Monocytes control natural killer cell differentiation to effector phenotypes

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Natural killer (NK) cells play a major role in immunologic surveillance of cancer. Whether NK-cell subsets have specific roles during antitumor responses and what the signals are that drive their terminal maturation remain unclear. Using an in vivo model of tumor immunity, we show here that CD11b^{hi}CD27^{low} NK cells migrate to the tumor site to reject major histocompatibility complex class I negative tumors, a response that is severely impaired in *Txb21*$^{-/-}$ mice. The phenotypical analysis of *Txb21*-deficient mice shows that, in the absence of *Txb21*, NK-cell differentiation is arrested specifically at the CD11b^{hi}CD27^{hi} stage, resulting in the complete absence of terminally differentiated CD11b^{hi}CD27^{low} NK cells. Adoptive transfer experiments and radiation bone marrow chimera reveal that a *Txb21*$^{+/+}$ environment rescues the CD11b^{hi}CD27^{hi} to CD11b^{hi}CD27^{low} transition of *Txb21*$^{-/-}$ NK cells. Furthermore, in vivo depletion of myeloid cells and in vitro coculture experiments demonstrate that spleen monocytes mediate the terminal differentiation of peripheral NK cells in a *Txb21*- and IL-15Rα–dependent manner. Together, these data reveal a novel, unrecognized role for *Txb21* expression in monocytes in promoting NK-cell development and help appreciate how various NK-cell subsets are generated and participate in antitumor immunity. (*Blood*. 2011; 117(17):4511-4518)

Introduction

Natural killer (NK) cells are innate lymphocytes that participate in the regulation of immune responses and in immunologic surveillance of cancer.[1] NK cells develop mainly in the bone marrow (BM) and are distributed throughout the body in both lymphoid and nonlymphoid tissues.[2] It is unclear, however, whether this wide distribution is the result of their recirculation, the existence of NK subsets with different homing capacities, or their development at multiples sites.

Surface markers and gene expression profiles have been used to define various NK-cell subpopulations. Based on the expression of the integrin CD11b and the tumor necrosis factor receptor superfamily molecule CD27, mature murine NK cells have been classified into distinct populations.[3] The precursor/product relationship between these subsets has been investigated using a number of approaches to conclude that CD11b^{low}CD27^{low}, CD11b^{low}CD27^{hi}, CD11b^{hi}CD27^{hi}, and CD11b^{hi}CD27^{low} represent discrete and sequential stages of in vivo maturation following the pathway double-negative to CD11b^{low}CD27^{hi} to double-positive to CD11b^{hi}CD27^{low}.[4] Functional features of CD11b^{hi}CD27^{hi} and CD11b^{hi}CD27^{low} NK cells have been studied mostly in vitro. For example, CD11b^{hi}CD27^{hi} NK cells responded to either IL-12 or IL-18, key cytokines derived from antigen-presenting cells, by rapidly producing interferon-γ (IFN-γ), whereas CD11b^{hi}CD27^{low} NK cells produced IFN-γ only when stimulated with a combination of IL-12 and IL-18. Furthermore, CD11b^{hi}CD27^{hi} NK cells showed greater responsiveness to dendritic cells (DCs), producing higher amounts of IFN-γ on in vitro coculture with BM-derived DCs, compared with CD11b^{hi}CD27^{low} cells.[3] Whether NK-cell subsets have specific roles during immune responses and what the signals are driving the final stages of differentiation remain unknown.

T-bet is a tyrosine and serine phosphorylated protein belonging to the T-box family specifically expressed in the hematopoietic cell compartment.[5] T-bet is responsible for direct transactivation of the IFN-γ gene on CD4 Th1 T cells[6] and specifies a transcriptional program that imprints homing of T cells to proinflammatory sites.[7] T-bet is also expressed in NK cells, where it plays a role in maturation and homeostasis.[8] Functionally, *Txb21*$^{-/-}$ NK cells exhibit a small decrease in prototypic target cell killing, consistent with a modest decrease in IFN-γ, perforin, and granzyme B.[8] It remains to be established whether the phenotype, function, and anatomic distribution of NK-cell subsets is modulated by T-bet and whether extrinsic signals complement the defective NK-cell development reported in *Txb21*-deficient mice.

In the past few years, relevant interactions occurring between NK cells and cells of the monocyte/macrophage/DC lineages have been extensively investigated. In vitro studies have shown that the NK-cell crosstalk with DC results in activation of both NK cells and DCs.[9-11] In addition, the transient in vivo depletion of DCs results in lack of homeostatic proliferation of NK cells in lymphopenic conditions, which is mediated by DC-derived IL-15.[12] Functional interactions between NK cells and DCs during viral infections have also been highlighted. For example, the presence of Ly49H^{+} NK cells is required to retain CD8α^{+} DCs in the spleen, whereas CD8α^{+} DCs are required for the expansion of Ly49H^{+} NK cells that occurs at the late stage of acute viral infection.[13] Moreover, NK cells limit viral-induced immunopathology by

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eliminating activated macrophages of the spleen,[14] and mutual activation of NK cells and monocytes contribute to the initiation and maintenance of immune responses at sites of inflammation.[15] Whether myeloid cells mediate NK-cell differentiation in peripheral lymphoid organs and whether the expression of T-bet in monocyte/macrophage/DC plays any role in these events remain largely unknown.

In this study, we show that CD11b^{hi}CD27^{low} NK cells are critically involved in the rejection of major histocompatibility complex (MHC) class I-negative tumors. The observation that this response is severely impaired in *Txb21*$^{-/-}$ mice that completely lack CD11b^{hi}CD27^{low} NK cells prompted us to investigate the signals involved in the terminal differentiation of NK cells. We demonstrate that the CD11b^{hi}CD27^{hi} to CD11b^{hi}CD27^{low} NK-cell transition depends on the interaction of NK cells with spleen monocytes. Mechanistically, we find that the expression of *Txb21* and IL-15Rα on monocytes is determinant to allow NK cells to proceed to the final stage of differentiation. Thus, our work dissects critical steps in peripheral NK-cell differentiation and demonstrates that the interaction between NK cells and monocytes could be relevant in antitumor immunity.

Methods

Mice

C57BL/6 and BALB/c mice were purchased from Harlan-United Kingdom. C57BL/6 and BALB/c *Txb21*$^{-/-}$ mice were purchased from Taconic Farms. The CD11c-DTR mice carry a transgene encoding the simian DT receptor (DTR)-gfp fusion protein under control of the murine CD11c promoter.[16] Transgenic mice designed for inducible depletion of CSF-1 receptor (CD115) expressing cells (MaFIA) have been described.[17] The transgene in MaFIA mice is under control of the *c-fms* promoter that regulates expression of the CSF-1 receptor. These mice express eGFP and a membrane-bound suicide protein composing the human low-affinity nerve growth factor receptor, the FK506 binding protein, and the cytoplasmic domain of Fas. AP20187 is a covalently linked dimer (Ariad Pharmaceuticals) that cross-links the FK506 binding protein region of the suicide protein and induces caspase 8–dependent apoptosis as described.[17] CD11c-DTR and MaFIA mice were originally obtained from The Jackson Laboratory. Mice were bred and maintained under sterile conditions in the Biologic Services Unit (New Hunts House) of King's College London. Mouse handling and experimental procedures were conducted in accordance with national and institutional guidelines for animal care and use.

Flow cytometric analysis

Cells were stained with the following antibodies: Pacific Blue–, or fluorescein isothiocyanate (FITC)–labeled (2C11); AlexaFluor-700–, FITC-, or phycoerythrin-labeled anti-CD11b (M1/70); phycoerythrin-labeled anti-CD27 (LG.3A10); FITC-labeled anti-Ly6G (1A8); FITC-labeled anti-IAb (AF6-120.1); FITC-labeled CD19 (1D3); biotinylated KLRG-1 (2F1); and FITC-labeled CD11c (HL3) from BD Biosciences; polyclonal goat anti–IL-15Rα, from R&D Systems; Alexa647- and FITC-labeled anti-NKp46 (29A1.4); phycoerythrin-labeled anti-CD115 (AFS98); and Alexa647-labeled anti–T-bet (4B10) from eBioscience. All samples were analyzed on a FACS-LSRII or sorted in a FACSAria (both BD Biosciences).

Short-term in vivo NK-cell differentiation assay

Txb21$^{-/-}$ spleen cells were labeled with 2.5μM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Invitrogen), and 25×10^6 cells were injected intravenously into *Txb21*$^{+/+}$ or *Txb21*$^{-/-}$ mice. The expression of CD27 and CD11b in NKp46$^+$CD3$^-$ NK cells was evaluated 48 hours after transfer.

BM chimera

Recipient mice were irradiated with 600 cGy 8 and 4 hours before the procedure. BM cells were harvested aseptically from the tibia and femurs of donor *Txb21*$^{+/+}$ and *Txb21*$^{-/-}$ mice, and 2×10^6 cells each were coinjected intravenously in the recipients. Irradiated mice were maintained on trimethaprim-sulphamethoxazole–treated water in sterile cages for 6 weeks before analysis.

In vivo modulation of myeloid cell numbers

For depletion of myeloid cells, 2 alternative approaches were used. First, 250 μL of clodronate-loaded liposomes was injected intravenously into C57BL/6 mice. Clodronate was a gift from Roche and was incorporated into liposomes as previously described.[18] The doses used fully eliminate blood monocytes[19] and splenic and liver macrophages.[18] Second, CD115 MaFIA mice were injected with dimerizer AP20187, a gift from Ariad Pharmaceuticals. Lyophilized AP20187 was dissolved in 100% ethanol at a concentration of 13.75 mg/mL (1nM) stock solution and stored at −20°C. MaFIA mice received 0.55 mg/mL of AP20187 containing 4% ethanol, 10% PEG-400, and 1.7% Tween-20 in water. A total of 10 mg/kg AP20187 was administered intraperitoneally on days −4, −3, −2, and −1, and spleens were harvested and labeled as indicated 24 hours after the last injection. For monocyte and macrophage expansion, mice were injected with 10 μg CSF-1 for 5 consecutive days; mice were killed 24 hours after last injection.

NK-cell and monocyte coculture

Spleen NK cells were negatively enriched by an NK-cell isolation kit (Miltenyi Biotec). The purity of the sorted population was typically more than 90% DX5$^+$ cells. In some experiments, spleen NKp46$^+$CD3$^-$CD27$^+$ *Txb21*$^{-/-}$ NK cells were sorted using FACSAria. The purity of the sorted population was more than 98%. Monocytes were enriched with the StemCell kit and identified as CD11b$^+$ to a more than 95% purity (supplemental Figure 4C, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). In some experiments, mouse monocytes were identified as Lin$^-$ CD11b$^+$ and sorted with a FACSAria to a purity of more than 99%. Lineage cocktail contained anti-CD3/CD19/NKp46/MHC class II/CD11c/Ly6G (supplemental Figure 4D). NKp46$^+$CD3$^-$CD27$^+$ *Txb21*$^{-/-}$ NK cells, and monocytes were cocultured at a 1:1 ratio (1×10^5 each) in round-bottom 96-well plates in the presence of 100 ng/mL of mouse IFN-γ, IL-2, IL-15 cytokines or IL-15/IL15Rα complexes (R&D Systems). In some experiments, interaction between IL-15 and IL-15Rα was prevented by adding 5 μg/mL of anti-IL15Rα antibodies (R&D Systems) into cocultures. Human IL-15 (R&D Systems) was precomplexed with murine IL-15Rα-human IgG1-Fc fusion protein (R&D Systems) as described[20] and incubated at 1 μg/10^6 cells with *Txb21*$^{-/-}$ monocytes.

Immunofluorescence on tissue sections

Immunofluorescence was performed on 7-μm-thick serial frozen sections. Sections were fixed with acetone, saturated in phosphate-buffered saline containing 0.1% (volume/volume) Triton-X and 10% (volume/volume) donkey serum before staining with rat anti–mouse-CD115 (AFS98; eBioscience), purified polyclonal goat anti–mouse NKp46 (R&D Systems), and anti–CD3-APC (2C11; BD Biosciences), followed by secondary donkey anti–goat IgG–Alexa-488 (Invitrogen) and donkey anti–rat DLR546 (Jackson ImmunoResearch). After staining, slides were dried and mounted with Prolong Gold (Invitrogen) and examined with an LSM 510 confocal microscopy (Zeiss LSM Exciter). Pictures were acquired using Zen and LSM image browser software (Carl Zeiss).

Tumor clearance in vivo

Live tumor cells (2×10^5 RMA and 2×10^5 RMA-S) were labeled with 5μM 5,6-(4-chloromethyl)-benzoyl-1-amino-tetramethylrhodamine (CMTMR, Invitrogen) or 2.5μM CFSE, respectively, and injected intraperitoneally in a volume of 0.2 mL. Forty-eight hours later, peritoneal cells were recovered and analyzed by flow cytometry for CMTMR and CFSE

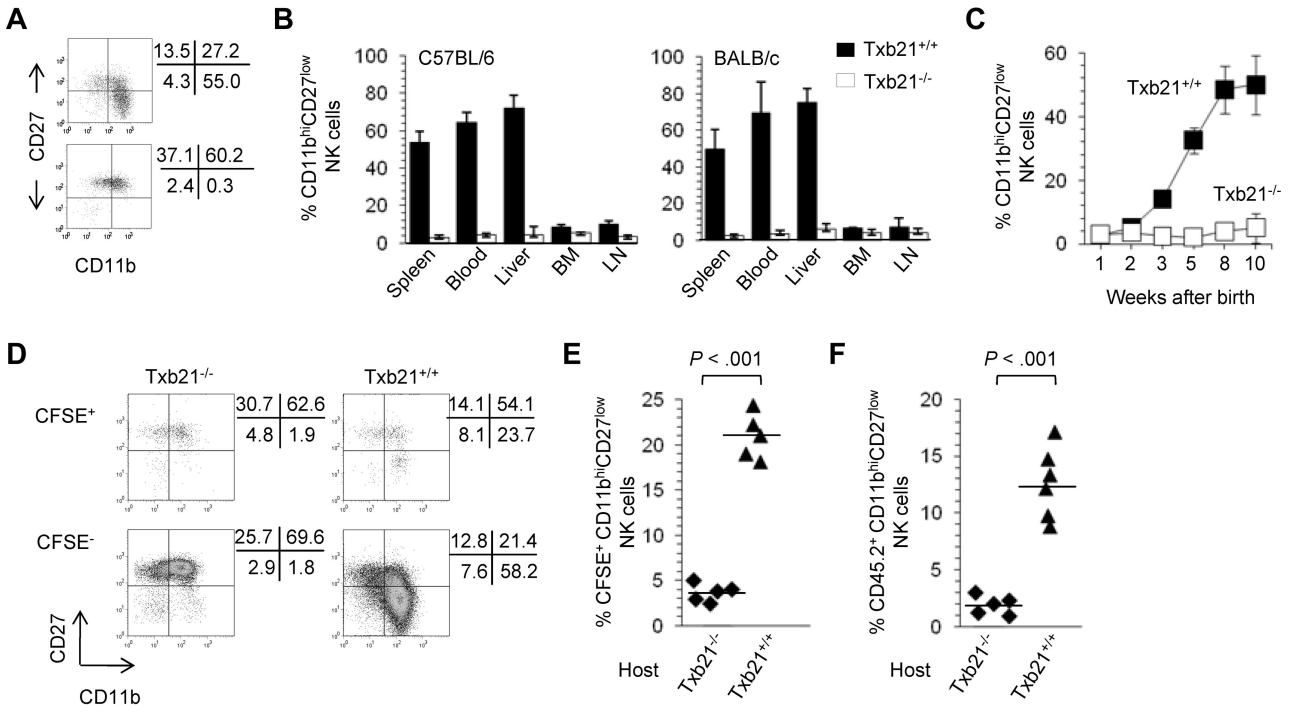

Figure 1. CD11b^{hi}CD27^{low} NK cells are absent in *Txb21*$^{-/-}$ mice but can be rescued in a *Txb21*$^{+/+}$ environment. (A) Expression of CD11b and CD27 in spleen NKp46$^+$CD3$^-$ NK cells of *Txb21*$^{+/+}$ (top panel) and *Txb21*$^{-/-}$ (bottom panel) C57BL/6 mice. (B) Frequency (mean ± SD) of CD11b^{hi}CD27^{low} NK cells of 6 mice per genotype in the indicated organs. (C) Frequency (mean ± SD) of CD11b^{hi}CD27^{low} NK cell of *Txb21*$^{+/+}$ and *Txb21*$^{-/-}$ C57BL/6 mice prepared from spleen of 5 mice per genotype at the ages indicated. (D) *Txb21*$^{-/-}$ spleen cells were labeled with CFSE and injected intravenously into *Txb21*$^{-/-}$ or *Txb21*$^{+/+}$ hosts. Shown is the expression of CD11b and CD27 in the transferred CFSE$^+$ (top panels) and host CFSE$^-$ (bottom panels) NKp46$^+$CD3$^-$ NK cells in *Txb21*$^{-/-}$ (left panels) and *Txb21*$^{+/+}$ (right panels) hosts. (E) Frequency of CFSE$^+$ CD11b^{hi}CD27^{low} NK cells in 5 *Txb21*$^{-/-}$ and 5 *Txb21*$^{+/+}$ mice. (F) Six *Txb21*$^{+/+}$ and 5 *Txb21*$^{-/-}$ CD45.1 host mice were irradiated and transferred with *Txb21*$^{-/-}$ CD45.2 BM cells. Shown is the frequency of donor CD45.2 CD11b^{hi}CD27^{low} NK cells. (E-F) Bars represent the means within the groups.

detection. In some experiments, tumor-bearing mice were injected intravenously with 5×10^6 of highly purified *Txb21*$^{+/+}$ or 5×10^6 *Txb21*$^{-/-}$ spleen NK cells. NK-cell depletion was achieved by injecting 200 μg intraperitoneally of anti-NK1.1 antibodies or isotype as controls, 2 days before tumor challenge.

Statistical analysis

Data, presented as mean ± SD, were analyzed with a paired Student *t* test using the SPSS software Version 17. $P < .05$ was considered significant.

Results

Defective NK-cell development in *Txb21*$^{-/-}$ mice is rescued by a *Txb21*$^{+/+}$ environment

Previous studies have shown that T-bet regulates differentiation and peripheral homeostasis of NK cells.[8] However, whether T-bet identifies checkpoints during NK-cell development in peripheral organs is unknown. To address this issue, we analyzed the expression of CD11b and CD27 in the NK cells of *Txb21*$^{+/+}$ and *Txb21*$^{-/-}$ mice. Figure 1A and supplemental Figure 1 show striking differences in the overall distribution of NK-cell subsets in 8- to 10-week-old *Txb21*$^{-/-}$ mice compared with *Txb21*$^{+/+}$ mice. Indeed, CD11b^{hi}CD27^{low} NK cells are virtually absent from the spleen of *Txb21*$^{-/-}$ mice. Furthermore, CD11b^{hi}CD27^{low} NK cells could not be detected in the blood and in any other lymphoid organ tested both in C57BL/6 and BALB/c backgrounds (Figure 1B). It has been reported that the proportion of CD11b^{hi}CD27^{low} NK cells increases with age.[21] To evaluate the role of T-bet in the ontogenetic appearance of CD11b^{hi}CD27^{low} NK cells, we next compared the development of NK cells in the spleen of *Txb21*$^{+/+}$ and *Txb21*$^{-/-}$ mice. We observed a gradual increase in the percentage of spleen CD11b^{hi}CD27^{low} *Txb21*$^{+/+}$ NK cells to reach a plateau at 8 weeks of age, with significant changes between 2 and 3, 4 and 5, and 5 and 8 weeks (Figure 1C). In sharp contrast, terminally differentiated CD11b^{hi}CD27^{low} *Txb21*$^{-/-}$ NK cells were never detected in the spleen (Figure 1C) and in any other organ tested (not shown), at any time point studied up to 2 months after birth.

Because T-bet is expressed in cells of the myeloid and lymphoid lineages,[5] we next assessed the role of NK cell–extrinsic *Txb21* in the generation of the CD11b^{hi}CD27^{low} NK-cell subset. We first used a short term in vivo differentiation assay, where CFSE-labeled *Txb21*$^{-/-}$ spleen cells were adoptively transferred into *Txb21*$^{+/+}$ or *Txb21*$^{-/-}$ mice, to evaluate the appearance of CD11b^{hi}CD27^{low} NK cells in the spleen of the host. Figure 1D-E shows that, 2 days after transfer, *Txb21*$^{-/-}$ CD11b^{hi}CD27^{low} NK cells can be readily detected in the spleen of *Txb21*-sufficient recipients, but not in *Txb21*-deficient recipients. We also performed competitive BM chimera experiments where lethally irradiated *Txb21*$^{+/+}$ hosts were reconstituted with a 1:1 mixture of *Txb21*$^{+/+}$ and *Txb21*$^{-/-}$ BM cells and confirmed that CD11b^{hi}CD27^{low} *Txb21*$^{-/-}$ NK cells can be detected in the blood and spleen of *Txb21*$^{+/+}$, but not *Txb21*$^{-/-}$, hosts 6 weeks after BM reconstitution (Figure 1F). Together, these data demonstrate that the NK-cell differentiation program has a checkpoint between CD11b^{hi}CD27^{hi} and CD11b^{hi}CD27^{low} stages that cannot be completed in the absence of T-bet and that environmental signals contribute to terminal NK-cell development.

Spleen monocytes promote NK-cell differentiation in a T-bet-dependent manner

The complex process of NK-cell differentiation occurs at several distinct tissue sites, including the BM, liver, thymus,[2] and lymph

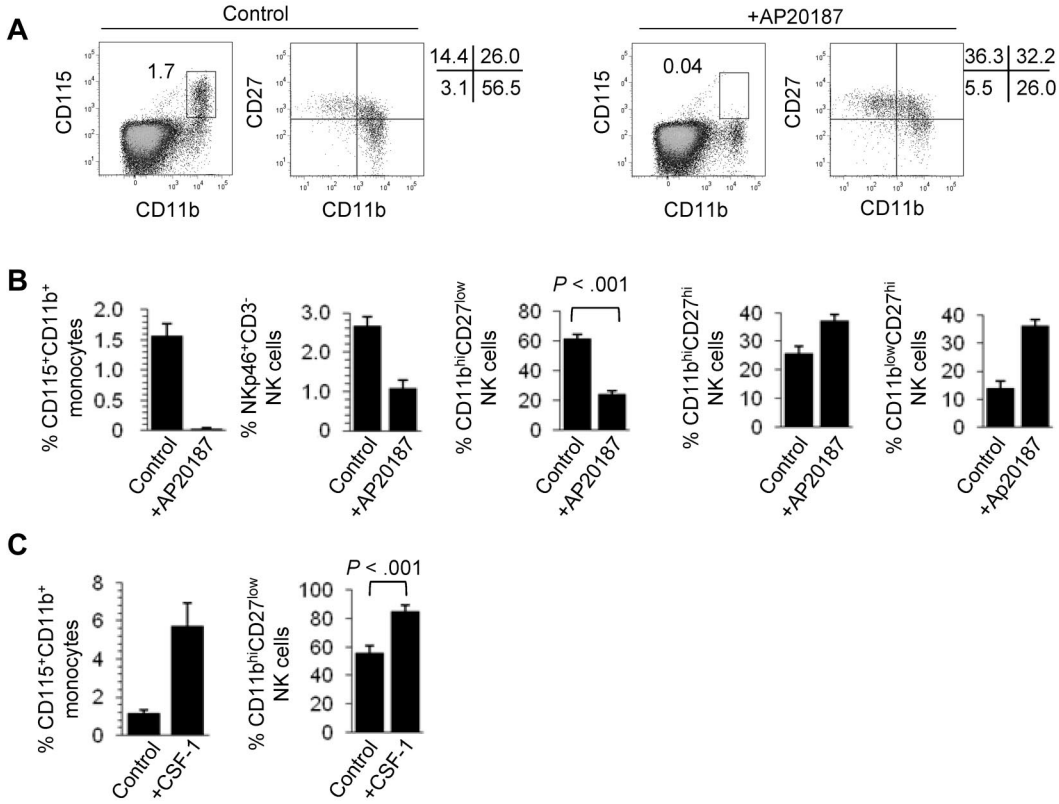

Figure 2. Monocytes control CD11b^{hi}CD27^{low} NK-cell numbers. (A) Frequency of CD115$^+$CD11b$^+$ monocyte and expression of CD11b and CD27 in NKp46$^+$CD3$^-$ NK cells in the spleen of control (left panels) and AP20187-treated (right panels) MaFIA mice. (B) Mean frequency ± SD of spleen CD115$^+$CD11b$^+$ monocytes, NKp46$^+$CD3$^-$ NK cells, CD11b^{hi}CD27^{low} NK cells, CD11b^{hi}CD27^{hi} NK cells, and CD11b^{low}CD27^{hi} NK cells, of 3 experiments with 4 mice per experiment. (C) CD115$^+$CD11b$^+$ monocyte and of CD11b$^+$CD27^{low} NK-cell frequency in the spleen of control C57BL/6 mice or mice injected with CSF-1. Data are mean ± SD of 2 independent experiments with 3 mice per group.

nodes and tonsils.[22] However, very little is known about the cellular mechanisms leading to the formation of distinct NK-cell subsets. To identify the cellular components and anatomic distribution that limit NK-cell differentiation in *Txb21*$^{-/-}$ mice, we adoptively transfer CFSE-labeled *Txb21*$^{-/-}$ cells into splenectomized mice (supplemental Figure 2A). The absence of CD11b^{hi}CD27^{low} NK cells in the blood of splenectomized mice strongly suggests that CD27^{hi} to CD27^{low} transition may occur in the spleen.

Next, we sought to identify the T-bet-expressing cells that contribute to NK-cell differentiation. T-bet is expressed on γc-dependent lymphocytes, such as conventional T cells, NKT cells, NK cells, γδT cells, and B cells[5] and on CD11c$^+$ DCs.[23] We hypothesized that the deletion of the cell component involved in NK CD27^{hi} to CD27^{low} step should result in changes in the overall distribution of NK-cell subsets in the spleen. Therefore, we used genetic models to investigate whether γc-dependent lymphocytes and DCs were involved in promoting NK-cell differentiation. We observed that the transition from CD27^{hi} to CD27^{low} stage readily occurs when *Txb21*$^{-/-}$ NK cells were transferred into Rag2$^{-/-}$ Il2rg$^{-/-}$ *Txb21*$^{+/+}$ mice (supplemental Figure 2B). Furthermore, the administration of diphtheria toxin, which induces a transient depletion of DCs in CD11c-DTR-gfp mice,[16] did not modify the spleen NK-cell subset composition (supplemental Figure 2C). These data indicate that DCs and γc-dependent lymphocytes are not required for NK terminal differentiation.

The red pulp of the spleen is a natural reservoir of mouse monocytes[24] and the niche where splenic NK cells reside in the steady state.[25] Because monocytes,[6] but not macrophages,[23] express T-bet, we next tested whether monocytes modulate NK-cell differentiation. To deplete in vivo mouse monocytes, we used 2 approaches. First, we analyzed the phenotype of splenic NK cells in MaFIA mice where transient elimination of CSF-1 receptor (CD115)-expressing cells is induced by administration of AP20187 dimerizer. Figure 2A shows that the complete elimination of CD115$^+$CD11b$^+$ monocytes in AP20287-treated mice correlates with a strong reduction in the proportion of CD11b^{hi}CD27^{low} NK cells. Although the overall proportion of NK cells was reduced in AP20187-treated MaFIA mice, the frequency of CD11b^{hi}CD27^{hi} and CD11b^{low}CD27^{hi} increased in treated animals (Figure 2B). This, together with the fact that AP20187 does not modify the NK-cell subset composition in wild-type animals (supplemental Figure 3A), rules out nonspecific toxic effects of this compound. Second, we injected intravenously clodronate-loaded liposomes into wild-type C57BL/6 mice. This treatment resulted in the depletion of spleen monocytes (supplemental Figure 3B) and was accompanied by a strong reduction in the percentage of CD11b^{hi}CD27^{low} spleen NK cells (supplemental Figure 3C). Clodronate-loaded liposome treatment did not affect the overall frequency of conventional T cells (supplemental Figure 3C) or CD19$^+$CD3$^-$ B cells (not shown). To further demonstrate the role for CD115$^+$ cells in NK-cell differentiation, we used a complementary approach that consists of expanding in vivo the number of monocytes and investigating the overall distribution of NK-cell subsets in the spleen. Figure 2C shows that administration of CSF-1 (M-CSF), the ligand for the CSF-R, into wild-type results in a marked increase of CD115$^+$CD11b$^+$ monocytes in the spleen, which is accompanied by a significant expansion of CD11b^{hi}CD27^{low} NK cells. Together, these experiments suggest that the presence of

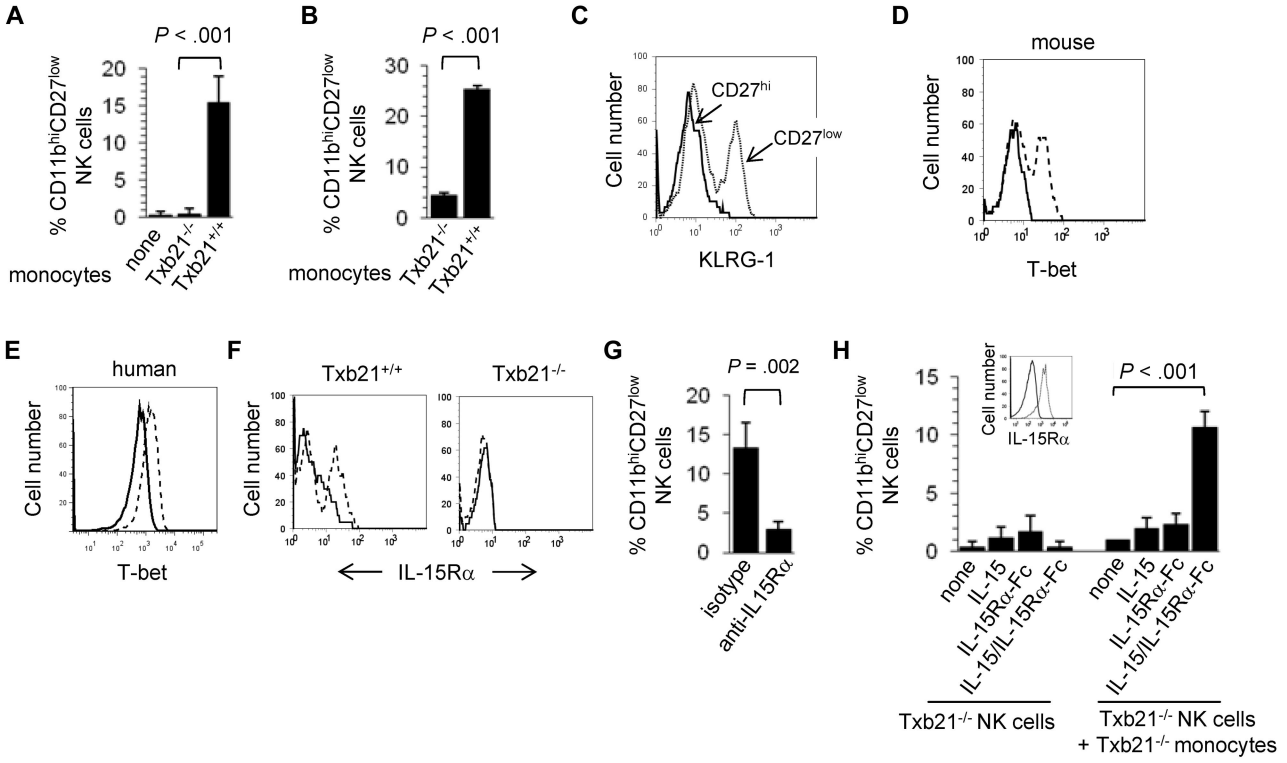

Figure 3. Mouse monocytes promote NK-cell differentiation. Mouse *Txb21*$^{+/+}$ and *Txb21*$^{-/-}$ monocytes were cocultured with *Txb21*$^{-/-}$ (A) or *Txb21*$^{+/+}$ (B) NKp46^{+}CD3^{-}CD27^{+} NK cells, and the appearance of CD27^{-} NK cells was evaluated 24 hours later. Shown is a representative experiment of 3. (C) KLRG-1 expression in *Txb21*$^{-/-}$ CD27^{hi} (solid line) and CD27^{low} (dotted line) NK cells cocultured with monocytes as in panel D. (D) Intracellular T-bet expression in enriched mouse monocytes cultured in the presence (dotted line) or the absence (solid line) of 100 ng/mL mouse IFN-γ. (E) Intracellular T-bet expression in CD14-enriched human peripheral blood mononuclear cell monocytes cultured in the presence (dotted line) or the absence (solid line) of 100 ng/mL human IFN-γ. (F) Surface expression of IL-15Rα (dotted lines) on mouse *Txb21*$^{+/+}$ (left panel) and *Txb21*$^{-/-}$ (right panel) mouse monocytes enriched and cultured as before; solid lines indicate isotype controls. (G) Enriched mouse monocytes and *Txb21*$^{-/-}$ NK cells were cocultured as before in the presence of blocking anti-IL-15Rα antibodies or isotype as controls. Shown is the frequency (mean ± SD) of CD27^{low} NK cells 24 hours after culture, of 3 independent experiments. (H) *Txb21*$^{-/-}$ NK cells were cultured with IL-15, IL-15Rα-Fc, or IL-15/IL-15Rα-Fc complexes, in the presence or the absence of *Txb21*$^{-/-}$ monocytes. In the latter, monocytes were preincubated with cytokines or complexes before coculture with NK cells. Shown is the frequency of CD27^{low} NK cells 24 hours after culture. (Inset) The expression of IL-15Rα in the surface of *Txb21*$^{-/-}$ monocytes that were preincubated with IL-15/IL-15Rα-Fc complexes (dotted line); solid line indicates the staining of monocytes preincubated with phosphate-buffered saline.

spleen CD11b^{hi}CD27^{low} NK cells correlates with that of CD115^{+} monocytes but does not clarify whether monocytes are involved in NK-cell survival or differentiation. To address this point, we next set up short-term cultures where monocytes were enriched from *Txb21*$^{+/+}$ and *Txb21*$^{-/-}$ mice (supplemental Figure 4) and were cocultured with sorted double-negative (CD11b^{low}CD27^{hi}) plus double-positive (CD11b^{hi}CD27^{hi}) NK cells. Remarkably, *Txb21*$^{+/+}$, but not *Txb21*$^{-/-}$, monocytes promoted the differentiation of *Txb21*$^{-/-}$ (Figure 3A) and *Txb21*$^{+/+}$ (Figure 3B) CD11b^{hi}CD27^{hi} to CD11b^{hi}CD27^{low} NK cells. For *Txb21*$^{-/-}$ NK cells, CD27 loss was accompanied by the up-regulation of the killer cell lectin-like receptor G1 (KLRG-1; Figure 3C), a surface molecule that is up-regulated on maturation and is selectively expressed by CD11b^{hi}CD27^{low} but not CD27^{hi} or double-negative NK cells. To further support the notion that physiologically relevant cross-talk occurs between monocytes and NK cells in vivo, we performed immunohistologic studies to show that monocytes and NK cells are found in close proximity in the red pulp of the spleen (supplemental Figure 5).

It has been proposed that T-bet influences the generation of type I immunity, not only by controlling Th1 lineage commitment in the adaptive immune system[6] but also by a direct influence on the transcription of the IFN-γ gene in myeloid cells, including human monocytes.[26] In turn, monocyte-derived IFN-γ stimulates activation of T-bet expression as a positive loop feedback to sustain IFN-γ production. We next confirmed that T-bet expression is inducibly expressed in monocytes freshly isolated from mouse spleens (Figure 3D) and in human CD14^{+} monocytes isolated from peripheral blood mononuclear cells (Figure 3E) that were cultured in the presence of IFN-γ.

NK cells do not survive in environments where IL-15Rα or the cytokine IL-15 are missing.[27] Furthermore, it is well documented that mice deficient in the γ-common chain (γc) of the IL-15R (CD132) lack NK cells.[28] It has been suggested that IL-15 needs to be *trans*-presented (chaperoned to the IL-15R) and exposed in the cell surface to modulate NK-cell survival.[27] Relevant to our study, IL-15Rα is expressed on myeloid cells, including DCs and macrophages.[29] Therefore, we next tested whether IL-15Rα is also expressed on monocytes. Figure 3F shows that surface expression of IL-15Rα can be readily detected in *Txb21*$^{+/+}$ but not *Txb21*$^{-/-}$ monocytes cultured in the presence of IFN-γ. Furthermore, preventing in vitro the interaction between IL-15Rα and IL-15 blocked the monocyte-mediated appearance of CD11b^{hi}CD27^{low} NK cells (Figure 3G). To further investigate whether IL-15 was sufficient to induce NK-cell maturation, we set in vitro experiments where *Txb21*$^{-/-}$ NK cells were plated in medium containing IL-15, IL-15Rα-Fc fusion protein, or IL-15/IL-15Rα-Fc complexes.[20] Figure 3H shows that IL-15Rα can be detected in the surface of *Txb21*$^{-/-}$ monocytes incubated with IL-15/IL-15Rα-Fc complexes but not when incubated with IL-15 or IL-15Rα-Fc (not shown). In

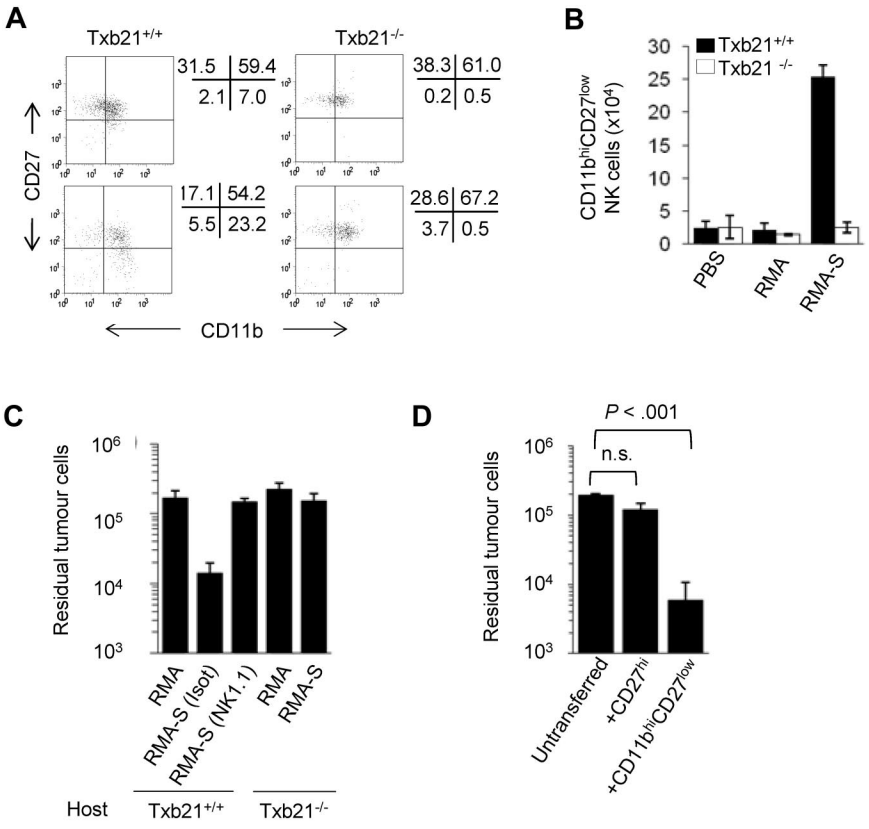

Figure 4. *Txb21*-deficient NK cells fail to reject MHC class I-negative tumors. (A) CD11b and CD27 expression in peritoneal NKp46$^+$CD3$^-$ NK cells in *Txb21*$^{+/+}$ (left panels) and *Txb21*$^{-/-}$ (right panels) mice injected with RMA (top panels) or RMA-S (bottom panels) 48 hours after tumor inoculation. (B) Absolute CD11b^{hi}CD27^{low} NK-cell number (mean ± SD) in the peritoneum of tumor injected mice from 2 independent experiments with 5 mice per group. (C) RMA and RMA-S tumor cells were labeled with CMTMR and CFSE, respectively, and coinjected into *Txb21*$^{+/+}$ and *Txb21*$^{-/-}$ C57BL/6 mice. Forty-eight hours after tumor challenge, peritoneal cells were recovered and analyzed by flow cytometry for CFSE and CMTMR. Data are mean ± SD residual tumor cells of 3 independent experiments, including 3 mice per group. (D) A total of 2×10^5 RMA-S-CFSE tumor cells were injected into *Txb21*$^{-/-}$ mice as in panel C, and mice were transferred intravenously with spleen CD27^{hi} or CD11b^{hi}CD27^{low} NK cells enriched from *Txb21*$^{+/+}$ mice. Forty-eight hours after tumor injection, peritoneal cells were recovered and analyzed by flow cytometry for CFSE. Data are mean ± SD of 2 independent experiments, including 4 mice per group.

the absence of monocytes, IL-15 and IL-15Rα complexes promoted survival and proliferation of *Txb21*$^{-/-}$ NK cells but not CD27 down-modulation (Figure 3H). In contrast, we observed the appearance of CD27^{low} NK cells when these were cocultured with *Txb21*$^{-/-}$ monocytes preincubated with IL-15/IL-15Rα-Fc complexes. Altogether, these results suggest that T-bet and IL-15Rα expressed by monocytes play a critical role in promoting terminal differentiation of NK cells.

CD11b^{hi}CD27^{low} NK cells are recruited to the tumor site and reject MHC class I-negative tumors

Whether NK-cell subsets defined by CD11b and CD27 expression have distinct roles on tumor immunity remains poorly understood. Because CD11b^{hi}CD27^{low} NK cells express high levels of Ly49 receptors recognizing MHC class I ligands,[3] we hypothesized that they could be involved more specifically in the rejection of MHC class I-negative tumors. To test this hypothesis, we used a well-established in vivo model of tumor rejection that depends on NK cells and their recruitment to the peritoneum.[30] Recruitment and activation of host NK cells occur after inoculation of tumor cells, provided that the tumor cells lacked critical host MHC class I molecules. In both *Txb21*$^{+/+}$ and *Txb21*$^{-/-}$ mice, most peritoneal NK cells are CD11b^{low}CD27^{hi} or CD11b^{hi}CD27^{hi} (Figure 4A). After the injection of MHC class I-negative RMA-S cells, but not MHC class I–positive RMA cells, the proportion (Figure 4A) and absolute number (Figure 4B) of CD11b^{hi}CD27^{low} NK cells greatly increased in the peritoneum of *Txb21*$^{+/+}$ mice 48 hours after tumor challenge. In contrast, CD11b^{hi}CD27^{low} NK cells were not detected at the tumor site of equivalently challenged *Txb21*$^{-/-}$ mice, ruling out the possibility that the CD27^{hi} to CD27^{low} NK-cell transition occurs during antitumor responses. To evaluate the ability of recruited NK cells to clear tumors, NK cell–sensitive RMA-S cells were coinjected with the same number of NK cell–resistant RMA cells as a control. Figure 4C shows that *Txb21*$^{+/+}$, but not *Txb21*$^{-/-}$, mice rejected RMA-S cells. As expected, the response in wild-type animals was lost after the administration of NK cell–depleting anti-NK1.1 antibodies.[30]

We next investigated the overall anatomic distribution of NK-cell subsets in tumor-bearing mice. The peritoneal injection of RMA-S induced a reduction in the proportion of CD11b^{hi}CD27^{low} NK cells in the spleen concomitant with an increase in the peritoneum (supplemental Figure 6A). Furthermore, adoptively transferred spleen CD11b^{hi}CD27^{low} NK cells migrated to the peritoneum of mice injected with RMA-S cells but not the peritoneum of mice challenged with RMA cells or to peripheral lymph nodes (supplemental Figure 6B). Notably, the adoptive transfer of highly purified spleen *Txb21*$^{+/+}$ CD11b^{hi}CD27^{low} but not CD27^{hi} NK cells restored the ability of *Txb21*$^{-/-}$ hosts to control RMA-S growth (Figure 4D). Together, this set of experiments underlines the relevance of the CD11b^{hi}CD27^{low} NK-cell subset in controlling the growth of NK cell–sensitive tumors developing in the peritoneal cavity.

Discussion

This study demonstrates that a functional interaction between NK cells and monocytes has profound implications in the NK-cell differentiation program, leading to the generation of NK-cell subsets relevant to control tumor growth. We have shown that the

NK-cell differentiation program cannot be completed in the absence of T-bet because of a checkpoint between CD27^{hi} and CD27^{low} stages, and we propose monocytes as a key player in overcoming the defective NK-cell development observed in *Txb21*$^{-/-}$ mice. We also show that monocytes deliver T-bet-dependent signals, including the expression of the IL-15Rα chain and IL-15 *trans*-presentation, for *Txb21*-deficient NK cells to continue their program of differentiation. The impossibility of rejecting MHC class I-negative tumors by *Txb21*$^{-/-}$ mice was restored by adoptive transfer of CD11b^{hi}CD27^{low} NK cells, thus highlighting the relevance of this NK-cell subset in controlling malignancy.

Recently, the basic leuzine zipper transcription factor E4bp4 was identified as the master regulator of NK-cell differentiation as *E4pb4*-deficient mice completely lack NK cells.[31] Our data suggest that other transcription factors may help to identify check points of functionally distinguishable NK-cell subsets and provide further support for a model where peripheral NK-cell differentiation can be monitored by the expression of CD11b and CD27.[3,4,32]

Previous studies underlined the relevant role for DCs and macrophages in promoting NK-cell survival[29,33] and homeostatic proliferation.[12] The reduction in the proportion of splenic NK cells after the AP20187-induced depletion of CD115^{+} monocytes and macrophages in MaFIA mice reinforces the current idea that interactions between myeloid cells and NK cells are necessary for NK-cell maintenance. Notably, AP20187 treatment impacted the overall distribution of NK-cell subsets in the spleen with a strong reduction in CD11b^{hi}CD27^{low} NK cells. Reciprocally, CD11b^{hi}CD27^{low} NK cells increased when wild-type animals were administered with CSF-1, a cytokine that promotes monocyte/macrophage differentiation and survival.[34] Furthermore, our in vitro experiments demonstrated that monocytes can drive NK-cell terminal differentiation, but it does not exclude a role of other myeloid, *Txb21*$^{+/+}$ cells. New genetic models specifically targeting these subpopulations will allow the dissection of cellular interactions required for NK cells to complete their differentiation program.

Several lines of evidence support the idea that expression of T-bet in NK cells may not be sufficient to complete the CD11b^{hi}CD27^{hi} to CD11b^{hi}CD27^{low} NK-cell transition. Indeed, CD11b^{hi}CD27^{low} *Txb21*$^{+/+}$ NK cells are barely detected in mice where IL-15Rα expression is restricted to CD11c^{+} DCs[33] or in mice bearing lineage-specific deletion of IL-15Rα in CD11c^{+} DCs, in macrophages or in both,[29] suggesting that NK-cell terminal differentiation requires extrinsic inputs. Our data and previous reports demonstrate that *Rag2*$^{-/-}$-dependent[21] and γc-dependent (this study) lymphocytes do not modulate terminal NK-cell differentiation. Instead, our results are consistent with a role for spleen monocytes, anatomically distributed in the same niche as spleen NK cells, in facilitating the CD11b^{hi}CD27^{hi} to CD11b^{hi}CD27^{low} NK-cell transition. The addition of cytokines (IFN-γ, IL-2, and IL-15) in the in vitro culture medium of sorted *Txb21*$^{-/-}$ CD11b^{hi}CD27^{hi} NK cells cannot by themselves modulate this step, indicating that a NK/monocyte cell-to-cell contact is required. Importantly, expression of T-bet and IL-15Rα in monocytes is determinant in modulating the CD27^{hi} to CD27^{low} NK-cell step, regardless of T-bet expression in NK cells. It should be noted that, under the in vivo and in vitro conditions tested here, the frequency of *Txb21*$^{-/-}$ NK cells that differentiate from CD27^{hi} to CD27^{low} stage remains lower than the *Txb21*$^{+/+}$ counterparts, suggesting that expression of T-bet in NK cells may still be required to complete fully their differentiation program. The ectopic expression of T-bet retrovirus in CD4 Th2 T cells, which do not normally express T-bet, induced surface expression of CD122 (IL-2Rβ chain),[35] and it has been shown that CD122 is a direct T-bet target gene in T cells.[36] Nevertheless, whether T-bet directly targets any of the IL-15/IL-15R gene components in monocytes and whether expression of IL15/IL-15R molecules IL-15 is directly or indirectly modulated by T-bet remain to be determined.

There is not a clear consensus as to whether functional specialization among NK-cell subsets exists. The infiltration of NK cells appears to have a prognostic value in human carcinoma malignancies as a higher amount of NK cells correlates with a better prognosis.[37] However, whether specific NK-cell subpopulations infiltrate tumor masses is unclear. Several studies have identified a predominant presence of CD56^{bright}CD16^{-} NK cells, the probable counterpart of mouse CD27^{+} NK-cell subset,[38] in nonsmall lung carcinomas[39] and in pancreatic metastatic lesions.[40] Other studies have observed CD57^{+} NK cells highly represented in squamous lung cancer[37] and colorectal[41] and gastric[42] carcinomas. In the mouse system, gene expression profiles[4] and functional studies[3,4] do not allow the attribution of unique functions to CD27^{hi} and CD11b^{hi}CD27^{low} NK-cell subsets. Our data show that CD11b^{hi}CD27^{low} NK cells migrate efficiently to inflamed tissues to restrain tumor growth. Cell migration depends on the functional expression of chemokine receptors; thus, a division of labor based on the expression of chemokine receptors may be proposed. Indeed, CXCR3 and CX3CR1 are expressed exclusively on CD27^{hi} and CD11b^{hi}CD27^{low} NK-cell subsets, respectively,[25] suggesting that different NK-cell subsets may be independently recruited into distinct inflammatory settings. The tumor-induced specific recruitment of CD11b^{hi}CD27^{low} NK-cell subset reported here and the defective antitumor responses described in CX3CR1-deficient mice[43] support this idea. It should also be considered that the tumor microenvironment, preferentially enriched for CXCR3 ligands rather than CX3CR1 ligands, and the anatomic location of the tumor, may dictate the recruitment of CD11b^{hi}CD27^{hi} NK cells[44] or CD11b^{hi}CD27^{low} NK cells (this study). The experiments of our study provide clear evidence for a division of labor among NK-cell subsets and demonstrate that incomplete NK-cell differentiation observed in *Txb21*-deficient mice may lead to a deficit in effector antitumoral NK-cell subsets. Furthermore, our findings may also explain the increased NK cell-dependent incidence of pulmonary[45] and hepatic[46] metastasis reported in splenectomized mice.

Previous dissection of NK-cell biologic differentiation and function has been complicated by the lack of deficiency models selectively deleting defined NK-cell subsets. We propose that the expression of T-bet on monocytes is a critical determinant in the functional differentiation of NK-cell subsets relevant to control tumor outgrowth. The intellectual challenge is to understand how the various NK-cell subsets regulate defined functions and how the hematopoietic compartment shapes NK-cell maturation versus survival. Understanding the intricacies of NK-cell development will help appreciate how various NK-cell subsets are generated and participate in antitumor immunity, ultimately offering opportunities for selective intervention.

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Authorship

Contribution: K.S., N.P., C.L., and T.W. performed research and analyzed and interpreted data; N.v.R. and A.H. provided reagents and tools; T.W., F.G., and G.M.L. provided essential advice; A.M.-F. designed research, analyzed and interpreted data, and wrote the manuscript; and all authors read and commented on the draft versions of the manuscript.

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