (data not shown). Tumor lysates of day 5 and day 10 RMA-S/CXCL10 tumors contained around 5-fold elevated levels of CXCL10 compared with RMA-S/vc tumors (Fig. 5*A*). Analysis of tumor-infiltrating cells after injection of mixtures of RMA-S/vc and RMA-S/CXCL10 cells at different ratios revealed that the more CXCL10-producing cells were injected, the more NK cells were detectable (Fig. 5*B*). Of note, similar cell surface expression of the markers CD11b, B220, MHC class II, and CD11c was observed on NK cells isolated from RMA-S/CXCL10 tumors compared with RMA-S/vc tumors (data not shown).

The median survival time of recipient mice increased with higher proportions of injected RMA-S/CXCL10 cells (Fig. 5*C*) that correlated with higher numbers of NK cells in tumors.

Survival of WT mice injected with RMA-S/CXCL10 cells was significantly prolonged compared with the RMA-S/vc injected group, and eventually, RMA-S/CXCL10 tumors were rejected in most recipients (Fig. 6*A*). Rejection was abrogated when mice were treated with an NK1.1<sup>+</sup> cell-depleting mAb (Fig. 6*A*). Furthermore, injection of RMA-S/CXCL10 cells in CXCR3<sup>-/-</sup> recipients resulted in progressive tumor growth (data not shown).

To substantiate the role of NK cells in the inhibition of RMA-S/CXCL10 tumor growth, we injected RMA-S/CXCL10 cells into RAG2<sup>-/-</sup> mice lacking T, B, and NKT cells. Growth of RMA-S/CXCL10 tumors was delayed compared with RMA-S/vc tumors, resulting in significantly increased median survival of RMA-S/CXCL10 injected RAG2<sup>-/-</sup> mice compared with recipients of RMA-S/vc cells (Fig. 6*B*). Depletion of NK1.1<sup>+</sup> cells abrogated this increase in survival (Fig. 6*B*). These data show that improved

host survival due to ectopic expression of CXCL10 by RMA-S cells involves NK cells.

Collectively, our results show that accumulation of NK cells in the tumor is dependent on the amounts of CXCL10 being present. Higher numbers of NK cells attracted via the CXCR3 receptor to RMA-S tumors correlated with improved host survival.

## Discussion

In this study, we investigated factors involved in NK cell accumulation in two NK cell-dependent tumor models: s.c. RMA-S lymphoma and s.c. B16-RAE-1 $\epsilon$  melanoma. Our study emphasizes the importance of IFN- $\gamma$  in this process. IFN- $\gamma$  that is predominantly produced by NK cells (data not shown) induces the production of CXCL9-10 by leukocytes in the tumor, leading to the recruitment of CD27<sup>high</sup> NK cells into the tumor. We show for the first time that CXCR3 expression, but not IFN- $\gamma$ R expression, on NK cells is required for their accumulation in tumor tissues. Intratumoral application of IFN- $\gamma$  or CXCL10 significantly prolonged the survival time that correlated with enhanced amounts of NK cells in the tumors.

Rejection of RMA-S tumors was reported to involve perforin or IFN- $\gamma$  (44). Here, we present that in the absence of IFN- $\gamma$  intratumoral NK cell accumulation in s.c. tumor models is reduced, correlating with a reduced survival rate of recipient mice (Fig. 1). Therefore, IFN- $\gamma$  controls the amounts of NK cells in s.c. RMA-S tumors that can directly attack MHC class I-deficient RMA-S cells. Previous studies reported that lack of MHC class I (22) and TNF- $\alpha$  (23) regulate migration of NK cells to the peritoneum after i.p. injection of RMA-S tumor cells. It is likely that the site of the tumor determines the factors being responsible for NK cell accumulation. It will be important to determine in future studies whether different subsets of NK cells are recruited by different cytokines and chemokines to distinct tumor sites.

The capacity of IFN- $\gamma$ R<sup>-/-</sup> NK cells to infiltrate tumors was not impaired, and thus far, no functional defects of IFN- $\gamma$ R<sup>-/-</sup> NK cells were reported (45, 46), suggesting that IFN- $\gamma$  did not directly affect

NK cell accumulation or function. Our data reveal that intratumorally injected IFN- $\gamma$  induced local expression of the CXCR3 ligands CXCL9, CXCL10, and CXCL11 (Fig. 3C). Furthermore, CXCR3 expression on NK cells was required for their accumulation in RMA-S tumors (Fig. 4B) and application of exogenous CXCR3 ligand CXCL10 enhanced NK cell accumulation in tumors *in vivo* (Fig. 3D). These results suggest that IFN- $\gamma$  treatment indirectly modulates NK cell accumulation by facilitating CXCR3-mediated NK cell accumulation in the tumor.

Because all cell types at the tumor site, including leukocytes, endothelial cells, and tumor cells, expressed the IFN- $\gamma$ R (data not shown) and were potentially able to respond to endogenously produced or exogenously applied IFN- $\gamma$ , we further dissected the source of CXCR3 ligands. These chemokines were mainly produced by the Ly5.1<sup>+</sup> leukocyte fraction in the tumor, which did not include Ly5.1<sup>-</sup> RMA-S cells (data not shown). Furthermore, RMA-S cells produced CXCR3 ligands *in vitro*, neither in the absence nor in the presence of IFN- $\gamma$  (data not shown). These data suggest that in untreated tumor-bearing mice or after exogenous application of IFN- $\gamma$  CXCR3 ligands are produced by cells other than tumor cells. We are currently attempting to identify the exact leukocyte subpopulations producing CXCR3 ligands in the tumors. Increased NK cell accumulation and prolonged survival were also observed when RMA-S cells were transduced to overexpress CXCL10 and after exogenous application of CXCL10, demonstrating that, independent of its source, higher levels of CXCL10 in the tumor correlated with higher amounts of tumor-infiltrating NK cell and better survival.

We have evidence that injection of recombinant IFN- $\gamma$  resulted in significantly increased NK cell numbers in tumors of CXCR3<sup>-/-</sup> mice, although not to the same extent as in WT mice (data not shown). Furthermore, application of IFN- $\gamma$  also increased survival of CXCR3<sup>-/-</sup> mice after RMA-S cell inoculation (data not shown). These observations imply that exogenous IFN- $\gamma$  application modulates intratumoral NK cell accumulation and host survival not only by induction of CXCR3 ligands but also by additional mechanisms.

Wald and colleagues (47) reported that i.p. injection of high doses of IFN- $\gamma$  induced NK cell mobilization from the bone marrow and, in an CXCR3-dependent manner, from the spleen resulting in increased numbers of NK cells in the periphery. The most pronounced increase in numbers of circulating NK cells was observed 4 to 7 hours after a second injection of IFN- $\gamma$ . In our experiments, we detected elevated levels of NK cells in the tumors analyzed on day 5 after IFN- $\gamma$  treatment (Fig. 2B). In parallel, no differences were detected in the numbers of NK cells in spleens, blood, or LN between mice injected with tumor cells alone or in combination with IFN- $\gamma$  (data not shown). Because our experiments were performed 5 days after tumor cell inoculation and IFN- $\gamma$  treatment, we cannot exclude that enhanced NK cell numbers in tumors after IFN- $\gamma$  treatment were due to their increased initial mobilization from bone marrow or spleens. Whether enhanced NK cell proliferation and/or survival are responsible for the increased NK cell accumulation in tumors of IFN- $\gamma$  treated mice is currently unknown.

Mature mouse NK cells were recently divided into functionally distinct subsets, as defined by expression levels of CD27 (37). In this study, CD27<sup>high</sup> NK cells displayed higher cytolytic activity and enhanced cytokine production compared with CD27<sup>low</sup> cells. Under steady-state conditions, expression of the chemokine receptors CXCR3 and CX3CR1 on mouse NK cell subsets is mutually

exclusive. CXCR3 is confined to the CD27<sup>high</sup> and CX3CR1 to the CD27<sup>low</sup> subset (16). Hence, it is possible that the tumor microenvironment, containing either the CX3CR1 ligand or CXCR3 ligands or both, is decisive for which NK cell subsets are preferentially recruited. Of note, CXCR3 ligands (Fig. 3C), but not the CX3CR1 ligand CX3CL1 (data not shown), are abundant in the RMA-S tumor tissue. Accordingly, the cytolytic and cytokine-producing CD27<sup>high</sup> NK cell subset, which under steady-state conditions expresses CXCR3 (37), preferentially accumulated in the tumor tissue (Fig. 3A).

Despite the reduction in NK cell numbers in tumors in CXCR3<sup>-/-</sup> mice, survival time in these mice remained unaffected (Fig. 4C). This observation can be explained by several reasons. First, other chemokine receptors might enable NK and T cells to accumulate in tumors of CXCR3<sup>-/-</sup> mice, thereby reaching a certain threshold number of immune cells sufficient for the initiation of an antitumor response, even in the absence of CXCR3. Nevertheless, attracting more NK cells into the tumors by ectopic expression of CXCR3 ligands correlated with significantly prolonged host survival (Fig. 6). Second, we have evidence that fewer regulatory T cells (Treg) that were shown to be recruited via the CXCR3 receptor (48) are present in the tumors in CXCR3<sup>-/-</sup> mice compared with WT mice (data not shown). Therefore, it is possible that tumor growth in CXCR3<sup>-/-</sup> mice was also influenced by reduced numbers of immunosuppressive Treg in the tumors that were reported to inhibit NK cell function (49).

Titration of injected RMA-S/CXCL10 cells showed that intratumoral NK cell accumulation increased along with CXCL10 amounts (Fig. 5B). Furthermore, an increased CXCL10 production correlated with prolonged survival of mice (Fig. 5C). Median survival of RMA-S/CXCL10-injected animals was significantly increased compared with RMA-S/vc-injected animals for WT and RAG2<sup>-/-</sup> recipients. This was abrogated when NK1.1<sup>+</sup> cells were depleted (Fig. 6A and B). In WT mice, it is possible that not only NK cells but also NKT cells were involved in tumor rejection. The contribution of NKT cells, however, was further excluded by the use of RAG2<sup>-/-</sup> mice that also lack NKT cells. Interestingly, upon depletion of NK cells, a longer median survival was observed for RAG2<sup>-/-</sup> mice injected with RMA-S/CXCL10 compared with RMA-S/vc-injected mice. These results suggest that CXCL10 exerts a tumor-inhibiting effect even in the absence of T, B, NKT, and NK cells (Fig. 6B). Whether CXCL10 mediates enhanced infiltration of other tumor-eradicating immune cells or whether it acts as an antiangiogenic factor (50) in our model is currently unknown.

In concordance with previous reports (13, 22), we observed that lower numbers of NK cells accumulated in RMA tumors compared with RMA-S tumors (Figs. 2A and 3D). Importantly, intratumoral injection of recombinant CXCL10 or IFN- $\gamma$  resulted in enhanced NK cell accumulation also in RMA tumors (Figs. 2A and 3D). These observations indicate that chemokine-mediated immune cell migration overrides the influence of MHC class I expression by tumor cells on NK cell accumulation. Whether and how enhanced NK cell infiltration can also be beneficial for the antitumor immune response in NK cell-insensitive tumor models remains unsolved.

For successful NK cell-based immunotherapy, it is of great importance to target highly effective NK cells at the right time to the right place in the tumor. Of note, factors regulating this accumulation are highly influenced by the tumor entity, chemokine profile of the tumor, and the tumor



microenvironment. In this study, we report that CXCR3 on NK cells, IFN- $\gamma$ , and lack of inhibitory MHC class I ligands on tumor cells contribute to NK cell accumulation in the tumor. The knowledge of factors enhancing NK cell accumulation in tumors might help the design of more effective NK cell-based immunotherapies against cancer.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Natural Killer Cell Accumulation in Tumors Is Dependent on IFN- $\gamma$ and CXCR3 Ligands

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