IFN-γ Acts on T Cells to Induce NK Cell Mobilization and Accumulation in Target Organs[1]

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The mechanism(s) that regulates NK cell mobilization and the significance of this process to NK cell activity are unknown. After Con A-induced hepatitis, NK cells are mobilized from the spleen and bone marrow into the periphery in an IFN-γ-dependent fashion. Intraperitoneal administration of IFN-γ stimulates the mobilization of NK cells into the circulation, but not their cell death or proliferation. Increased number of circulating NK cells was coupled with their accumulation in the peritoneum, liver, and tumor-bearing lung tissue. Furthermore, increased number of NK cells in the lung reduced metastasis of Lewis lung carcinoma cells (3LL cell line) resulting in significantly extended NK-dependent survival. Mobilization of NK cells was specific and required the presence of T cells. Moreover, mobilization and migration of spleen NK cells in response to IFN-γ treatment is dependent on the chemokine receptor CXCR3. Mechanistic insights regarding the role of IFN-γ in the regulation of NK cell mobilization and their accumulation at sites of tumor metastasis may lead to the development of novel immunotherapy for cancer. ***The Journal of Immunology,*** **2006, 176: 4716–4729.**

Classical NK cells are non-T cell lymphocytes lacking the expression of TCRs. These cells express a variety of cell surface markers, including CD56 in humans and NK1.1 and DX5 in mice (1). NK cells are dependent on the bone marrow (BM)[4] for development and are present as mature populations in blood, spleen, and liver (1, 2). NK cells express unique chemokine receptors and adhesion molecule repertoires that are critical for their trafficking in the body. Upon stimulation, NK cells are recruited from the peripheral blood to sites of viral infection and tumor metastasis to exert their cytotoxic effect (3–5). IFN-γ and IFN-α were shown to be critical for NK-mediated clearance of liver murine CMV (MCMV) infection (6–8). More specifically, the chemokines CCL3, induced by IFN-α, and CXCL9 and CXCL10, induced by IFN-γ, play a crucial role in the recruitment of NK cells to the inflamed liver during MCMV infection (6–9). Trafficking of NK cells into tumors is also critical for their antitumor effect. The chemokine receptor, CX3CR1, was shown to be essential for NK-dependent antitumor responses in vivo (10). Recent reports also define a role for NK cells in adoptive immunotherapy after BM transplantation (BMT). The use of delayed lymphocyte infusion after allogeneic BMT resulted in less graft-vs-host disease with a significant graft-vs-leukemia response (11, 12).

To accumulate in large numbers in their target organ, NK cells should initially mobilize from their BM and spleen storages to the peripheral blood and subsequently be recruited to target tissues (1, 2). Very little is known about the mechanism(s) that regulates NK cell mobilization under normal and pathological conditions.

In this study, mechanisms regulating NK cell mobilization are presented. Furthermore, a link between NK cell mobilization and reduced metastasis is shown. BM- and spleen-resident NK cells were mobilized into the circulation by i.p. administration of IFN-γ and accumulated in the peritoneum and liver of treated mice. In mice injected with Lewis lung carcinoma cells (3LL cell line), IFN-γ treatment induced the mobilization of NK cells and their accumulation in the lung. Consequently, the treated mice had NK-dependent reduction in their metastatic load and prolonged survival. Understanding the mechanism(s) controlling NK cell mobilization and recruitment may enable the deliberate direction of NK cells to sites of viral infection and tumor metastasis.

Materials and Methods

Mice and experimental protocols

C57BL/6 control mice were maintained under specific pathogen-free conditions at the Hebrew University Animal Facility (Jerusalem, Israel). IFN-γ- and TNF-α-deficient mice on C57BL/6 background (The Jackson Laboratory) were maintained under specific pathogen-free conditions at the Weizmann Institute of Science Animal Facility (Rehovot, Israel). The animal care committee of the Hebrew University approved all experiments. C57BL/6 control mice and CXCR3 knockout (KO), CX3CR1 KO, and CCR1 KO mice backcrossed on C57BL/6 background (seven generations) were maintained at the National Institutes of Health Animal Facility (Bethesda, MD) by J. Farber. Con A (Sigma-Aldrich) was reconstituted in PBS at a concentration of 1.5 mg/ml. In all the experiments with Con A, mice were injected with Con A (0.3 mg per mouse) in a total volume of 200 μl

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[4] Abbreviations used in this paper: BM, bone marrow; BMT, BM transplantation; MCMV, murine CMV; KO, knockout; ^{18}F-FDG, ^{18}F-fluorodeoxyglucose; LN, lymph node.

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i.v. Cytokines and chemokines (IFN-γ, IFN-α, and IL-18; PeproTechAsia) were reconstituted in PBS. Cytokines and chemokines were injected at the indicated concentrations; control mice were injected with PBS. A total volume of 500 μl per mouse was injected i.p. 24 and 7 h before mice were sacrificed. All reagents used for in vitro and in vivo studies had endotoxin levels that were <0.1 ng/μg (1 EU/μg).

In vivo T cell depletion

Depletion of $CD3^+$, $CD4^+$, or $CD8^+$ cells was done in C57BL/6 mice using functional grade purified anti-CD3e, anti-CD4, and anti-CD8a (eBioscience). Mice were injected i.v. with 100 μg of each Ab 2 days before IFN-γ treatment. Depletion was confirmed by FACS analysis. In adoptive transfer experiments, 10^7 purified spleen $CD3^+$ cells were injected i.v. to SCID mice on BALB/c background. IFN-γ treatment was done 5 days after transplantation; the presence of T cells in the BM and spleen of transplanted mice was confirmed by FACS analysis.

In vivo model for tumor metastasis

C57BL/6 mice were injected i.v. with 1×10^6 3LL per mouse. Mice were treated every 4 days with two injections i.p. of IFN-γ (10,000 IU per injection) at 0 and 17 h. Human IL-2 (Proleukin; Chiron) was injected once i.p. into mice every 4 days (10,000 IU per injection). The IFN-γ-treated groups were injected i.p. once a week with anti-murine NK1.1 Abs (50 μg per mouse) or murine IgG Abs (50 μg per mouse). NK depletion was first done 24 h before the tumor cells were injected. Depletion was verified by FACS analysis using the anti-NK Abs, DX5. Four weeks after injection of cells, tumor growth was assessed. Mice were injected with 40 μCi of ^{18}F-fluorodeoxyglucose (^{18}F-FDG), and lungs were harvested 1 h postinjection of the radiotracer (Cyclotron Unit, Nuclear Medicine Department, Hadassah University Hospital). Harvested lungs were measured for ^{18}F-FDG uptake and weighed, paraffin-embedded histological specimens were prepared, and sections were examined blindly by a noninformed expert in pathology to assess the number of metastatic nodules. In experiments aimed at determining the percentage of NK cells in the lung, lymphocytes were isolated and analyzed by FACS as described below. For survival assay, mice were treated as described above for as long as they survived.

Lymphocyte isolation and quantization

Bones and spleen were splashed with PBS; blood was collected into heparin-containing tubes; and cells were treated with RBC lysis solution (0.155 M NH_4Cl, 0.01 M $KHCO_3$, 0.01 mM EDTA (pH 7.4)), washed, and resuspended with PBS containing 0.1% BSA (Biological Industries) and 0.01% sodium azide (FACS buffer). Peritoneum was washed with 3 ml of PBS and 30 μl of heparin. Cells were filtered through a cell-strainer FACS tube (BD Biosciences) and stained for cytometry analysis.

Liver and lung were weighed, meshed through 70-μM cell strainers (BD Falcon), washed with PBS twice (900 rpm), and loaded on Ficoll (Histopaque 1077-1; Sigma-Aldrich). Cells were washed again in PBS, centrifuged, resuspended in FACS buffer, and stained for flow cytometry analysis.

The total number and percentage of NK cells was calculated as follows: the number of $NK1.1^+CD3^-$ NK cells in 100 μl of blood was counted by FACS and multiplied by 15 assuming a total blood volume of 1.5 ml. The number and percentage of NK cells in the tibia and femur were determined by FACS, and the number of cells was multiplied by 5 assuming that these cells represent 20% of the total NK cells in the BM. The total number and percentage of isolated NK cells in the liver, peritoneum, and spleen also were determined by FACS. Isolated cells from all samples were suspended in 0.9 ml of FACS buffer, and the number of cells in 20 μl was determined by FACS and multiplied by a factor of 45. The total cell number in the blood spleen and BM did not change dramatically in the duration of the various treatments. In the blood of control mice, the total number of cells was $8.6 \times 10^6 \pm 3.4$; after Con A treatment, the number of cells was $7.6 \times 10^6 \pm 1.5$, and after IFN-γ was $10.4 \times 10^6 \pm 3.1$. In the spleen of control mice, the total number of cells was $82 \times 10^6 \pm 36$; after Con A treatment, the number of cells was $115 \times 10^6 \pm 34$, and after IFN-γ, the number was $67 \times 10^6 \pm 39$. In the BM of control mice, the total number of cells was $132 \times 10^6 \pm 24$; after Con A treatment, the number of cells was $95 \times 10^6 \pm 15$, and after IFN-γ, the number was $116 \times 10^6 \pm 39$. In the adoptive transfer experiments, splenocytes from BALB/c mice were isolated on Ficoll, and then $CD3^+$ T cells were negatively selected using the Pan-T cell isolation kit (purity >90%; Miltenyi Biotec).

Phenotypic analysis of lymphocytes and flow cytometry analysis

To assess the number of total NK cells in the BM, spleen, blood, and liver, lymphocytes derived from these organs were gated according to forward scatter and side scatter to exclude dead cells and include both activated and nonactivated lymphocytes. Then, cells were stained with anti-CD45.2 FITC, anti-NK1.1 PE, and anti-CD3 Cy3 mAbs; $CD45.2^+$ cells were gated and subpopulations of $NK1.1^+CD3^-$ (NK cells), $NK1.1^+CD3^+$ (NKT cells), and $NK1.1^-CD3^+$ (T cells) were defined. Lymphocytes were taken at a concentration of $>1 \times 10^6$ cells/ml in 0.1 ml of FACS buffer and stained with anti-CD45.2 FITC, NK1.1 PE, and CD3ϵ Cy3 mAbs (eBioscience), CXCR3 FITC (Joshua Farber laboratory), or isotype controls for 30 min and were washed with FACS buffer. NK cells from C57BL and KO mice of the same genetic background expressed similar levels of the NK cell marker NK1.1 before and after treatments with Con A, IFN-γ, and IL-18 (data not shown). NK cells from SCID mice of BALB/c background or BALB/c mice were identified using anti-DX5 Abs (eBiosience). Depletion was verified using anti-CD8a or anti-CD4 FITC (eBioscience). Immunostained cells were analyzed by flow cytometry using the FACS Caliber Flow Cytometer (BD Biosciences); the data were analyzed using software from CellQuest (version 3.3; BD Biosciences) or FlowJo (version 6.0; Tree Star).

Transwell migration assays

NK cell migration with the indicated concentrations of CXCL9 or CXCL10 (PeproTech) was done as described previously (13). Shortly, in vivo, mice were injected with IFN-γ (10,000 IU per mouse; PeproTech), and BM and spleen cells were collected 3 h after injection. The cells were loaded on Ficoll (Histopaque 1077-1; Sigma-Aldrich), washed, and resuspended in 1% FCS RPMI 1640 medium. Whole BM or spleen cells were loaded on the upper well, 5×10^5 cells per well. In vitro, BM and spleen cells were harvested from mice and divided into three groups. Cells were incubated with no IFN-γ, 50 IU/ml IFN- γ, and 125 IU/ml IFN- γ for 3 h at 37°C and afterward loaded on Transwells, 5×10^5 cells per well. Cells were collected after 3 h from the lower well, stained, and read by FACS.

Cell apoptosis assay

The apoptosis of BM and spleen NK cells after injection of IFN-γ was tested using the annexin V assay (IQP) according to the manufacturer's recommendation.

Cell proliferation assay

Spleen cells were isolated on Ficoll, diluted to 10^7 cells/ml, and labeled with 1 μg/ml CFSE (BCECF/AM; Calbiochem). A total of 3×10^7 labeled cells were injected per mouse i.v. 3 days before injection of IFN-γ. After injection of IFN-γ, cells were isolated, stained for NK1.1 (PE) and CD3e (PE-Cy5), and CFSE (FITC fluorescence) was measured using the FACS to assess cell proliferation.

ELISA

ELISAs for murine CXCL9 and CXCL10 was performed using the Quantikine kit (R&D Systems) according to the manufacturer's instructions. In the in vitro experiments, BM and spleen cells were purified on Ficoll and incubated with 125 IU/ml IFN-γ. After 24 h, the medium was collected, and CXCL9 and CXCL10 levels were determined. In the in vivo experiments, 24 h after IFN-γ injections, blood, spleen, liver, and BM were collected. BM and spleen were flashed with PBS containing protease inhibitor mixture (Roche Diagnostics). Liver extracts were obtained by mashing the tissue on 70-μM nylon mesh with PBS containing a protease inhibitor mixture, and CXCL9 and CXCL10 levels were determined.

Statistical analysis

Differences among groups of mice and different treatments in the number of NK cells were calculated using standard Student's t test. Statistical tests were two-sided. Values of $p < 0.05$ were considered to be statistically significant.

Results

Involvement of IFN-γ in the mobilization of NK cells during Con A-induced hepatitis

Con A-induced immune cell-mediated hepatitis is widely used to assess the potential role of cytokines and chemokines in the regulation of liver inflammation (14, 15). We used this model to determine the role of cytokines and chemokines in the mobilization of NK cells from the BM and spleen into the blood and their further recruitment to the liver. To identify the population of NK cells in the BM, spleen, blood, liver, and peritoneum, lymphocytes

derived from these organs were first gated according to forward scatter and side scatter to exclude dead cells and include both activated and nonactivated lymphocytes. Then, cells were stained with anti-CD45.2 FITC, NK1.1 PE, and CD3ϵ Cy3 mAbs; $CD45.2^+$ cells were gated and sub-populations of $NK1.1^+CD3^-$ (NK cells), $NK1.1^+CD3^+$ (NKT cells), and $NK1.1^-CD3^+$ (T cells) were defined (Fig. 1*A*). The number of NK cells in the BM (2.25×10^6), spleen (1.35×10^6), liver (6.75×10^5), blood (9×10^5), and peritoneum (1.35×10^4) was measured using the FACS analysis (Fig. 1*A*). After injection of Con A, the number of NK cells in the BM and spleen was reduced in a time-dependent manner (Fig. 1*B*). Eight hours after Con A injection, the number of NK cells in the BM and spleen was reduced to 1.2×10^6 and 6.75×10^5 cells, respectively. In parallel, the number of NK cells in the blood increased to 1.2×10^6 cells, and their number in the liver increased to 1.1×10^6. After 24 h, the number of NK cells in the BM and spleen was dramatically reduced to 6.75×10^5 and 2.9×10^5 cells, respectively. At the same time, the number of NK cells in the blood was reduced from 1.2×10^6 to 4×10^5, whereas the number of NK cells in the liver increased to 3.5×10^6. During the recovery phase, 96 h after Con A injection, the number of NK cells in the BM, spleen, and peripheral blood significantly rose, while their number in the liver was reduced (Fig. 1). These results show that the majority (>90%) of NK cells that were mobilized from the BM and spleen into the blood were subsequently recruited into the liver.

Con A induces a systemic activation of NK cells, which may lead to either NK cell proliferation or apoptosis. To assess the effect of Con A on the survival of NK cells, we collected the BM and spleen NK cells 8 and 24 h after treatment and tested for apoptotic NK cells using the annexin assay. We found no evidence for increased NK cell apoptosis (Fig. 2*A*). To test whether the increase in the numbers of NK cells in the liver was due to proliferation or recruitment of cells, adoptive transfer assays were performed. Total spleen cells were loaded with CFSE and injected to mice 3 days before Con A treatment. Twenty-four hours after treatment, the cells were collected from the liver, spleen, BM, and peripheral blood and stained for NK1.1 and CD3. As previously shown in Fig. 1, $CFSE^+NK1.1^+CD3^-$ cells were mobilized from the spleen and BM and accumulated in the liver (Fig. 2, *B* and *C*). No mobilization of $CFSE^+NK1.1^-CD3^+$ T cells from the BM and spleen was observed (Fig. 2*C*). Because the fluorescence intensity of Con A-induced $CFSE^+NK1.1^+$CD3-liver-infiltrating NK cells was similar to that observed in $CFSE^+NK1.1^+$CD3-liver-derived NK cells from untreated mice, we concluded that the accumulation

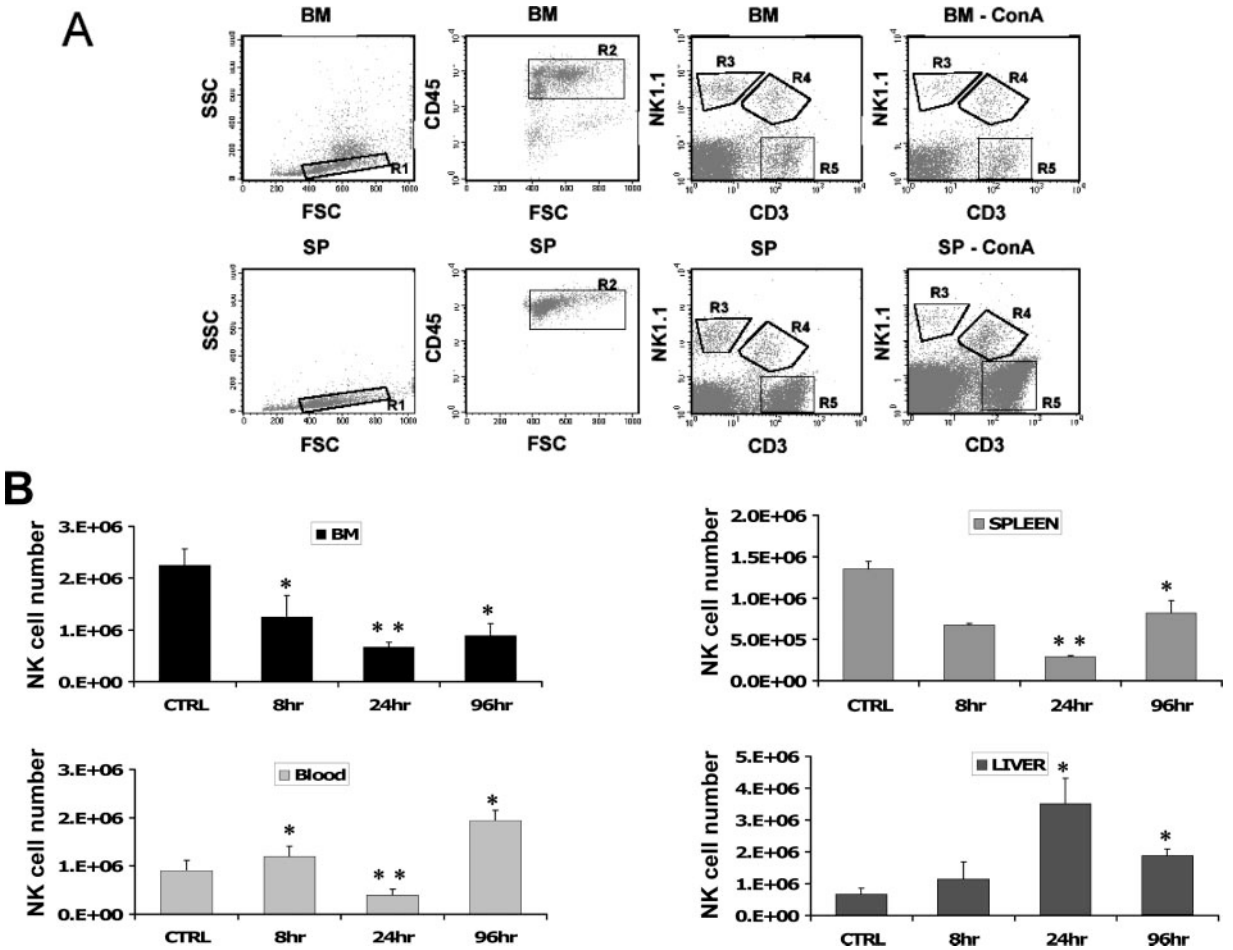

FIGURE 1. After Con A-induced hepatitis, NK cells are mobilized from the BM and spleen to the blood and then to the liver. *A*, A representative FACS analysis of BM- and spleen-derived lymphocytes stained with anti-CD45.2 (FITC), anti-NK1.1 (PE), and anti-CD3 (CY) is shown. Cells were gated according to forward scatter and side scatter (R1); then $CD45.2^+$ cells were gated (R2), and subpopulations of $NK1.1^+CD3^-$ (NK cells, R3), $NK1.1^+CD3^-$ (NKT cells, R4), and $NK1.1^-CD3^+$ (T cells, R5) were defined. *B*, Cells from the BM, spleen, PBL, and liver of Con A-treated and CTRL untreated mice were collected at 0, 8, 24, and 96 h postinjection. The absolute NK cell numbers derived from these tissues were analyzed as shown in *A*. The result presented represents one of four experiments performed. The results presented are the average ± SD. For each time point, six mice were analyzed. ∗, $p < 0.05$; ∗∗, $p < 0.01$.

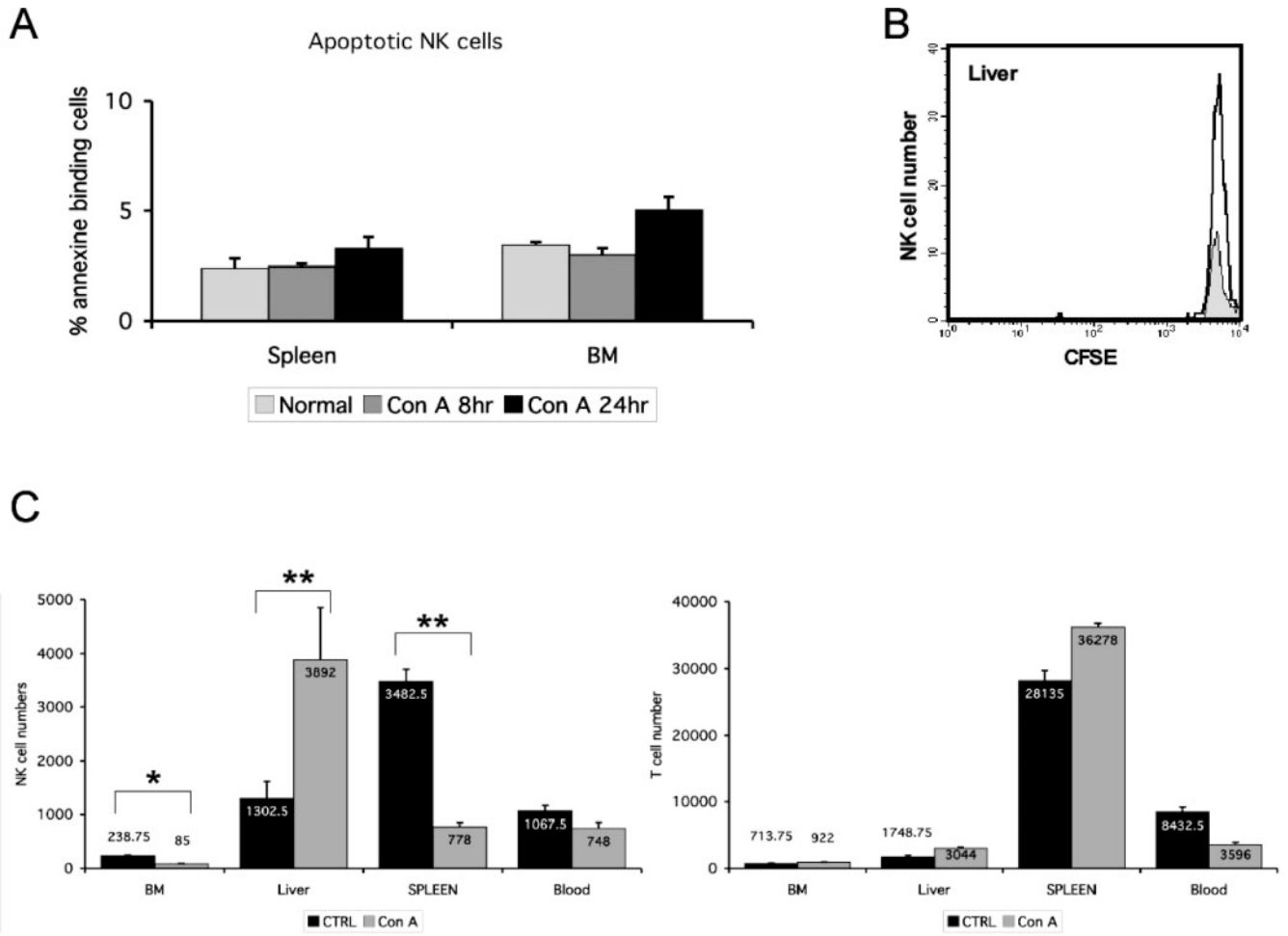

FIGURE 2. After Con A administration, CFSE-labeled NK cells are mobilized from the BM and spleen and accumulate in the liver. *A*, Apoptosis of NK cells in the BM and spleen, 8 and 24 h after Con A administration, was measured using annexin assay. *B* and *C*, CFSE-labeled splenocytes were injected into mice. Cell proliferation and tissue localization of CFSE-labeled NK cells were tested 24 h after Con A injection. The number and fluorescence intensity of CFSE-labeled liver-derived NK cells before (filled) and after Con A treatment (black line) are shown in *B*. The number of CFSE-labeled NK cells (*left panel*) and T cells (*right panel*) in the BM, liver, spleen, and blood is shown in *C*. The result presented represents one of two experiments performed. For each time point, five mice were analyzed. The results presented are the average ± SD. *, $p < 0.05$; **, $p < 0.01$.

of cells in the liver was due to cell migration rather than proliferation (Fig. 2*B*). Con A-induced liver damage and inflammation are mediated by the production of the cytokines TNF-α and IFN-γ (16–18). In addition to cytokines, the CCR1 chemokine receptor ligand, CCL3, and the CXCR3 chemokine receptor ligands, CXCL10 and CXCL9, play a role in the recruitment of NK cells to the inflamed liver (6, 7, 9). Considering the role of TNF-α and IFN-γ in the establishment of Con A-induced hepatitis and the suggested role of the CCR1 and CXCR3 ligands in the recruitment of NK cells to the liver, we analyzed whether mobilization of NK cells from the BM and spleen would be affected in mice deficient of these cytokines or chemokine receptors. After Con A injection, mobilization of NK cells from the BM and spleen was reduced in the IFN-γ KO mice, but not in the TNF-α KO mice (Fig. 3, *A* and *B*). Furthermore, the mobilization of NK cells from the spleen, but not from the BM, was partially yet significantly dependent on the presence of CXCR3, as evidenced by experiments in CXCR3 KO mice (Fig. 3*B*). CCR1 and CX3CR1 are chemokine receptors functionally expressed by NK cells; nevertheless, mobilization of NK cells was not affected by the absence of these receptors. (Fig. 3, *A* and *B*) (10, 19). These results suggest a possible role for the IFN-γ inducible chemokines CXCL9 and CXCL10 (CXCR3 ligands) in the mobilization of NK cells from the spleen. In contrast, IFN-γ-induced mobilization of NK cells from the BM is suggested to be CXCR3 independent. (Fig. 3*C*). Salazar-Mather et al. (6) reported a crucial role for CCL3 in the recruitment of NK cells to the liver during MCMV-induced hepatitis. In accordance with this study, we found that, in Con A-induced hepatitis, the recruitment of NK cells to the liver was reduced in CCR1 KO mice, but not in CXCR3 KO mice (Fig. 3*C*). It is therefore suggested that, in Con A-induced hepatitis, the molecular mechanisms regulating NK cell mobilization differ from those that regulate recruitment to the target organ.

IFN-γ administration induces the specific mobilization of NK cells from the BM and spleen

The cytokines IFN-γ and IFN-α play a role in the recruitment of NK cells to the inflamed liver (6, 7, 9). To further characterize the role of these cytokines in NK mobilization, IFN-γ and IFN-α were injected twice i.p. 24 and 7 h before mice were sacrificed, and the number of NK cells in the BM, spleen, peritoneum, and peripheral blood was assessed. Both IFN-γ and IFN-α stimulate the accumulation of rather small numbers of NK cells in the peritoneum (Fig. 4*A*). Furthermore, IFN-γ, but not IFN-α, induced the mobilization of large numbers of NK cells from the BM and spleen to the peripheral blood (Fig. 4, *C–E*).

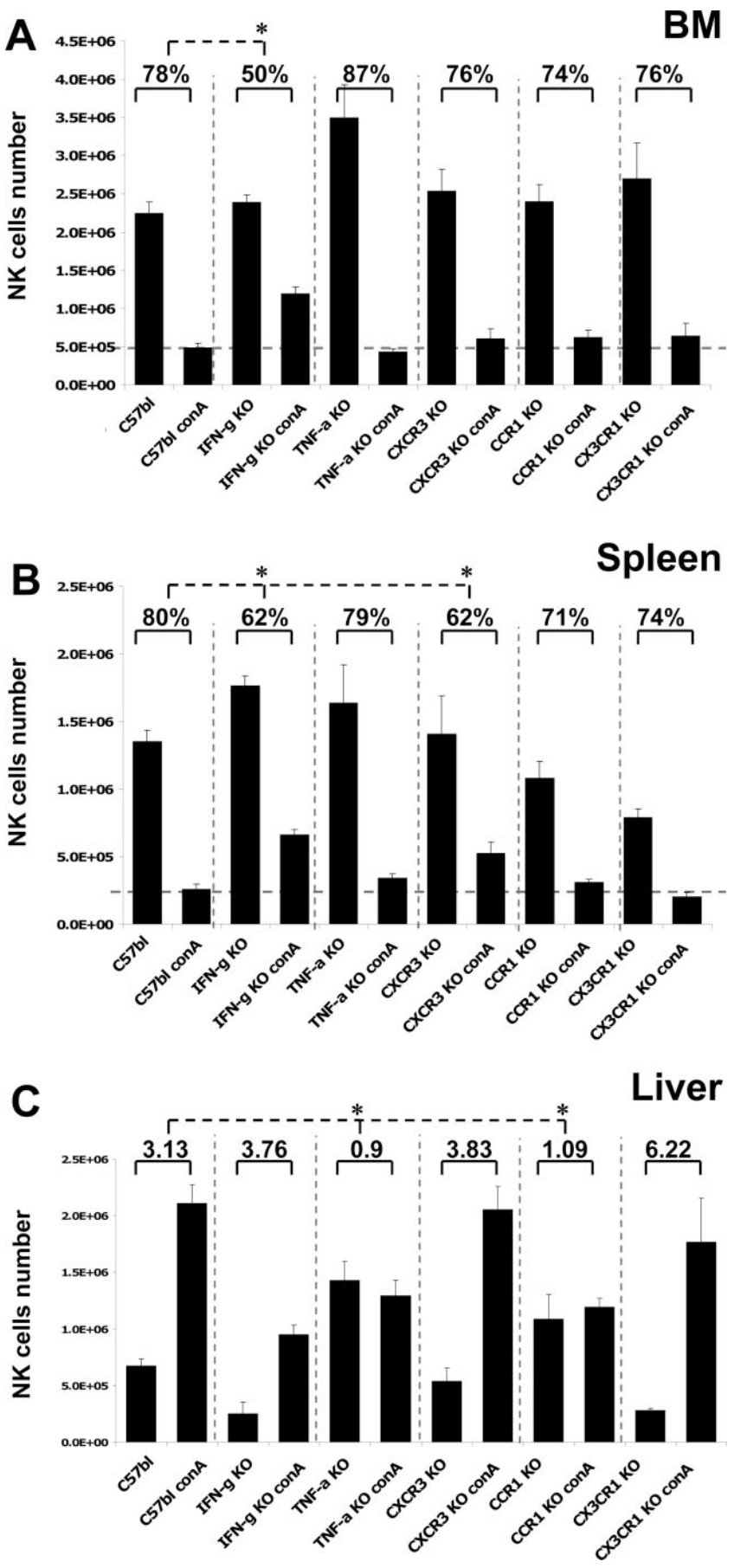

FIGURE 3. IFN-γ and CXCR3 play a role in the mobilization of NK cells from the BM and spleen. Con A-induced mobilization of NK cells and their subsequent recruitment to the liver were studied in IFN-γ, TNF-α, CXCR3, CCR1, and CX3CR1 KO mice. *A–C*, After Con A injection, NK cell numbers in the BM, spleen, and liver were counted. Percentage of mobilization is shown for each KO mice. *A* and *B*, Significant reduction in NK cell mobilization is indicated in IFN-γ and CXCR3 KO mice, compared with control C57BL mice (dotted line). Fold increase in the number of NK cells entering the liver after Con A stimulation is shown in *C*. Reduced recruitment of NK cells to the liver of TNF-α and CCR1 KO mice relative to control C57BL mice also is shown in *C* (dotted line). The results are the average ± SE of three experiments. For each strain of mice, 9–15 mice were analyzed. (∗, $p < 0.05$).

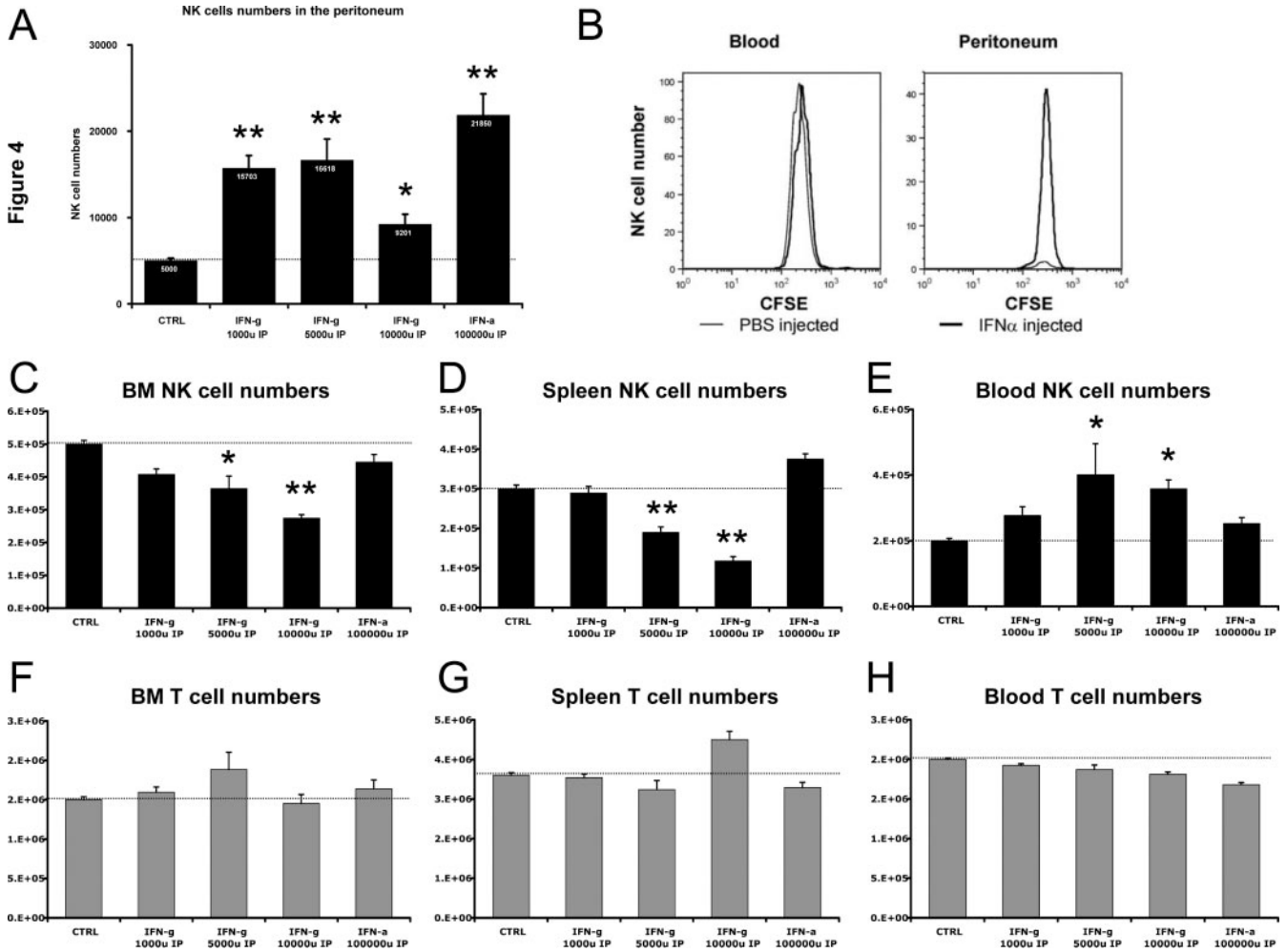

FIGURE 4. Intraperitoneal injection of IFN-γ induced specific mobilization of NK cells from the BM and spleen to the blood. *A–H*, IFN-γ (1,000–10,000 IU per mouse) and IFN-α (100,000 IU per mouse) were injected i.p. 24 and 7 h before harvesting, and the number of NK cells (*A–E*) and T cells (*F–H*) was counted in the peritoneum, BM, spleen, and blood. The results are the average $\pm$ SE of three experiments. For each point, 9–15 mice were analyzed (*, $p < 0.05$; **, $p < 0.01$). *B*, The number of CFSE-labeled NK cells to the peritoneum and blood of mice injected with IFN-α was tested. Cell proliferation and tissue localization of CFSE-labeled NK cells were tested 7 h after the second injection of IFN-α. The fluorescence intensity of CFSE-labeled blood and peritoneum-derived NK cells before (dotted line) and after IFN-α treatment (solid) is shown.

To test whether the increased number of NK cells in the peritoneum was due to proliferation or recruitment of cells, adoptive transfer assays were performed. Total spleen cells were loaded with CFSE and injected to mice 3 days before IFN-α treatment (100,000 U per injection). Twenty-four hours after treatment, the cells were collected from the peripheral blood and peritoneum and stained for NK1.1 and CD3. As shown in Fig. 4*A*, $CFSE^+NK1.1^+CD3^-$cells accumulated in the peritoneum of treated mice (Fig. 4*B*). However, as shown in Fig. 4*B*, no reduction in the number of CFSE-labeled NK cells in the blood was observed. Because the fluorescence intensity of IFN-α-induced $CFSE^+NK1.1^+CD3^-$ peritoneal-infiltrating NK cells was similar to that observed in $CFSE^+NK1.1^+CD3^-$ peritoneal-derived NK cells from untreated mice, we concluded that the accumulation of cells in the peritoneum was due to cell migration rather than proliferation (Fig. 4*B*). However, because the absolute number of recruitment cells was low, no reduction in the total number of cells in the blood, spleen, and BM was observed (Fig. 4, *A* and *B*).

IFN-γ induced the mobilization of NK cells from the BM and spleen in a dose-dependent manner (Fig. 4, *C* and *D*). Reduced numbers of NK cells in the BM and spleen were associated with an increased number of circulating NK cells (Fig. 4*E*). For example, 24 h after initiation of treatment with IFN-γ (5,000 IU per mouse), 6.3×10^5 of BM and 4.95×10^5 of spleen-derived NK cells were mobilized from these organs. Ninety percent of mobilized NK cells (1.0×10^6) were found in the blood. IFN-γ-induced mobilization of NK cells was specific for these cells, because no T cell mobilization was evident (Fig. 4, *F–H*). The increase in the number of circulating NK cells was mainly observed 4–7 h after the second injection of IFN-γ (5,000 and 10,000 IU per mouse) (Fig. 5*A*). At the same time, NK cell accumulation at the site of injection, i.e., the peritoneum, also was observed (Fig. 5*A*). IFN-γ induces systemic activation of NK cells, which may lead to NK cell proliferation or apoptosis. To assess the effect of IFN-γ on the survival of NK cells, we collected the BM and spleen NK cells 3 h after the second injection of 10,000 IU IFN-γ per mouse and tested for apoptotic NK cells using the annexin assay. We found no evidence for increased NK cell death (Fig. 5*B*). To test whether the increase in the number of NK cells in the blood and peritoneum was due to proliferation or recruitment of cells, adoptive transfer assays were performed. Total spleen cells were loaded with CFSE and injected to mice 3 days before IFN-γ treatment. Twenty-four hours after treatment, the cells were collected from the liver, spleen, BM, peritoneum, and peripheral blood and stained for NK1.1 and CD3.

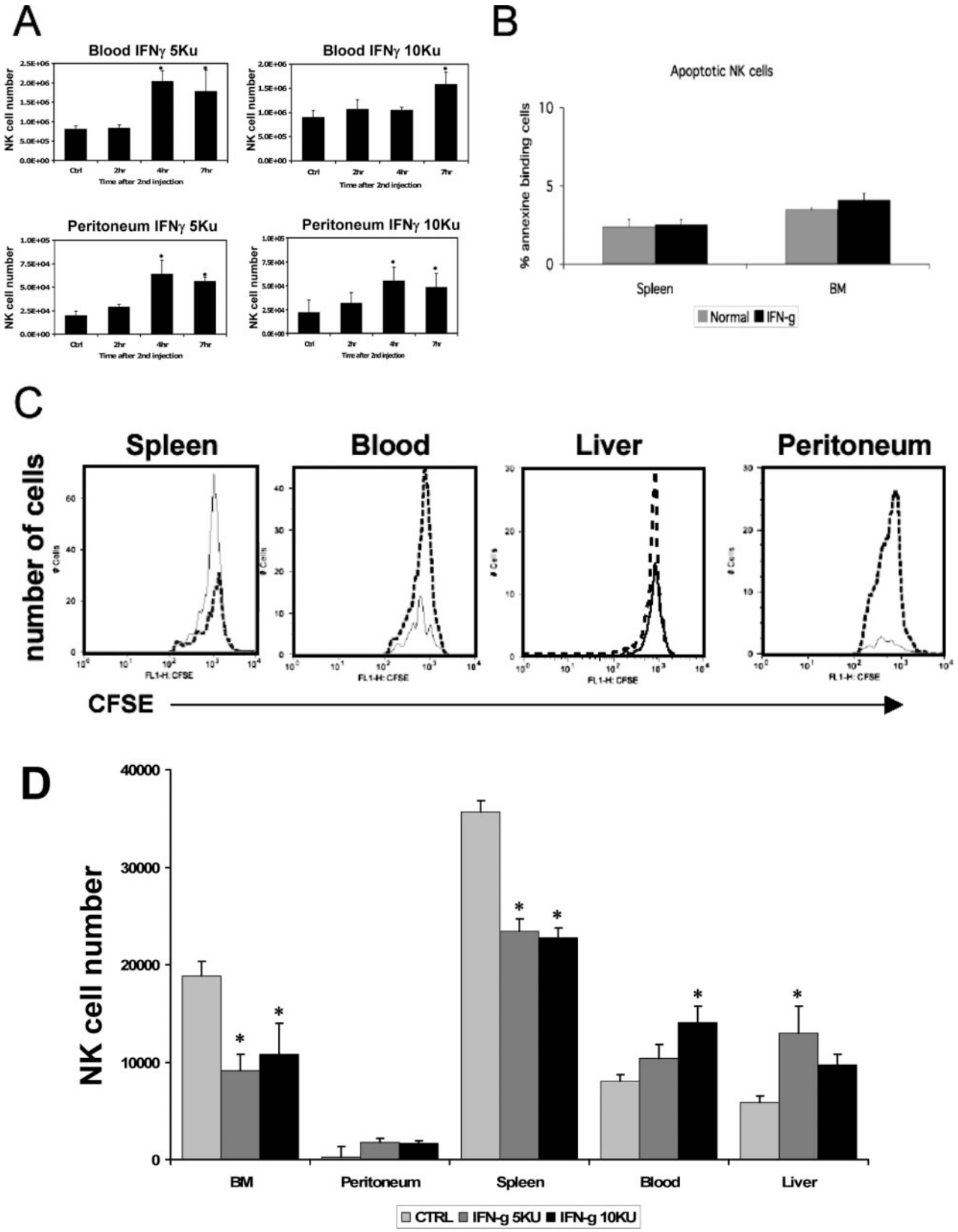

FIGURE 5. After IFN-γ administration, CFSE-labeled NK cells are mobilized from the BM and spleen into the blood and further accumulated in the liver and peritoneum. *A*, Time-dependent NK cell mobilization to the blood and accumulation in the peritoneum after the second injection of 5,000 and 10,000 IU per mouse of IFN-γ. *B*, Apoptosis of NK cells in the BM and spleen, 3 h after the second injection of IFN-γ, was measured using annexin assay. *C* and *D*, CFSE-labeled splenocytes were injected into mice. Cell proliferation and tissue localization of CFSE-labeled NK cells were tested 7 h after the second injection of IFN-γ. The number and fluorescence intensity of CFSE-labeled spleen, blood, liver, and peritoneum-derived NK cells before (solid line) and after IFN-γ treatment (dotted line) is shown in *C*. The number of CFSE-labeled NK cells in the BM, liver, spleen, peritoneum, and blood, before (CTRL) and after IFN-γ treatment (5,000 IU per mouse (5KU), 10,000 IU/mouse (10KU)) is shown in *D*. The results are the average ± SE of three experiments. For each time point, 9–12 mice were analyzed. (∗, $p < 0.05$).

As previously shown in Fig. 1, $CFSE^+NK1.1^+CD3^-$ cells were mobilized from the spleen and BM and accumulated in the blood and the peritoneum (Fig. 5, *C* and *D*). Surprisingly, we found that, in addition to their accumulation in the peritoneum, NK cells also accumulated in the liver of these mice (Fig. 5, *C* and *D*). Because the fluorescence intensity of $CFSE^+NK1.1^+CD3^-$ NK cells accumulating in the blood, peritoneum, and liver after IFN-γ treatment is similar to that observed in $CFSE^+NK1.1^+CD3^-$ NK cells derived from the organs of untreated mice, we concluded that the accumulation of cells in these organs was due to cell trafficking rather than cell proliferation (Fig. 5, *C* and *D*).

T cells are required for IFN-γ-induced mobilization of NK cells from the BM and spleen

IFN-γ induces the mobilization of NK cells, but not of T cells from the BM and spleen. These results may suggest a direct effect of IFN-γ on NK cells. To further analyze this hypothesis, we injected 10,000 IU per mouse of IFN-γ i.p. into SCID mice and into their control BALB/c mice and tested the mobilization of NK cells. Administration of IFN-γ into BALB/c mice induced the mobilization of NK cells from both spleen and BM (Fig. 6*A*). However, the ability of IFN-γ to induce the mobilization of NK cells from the spleen was attenuated in comparison to that observed in C57BL/6 mice (Fig. 6*A*). Surprisingly, we found that IFN-γ administration into SCID mouse failed to induce the mobilization of NK cells from the BM and spleen (Fig. 6*A*). SCID mice are depleted from T and B cells. To test and substantiate the role of T cells in the process of mobilization, we adoptively transferred purified $CD3^+$ spleen T cells from BALB/c mice into SCID mice. Transplantation of $CD3^+$ T cells into SCID mice restored their ability to mobilize NK cells in response to IFN-γ (Fig. 6*A*). To further study the role of T cells in the mobilization of NK cells, we depleted $CD3^+$, $CD4^+$, and $CD8^+$ T cells from C57BL/6 mice using Abs against these cell surface receptors (Fig. 6*B*). Depletion of CD3 cells completely inhibited the mobilization of NK cells. Near total depletion of $CD4^+$ cells inhibited ~80% of NK cell

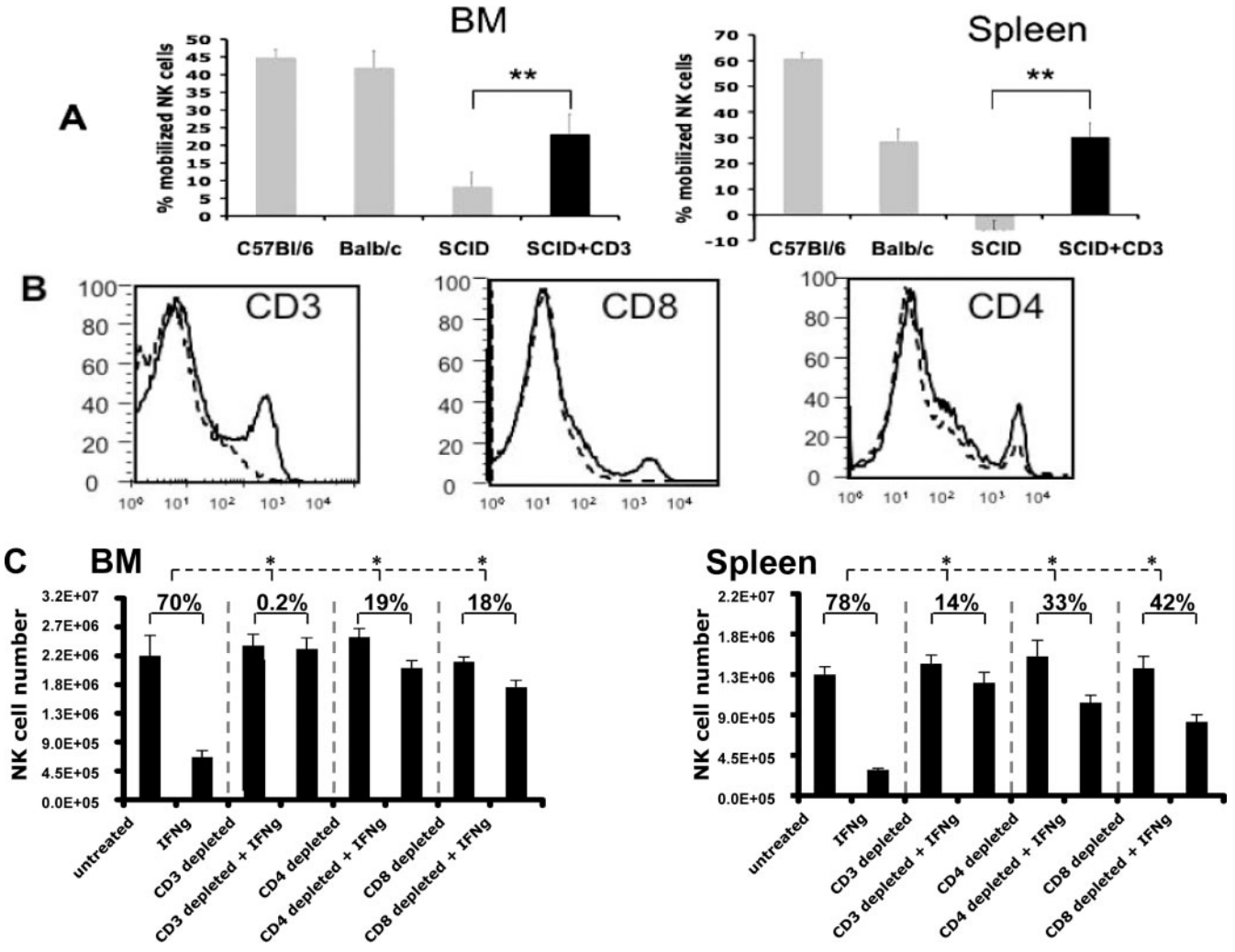

FIGURE 6. T cells in the spleen and BM are essential for the mobilization of NK cells after IFN-γ administration. *A*, IFN-γ (10,000 IU per mouse)-induced NK mobilization was conducted in C57BL/6, BALB/c, and SCID mice on the genetic background of BALB/c. Percentage of mobilized NK cells of total NK cells is shown. Purified $CD3^+$ T cells were transplanted into SCID mice, and the number of mobilized cells was tested after IFN-γ administration. The results are the average ± SE of two experiments. For each time point, 12 mice were analyzed (**, $p < 0.01$). *B* and *C*, $CD3^+$, $CD4^+$, and $CD8^+$ cells were depleted from C57BL/6 mice using Abs against these receptors. Expression of CD3, CD4, and CD8 Ags on spleen cells before (solid line) and after (dotted line) depletion is shown in *B*. The number of spleen and BM NK cells in mice depleted of T cells and treated with IFN-γ is shown in *C*. The percentage of mobilized NK cells for each treatment also is shown in *C*. Significant reduction in NK cell mobilization is shown for all treatments, compared with control untreated mice (dotted line). The results are the average ± SE of two experiments. For each time point, six to nine mice were analyzed. *, $p < 0.05$.

mobilization (Fig. 6*C*). Mobilization of NK cells also was partially inhibited by depletion of CD8 cells (Fig. 6*C*). These results suggest a critical role for both CD4 and CD8 T cells in the mobilization of NK cells after administration of IFN-γ.

Involvement of the IFN-γ-inducible chemokines CXCL9 and CXCL10 and their receptor CXCR3 in the mobilization of NK cells from the BM and spleen

The IFN-γ-inducible, CXCR3 chemokine receptor ligands, CXCL10 and CXCL9, play a role in the recruitment of NK cells to the inflamed liver (6, 7, 9). Moreover, it was reported that CXCR3-mediated trafficking of NK cells to draining lymph nodes (LN), is critical for induction of Th1 response (20). To study the role of CXCR3 in the IFN-γ-dependent mobilization of NK cells from the spleen, IFN-γ (10,000 IU per mouse) was injected into the peritoneum of CXCR3 KO mice and control mice. IFN-γ failed to stimulate the mobilization of NK cells from the spleen of CXCR3 KO mice. In contrast, it induced the mobilization of NK cells from the BM into the periphery (Fig. 7*A*).

Further evidence for the role of CXCR3 in the mobilization of NK cells from the spleen was found when we tested the effect of IFN-γ-inducing factors, IL-18 on NK cell mobilization. IL-18 is a potent NK cell activator (21–24). Intraperitoneal administration of IL-18 into C57BL/6 control mice induced NK cell mobilization from the BM and spleen similar to the mobilization induced by IFN-γ (Fig. 7, *B* and *C*). In contrast, administration of IL-18 into IFN-γ KO mice failed to induce NK cell mobilization from the BM and induced a significantly reduced mobilization of NK cells from the spleen. In accordance with the results obtained with IFN-γ administration into CXCR3 KO mice, the IL-18-induced mobilization of NK cells from the spleen, but not from the BM, was mainly dependent on CXCR3 (Fig. 7, *B* and *C*). In summary, IL-18-induced NK cell mobilization from the spleen in an IFN-γ/CXCR3-dependent pathway. In contrast, BM-resident NK cells were mobilized by IL-18/IFN-γ pathway in a CXCR3-independent way.

BM- and spleen-resident NK cells differ in CXCR3 expression and in migration to its ligands

The IFN-γ/CXCR3 pathway is critically involved in the mobilization of NK cells from the spleen, but not from the BM. It is therefore possible that BM- and spleen-resident NK cells differ in their expression of CXCR3 or in their responsiveness to the CXCR3 ligands, CXCL9 and CXCL10.

To address this issue, BM- and spleen-resident NK cells ($NK1.1^+CD3^-$) were stained for CXCR3. The percentages of BM- and spleen-derived NK cells that express CXCR3 are 60 and 45%, respectively. BM-resident NK cells express 4-fold higher

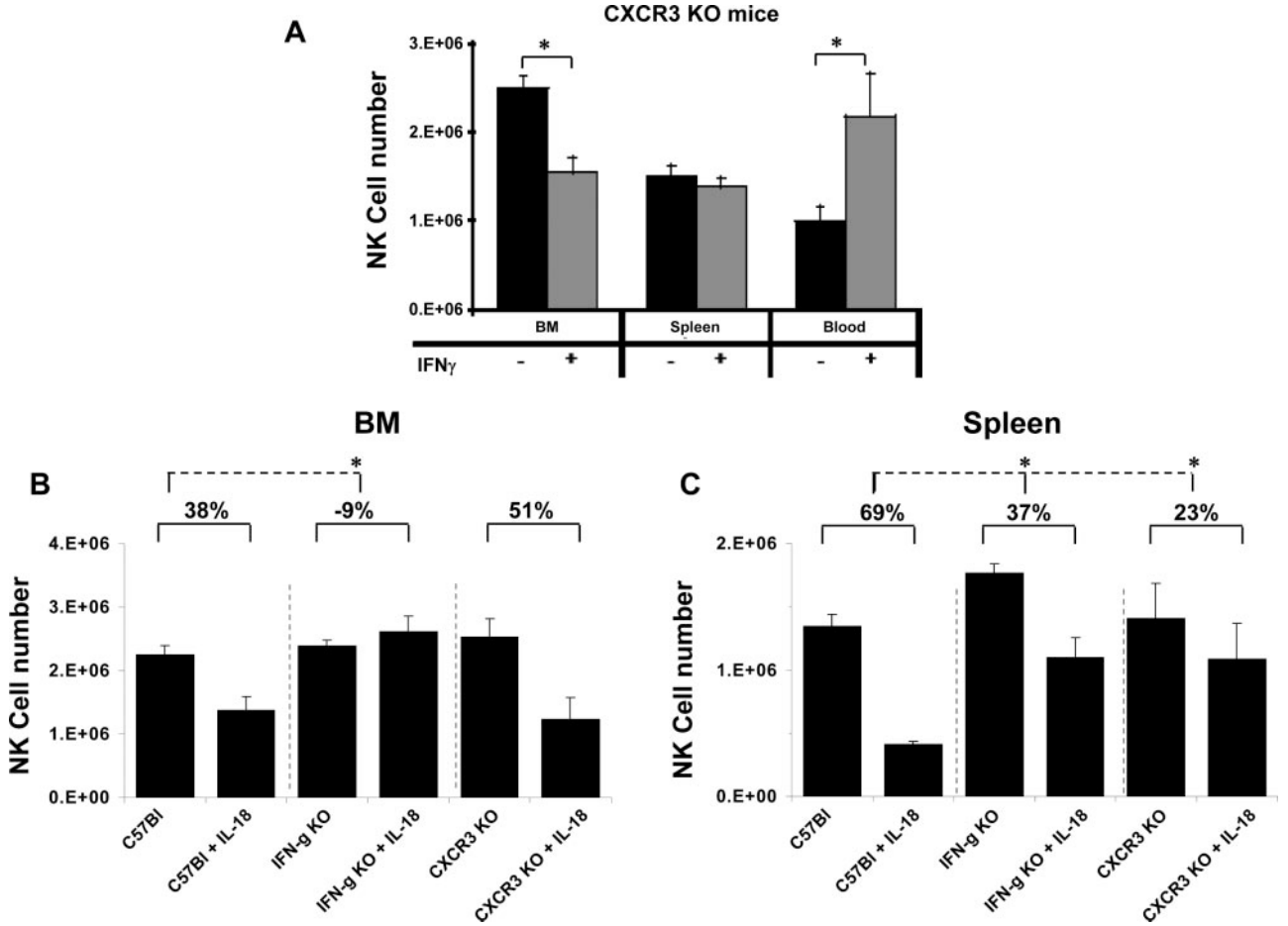

FIGURE 7. Administration of IL-18 and IFN-γ induces CXCR3-dependent mobilization of spleen NK cells. *A*, IFN-γ-induced mobilization of NK cells in CXCR3 KO was studied. The numbers of NK cells in the BM, spleen, and blood after IFN-γ injection is shown (■, nontreated mice; (▨ bar, IFN-γ-injected mice). *B* and *C*, IL-18-induced mobilization of NK cells in C57BL/6 mice, IFN-γ KO mice, and CXCR3 KO mice was studied. The number of NK cells in the spleen (*B*) and BM (*C*) after IL-18 injection is shown. The percentage of mobilized NK cells for each treatment also is shown in *B* and *C*. Significant reduction in NK cell mobilization is shown for IFN-γ and CXCR3 KO mice, compared with control C57BL mice (dotted line). The results are the average of two experiments. For each strain of mice, six to nine mice were analyzed. (*, $p < 0.05$; **, $p < 0.01$).

levels of CXCR3 (mean fluorescence intensity = 48) than spleen NK cells (mean fluorescence intensity = 11.5) (Fig. 8*A*). Furthermore, BM-derived NK cells also express variable levels of CXCR3, compared with the uniform levels of CXCR3 expressed by spleen NK cells (Fig. 8*A*). In correlation with CXCR3 levels of expression, freshly isolated BM-derived NK cells migrated better than spleen-derived NK cells in response to various concentrations (50–500 ng/ml) of CXCL10 and CXCL9 (Fig. 8*B*). We found that, 3 h after in vivo administration of 10,000 IU IFN-γ, the migration of BM-resident NK cells in response to CXCL9 and CXCL10 was dramatically reduced. Interestingly, IFN-γ stimulated spleen-resident NK cell migration in response to CXCL9, but not to CXCL10 (Fig. 8*B*).

The differential response of spleen- and BM-derived NK cells in response to CXCL9 and CXCL10 was further tested in vitro by incubating the BM and spleen cells for 3 h in the presence of IFN-γ. In accordance with the in vivo results, we found that, after incubation with IFN-γ, the migration of spleen NK cells in response to CXCL9 was increased, while migration in response to CXCL10 is decreased (Fig. 8*C*). BM-derived NK cell migration in response to CXCL9 and CXCL10 was reduced (Fig. 8*C*). These results suggest that, after IFN-γ stimulation, CXCL9, but not CXCL10, plays a key role in the mobilization of spleen NK cells. In support of these findings, we have found that, after IFN-γ injection, the levels of CXCL9 in the blood and liver increased dramatically compared with CXCL10 (Fig. 8*D*). To further characterize the role of local production of CXCL9 in the BM and spleen on CXCR3 function, we injected IFN-γ (10,000 IU per mouse i.p.) and tested for CXCL9 expression in these organs. In addition, spleen- and BM-derived cells were incubated in vitro with IFN-γ (125 IU/ml for 24 h) and tested for CXCL9 levels in the supernatant. We found that both in vitro and in vivo treatment with IFN-γ induced significantly higher levels of CXCL9 than CXCL10 in the BM and the spleen (Fig. 8, *E* and *F*). It was demonstrated for T cells that of the three CXCR3 ligands, CXCL11 and CXCL10 were the most potent inducers of CXCR3 internalization, whereas CXCL9 induced only a poor response (25). In accordance, we found that 3 h after in vivo administration of 10,000 IU IFN-γ, the levels of CXCR3 cell surface expression on BM and spleen NK cells was almost unchanged (Fig. 8*A*). Overall, it is suggested that

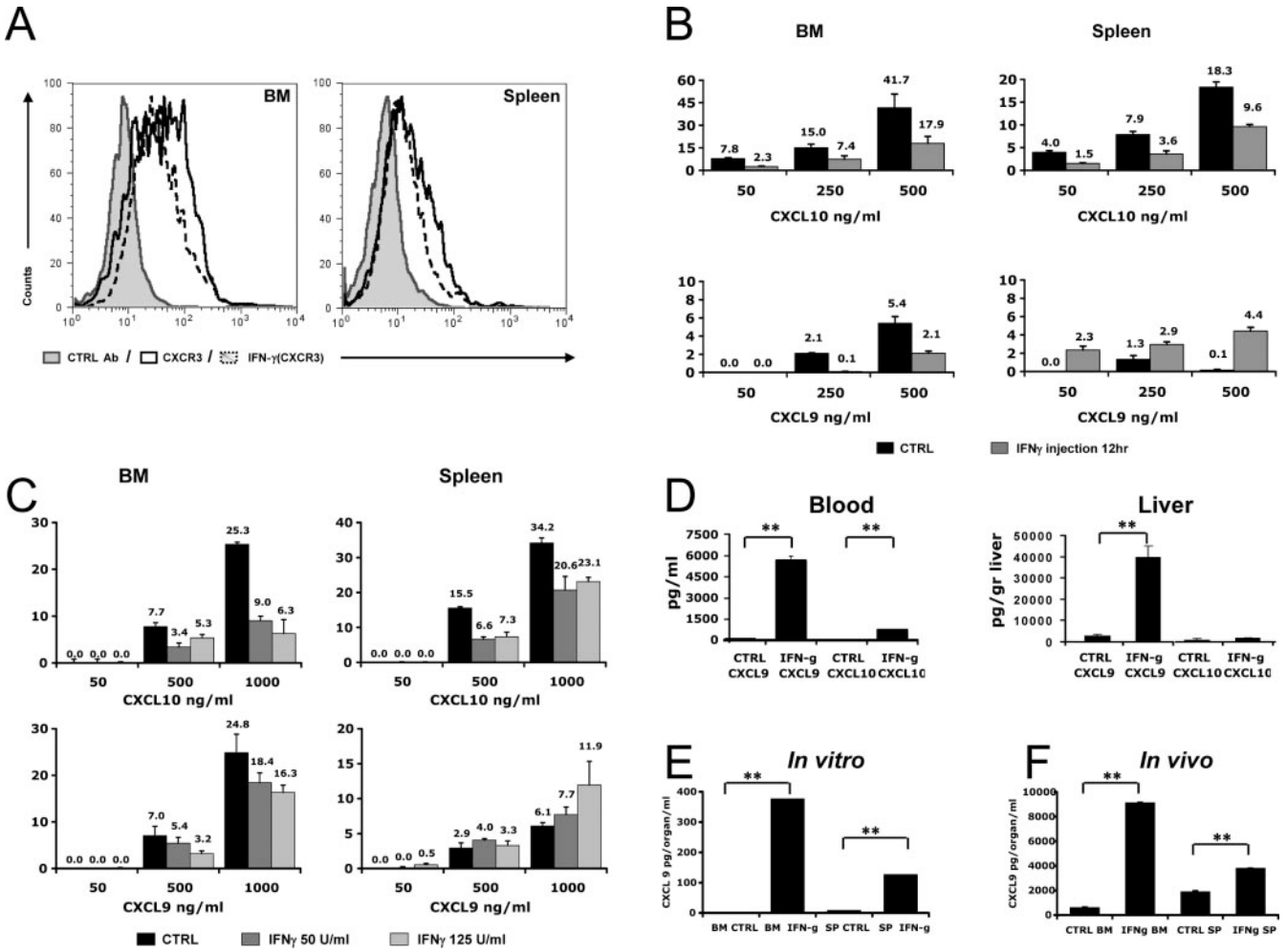

FIGURE 8. BM and spleen resident NK cells differ in CXCR3 expression and in migration to its ligands. *A*, CXCR3 expression by BM- and spleen-resident NK cells (gray, CTRL Ab; black line, CXCR3; dashed black line, CXCR3, 3 h after IFN-γ injection). *B*, BM- and spleen-resident NK cells were collected 3 h after i.p. injection of 10,000 IU of IFN-γ to C57BL/6 mice. Migration to increasing concentrations of CXCL10 and CXCL9 was studied. NK cells from noninjected mice were used as controls. *C*, BM- and spleen-derived NK cells were collected and incubated with 50 and 125 IU/ml IFN-γ for 3 h. After incubation, migration to increasing concentrations of CXCL10 and CXCL9 was studied. Nontreated NK cells were used as controls. *D*, CXCL9 and CXCL10 levels in the serum and the liver 24 h postinjection with IFN-γ are shown (**, $p < 0.01$). *E*, Production of CXCL9 by BM-derived cells 24 h after IFN-γ (125 IU/ml) treatment in vitro (**, $p < 0.01$). *F*, Production of CXCL9 by BM and spleen cells 24 h after injection of IFN-γ (10,000 IU per mouse) (**, $p < 0.01$). The result represents one of three experiments performed. The results are the average ± SD. In *F*, in each time point, three mice were analyzed.

IFN-γ differentially regulates the secretion of the CXCR3 ligands, CXCL9 and CXCL10, from the liver, spleen and BM, and that this process may affect the ability of CXCR3 to induce NK cell mobilization from these organs and the recruitment of cells to the liver after IFN-γ administration.

IFN-γ-dependent mobilization of NK cells enhanced their antimetastatic effect

Administration of IL-12 was shown to inhibit systemic metastases of 3LL in an IFN-γ- and NK-dependent manner (26). NK cells play a role in reducing metastasis. To test the ability of NK cells to inhibit the development of metastases of 3LL, these cells were injected in low numbers (10^5 per mouse) i.v. into mice. Mice were treated weekly with PBS, anti NK1.1, or IgG control Abs. Depletion of NK cells with NK1.1 Abs resulted in reduced survival due to tumor development (Fig. 9*A*). These results suggest that NK cells play a critical role in the eradication of 3LL metastatic cells within the lung.

To test the role of IFN-γ-dependent mobilization of NK cells in inhibiting the development of metastases of 3LL, these cells were injected in high numbers (10^6 per mouse) i.v. into mice. The transplanted mice were then treated twice with IFN-γ (10,000 IU per mouse), 6 and 17 h after 3LL cell delivery. Every 4 days, IFN-γ was injected twice, and the survival of treated and nontreated mice

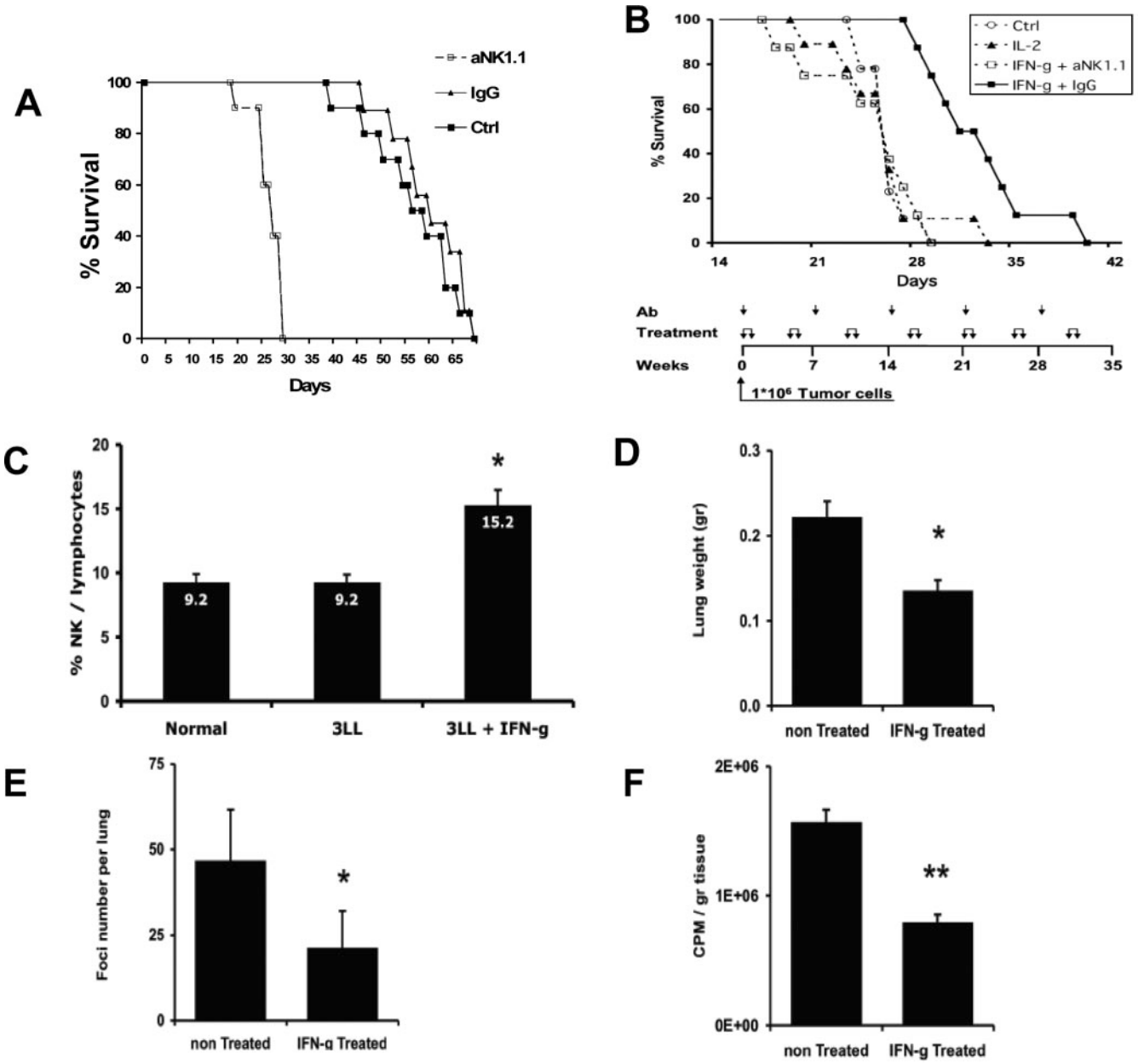

FIGURE 9. NK cell mobilization prolongs survival and reduces metastasis of mice injected with 3LL cells. *A*, Mice were injected with 105 3LL cells and treated with PBS (CTRL), anti-NK1.1, or IgG. Anti-NK1.1 Abs or IgG control Abs (Ab) were injected once a week. *B*, Mice were injected with 106 3LL cells and treated with PBS (CTRL), IL-2, IFN-γ plus anti-NK1.1, or IFN-γ plus IgG. Anti-NK1.1 Abs or IgG control Abs (Ab) were injected once a week. IFN-γ (10,000 IU per mouse) (treatment) was administered twice (at 0 and 17 h later) every 4 days. Human IL-2 (10,000 IU per mouse) (treatment) was administered once every 4 days. The survival of mice was monitored ($n = 10$ mice per group). *C–F*, Mice were injected with 106 3LL cells and treated with IFN-γ as described above. Four weeks after injection of 3LL cells, the mice were sacrificed, and their lungs were collected. Percentage of NK cells (*C*), number of metastatic foci (*D*), lung weight (*E*), and ^{18}F-FDG uptake (*F*) are shown ($n = 10$ mice per group). *, $p < 0.05$; **, $p < 0.01$.

was tested (Fig. 9*B*). In the experimental groups, the relative contribution of NK cells to antitumor immune response was evaluated by depletion of NK cells with NK1.1 Abs, whereas injection of IL-2 and IFN-γ plus IgG was used as control (Fig. 9*B*). IFN-γ, but not IL-2, treatment resulted in a significantly extended survival of mice, this effect was dependent on NK cells (Fig. 9*B*). To further evaluate the effect of IFN-γ on the metastatic process, IFN-γ-treated and nontreated mice were sacrificed 4 wk after 3LL cell delivery, and the number of metastatic nodules, ^{18}F-FDG uptake, lung weight, and percentage of NK cells were measured. Treatment of mice with IFN-γ increased the percentage of NK cells within the metastatic lungs (Fig. 9*C*). Furthermore, IFN-γ treatment reduced the number of metastatic nodules as well as their size (Fig. 9*D*). The reduced number of metastatic nodules and their smaller size could be demonstrated by lower ^{18}F-FDG uptake and weight of the treated lungs (Fig. 9, *E* and *F*). Overall, these results suggest that, after IFN-γ-induced NK cell mobilization, NK cell numbers in the periphery increased, leading to better eradication of metastatic cells within the lung.

Discussion

NK cell trafficking after Con A-induced hepatitis

Con A-induced hepatitis is a model for immune cell-mediated liver inflammation and damage (15–18). We show that recruitment of large numbers of NK cells to the liver in Con A-induced hepatitis is associated with a significant reduction in the number of these cells in the BM and spleen. Using annexin dye and CFSE labeling of NK cells, we demonstrate that administration of Con A induces NK cell mobilization and accumulation in the liver, but not NK cell death or proliferation. A sequential process, including initial mobilization from storage organs to the circulation and then recruitment to the liver, is presented. Although mobilization of NK cells is affected by IFN-γ and CXCR3 expression, recruitment to the liver is influenced by CCR1 expression. Administration of Con A to mice resulted in T cell and macrophage activation. This has been shown to rapidly elevate levels of T cell- and macrophage-derived cytokines in both plasma and liver tissues. TNF-α and IFN-γ, the cytokines known to mediate damage after Con A administration, were rapidly induced. Peak TNF-α expression in plasma and liver tissues was detectable within the first hr after challenge and gradually declined. In contrast, the levels of IFN-γ maintained at high levels for the first 12 h after injection of Con A only then gradually declined (18).

Our results suggest a critical role of TNF-α and CCR1, but not of IFN-γ, in the trafficking of NK cells to the liver. In contrast, mobilization of NK cells from the BM and spleen is partially dependent on the presence of IFN-γ and may be due to the persistence of high levels of this cytokine in the blood. Indeed, we found that mobilization of NK cells after one instillation of IFN-γ (20% mobilization; data not shown) is significantly inferior to a sequential administration of IFN-γ (60% mobilization).

Our results regarding the function of CCR1 in the recruitment of NK cells to the liver are in accordance with the reported role of CCL3, the ligand for CCR1, in the recruitment of NK cells to the liver during MCMV infection and with the reported role of CCR1 in the recruitment of NK cells to the lung during pulmonary granuloma formation (7, 27). Nevertheless, CCR1 is not involved in the mobilization of NK cells from storage organs. Unlike CCR1, we have found that CXCR3 is involved in Con A-induced NK cell mobilization from the spleen.

Sequential administration of IFN-γ induces NK cell mobilization

Because multiple cytokines and chemokines are produced during Con A-induced hepatitis, it was difficult to characterize the role of specific proinflammatory factors in this model (18, 28). IFN-α and IFN-γ were shown to be critical for NK cell trafficking to the liver during viral infections. Considering the role of these cytokines in the trafficking of NK cells, we now show their role in the mobilization of NK cells from their storage organs, the BM and spleen, and into the circulation and target organs, such as the liver and metastatic tumors. We found that administration of IFN-γ and IL-18, but not IFN-α, stimulated the mobilization of NK, but not T, cells from the BM and spleen into the circulation.

The doses of IFN-γ used to induce NK cell mobilization are similar to those used for treatment of cancers in humans. In studies conducted for the treatment of bladder cancer recurrence, 2.14×10^5 IU/kg was used per each instillation (29). In our study, we have used 4×10^5 IU/kg per each instillation. Furthermore, 8 h after injection of IFN-γ (4×10^5/kg), we found 610 pg/ml IFN-γ in the plasma of injected mice. Eight hours after Con A-induced hepatitis, the levels of IFN-γ in the plasma of injected mice reached 4000 pg/ml IFN-γ (18). These results suggest that the levels of IFN-γ that we used to induce NK mobilization are in the range of physiological doses that can be achieved by Con A administration. Using annexin dye and CFSE labeling of NK cells, we show that administration of IFN-γ i.p. induces NK cell mobilization into the circulation and their accumulation in the peritoneum, liver, and tumor metastatic sites, but not NK cell death or proliferation.

IL-18-induced mobilization of NK cells was mainly dependent on the production of IFN-γ. This finding is in accordance with the well-documented effect of IL-18 on IFN-γ production (30). IL-18- and IFN-γ-induced mobilization of spleen NK cells, but not BM NK cells, also was dependent on the expression of the chemokine receptor CXCR3. The chemokine receptor CXCR3 and its ligand CXCL9 were already shown to play a role in the recruitment of NK cells to the inflamed liver. Furthermore, it was reported recently that the chemokine receptor CXCR3-mediated trafficking of NK cells to draining LN, was critical for induction of Th1 response. NK cell depletion and reconstitution experiments showed that NK cells provide an early source of IFN-γ that is necessary for Th1 polarization (20). The IL-18/IFN-γ/CXCR3-dependent NK cell mobilization from the spleen may increase the number of NK cells in the periphery that are capable of responding to CXCR3 ligand released from the LN. In support of this hypothesis, we found that in BALB/c mice, NK cell mobilization from the spleen, but not from the BM, was significantly attenuated (50% reduction). This phenomenon may contribute to the reduced NK cell-dependent Th1 response in these mice. Overall, our results describe a novel role for the chemokine receptor CXCR3 in regulating the mobilization of mature NK cells from the spleen into the circulation.

The BM is the main site for NK cell production, and the heterogeneity of BM resident immature and mature NK cells is considerably high, compared with spleen and peripheral mature NK cells (1, 31). In accordance with these studies, we found that BM- and spleen-resident NK cells differ in their CXCR3 expression and function. BM-derived NK cells express higher and variable levels of CXCR3, whereas spleen NK cells express lower, but uniform levels of CXCR3. In correlation with CXCR3 levels of expression, freshly isolated BM-derived NK cells migrate better in response to CXCL10 and CXCL9 than spleen-derived NK cells. Differences

between BM- and spleen-derived NK cells are even more prominent after stimulation with IFN-γ. The migratory response of BM-derived NK cells to CXCL9 and CXCL10 was reduced after IFN-γ stimulation in vitro and in vivo. In contrast, spleen-derived NK cells increased their migration in response to CXCL9 after IFN-γ stimulation. High levels of CXCL9 produced in the BM microenvironment, compared with the spleen after IFN-γ stimulation (Fig. 8, *D* and *F*) may desensitize CXCR3 and/or may decrease the gradient of CXCL9 between the blood and BM microenvironment. Furthermore, CXCL9, but not CXCL10, levels in the circulation are dramatically elevated after IFN-γ injection. It is therefore possible that, in the setting of increased in vivo IFN-γ production, spleen NK cells are induced to respond to CXCL9 by mobilization and may therefore increase the numbers of circulating NK cells and augment the innate and adoptive immune response.

Administration of IFN-γ into SCID mice that are depleted from T and B cells failed to induce the mobilization of NK cells from the BM and spleen. Furthermore, depletion of $CD3^+$, $CD4^+$, and $CD8^+$ T cells from C57BL/6 mice inhibited the IFN-γ-induced mobilization of NK cells, thus suggesting a critical role for T cells in this process. Mobilization of subpopulations of immune cells is a complex process involving the interaction between different cells, cytokines, chemokines, adhesion molecules, and proteases (32). Similar to the involvement of T cells in IFN-γ-dependent mobilization of NK cells, it was demonstrated that G-CSF-induced mobilization of hemopoietic stem cells from the BM was mediated by neutrophils. More specifically, it was shown that elastase secreted by activated neutrophils modulated the CXCL12/CXCR4 chemokine/chemokine receptor axis to stimulate the mobilization of hemopoietic stem cells from the BM (33). The adhesion molecules and protease involved in the release of NK cells from their storage organs are yet to be identified.

Increased numbers of NK cells in the periphery is associated with better eradication of 3LL metastatic cells

IL-12 and IL-18 are associated with expression of the IFN-γ CXCL10 and CXCL9 genes and proteins in preclinical animal models for immunotherapy of tumors and in models of viral infection (34). In syngeneic A/J mice, SCK murine mammary carcinoma cells expressing mIL-18 and mIL-12 were less tumorigenic and formed tumors more slowly than control cells (35). Ab neutralization studies revealed that the antitumor effects of mIL-12 and mIL-18 required IFN-γ. Other studies have demonstrated that CXCL10 and CXCL9 are responsible, at least in part, for the antitumor effect of IL-12, mediated through IFN-γ (36). IFN-γ-inducible chemokines CXCL10 and CXCL9 expression in the tumor, the liver, and the kidney were inversely correlated with tumor size. Furthermore, administration in vivo of neutralizing Abs to CXCL10 and CXCL9 substantially reduced the antitumor effects of IL-12 inoculated locally into the tumors (37, 38). Our results suggest that, after IFN-γ induction, CXCL9 contributes to the antitumor effects of IL-12 not only through inhibiting angiogenesis, but also through stimulation of NK cell mobilization and further recruitment of these cells into the tumors. In support of this hypothesis, we found that, after IFN-γ administration, NK cell numbers in the periphery increased, leading to increased numbers of NK cells and better eradication of 3LL metastatic cells within the lungs of injected mice. In the setting of BMT, "alloreactive" NK cells are critical in eradicating acute myeloid leukemia and in preventing both graft rejection and graft-vs-host disease. We suggest that, by using IL-18 and IFN-γ for the deliberate mobilization of NK cells to the circulation, novel protocols for collection of alloreactive NK cells could be developed. Furthermore, the new mechanistic insights regarding the role of IFN-γ in the regulation of NK cell mobilization from the BM and spleen may lead to the development of new therapeutic approaches to augment antiviral and antitumor immunotherapy.

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Disclosures

The authors have no financial conflict of interest.

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