A novel *Ncr1*-Cre mouse reveals the essential role of STAT5 for NK-cell survival and development

Eva Eckelhart,[1] Wolfgang Warsch,[1] Eva Zebedin,[1] Olivia Simma,[1] Dagmar Stoiber,[1,2] Thomas Kolbe,[3] Thomas Rülicke,[3,4] Mathias Mueller,[3,5] *Emilio Casanova,[2] and *Veronika Sexl[1,6]

[1]Institute of Pharmacology, Center of Physiology and Pharmacology, Medical University of Vienna, Vienna, Austria; [2]Ludwig Boltzmann Institute for Cancer Research, Vienna, Austria; and [3]Biomodels Austria, [4]Institute of Laboratory Animal Science, [5]Institute of Animal Breeding and Genetics, and [6]Institute of Pharmacology and Toxicology, University of Veterinary Medicine, Vienna, Austria

We generated a transgenic mouse line that expresses the Cre recombinase under the control of the *Ncr1* (*p46*) promoter. Cre-mediated recombination was tightly restricted to natural killer (NK) cells, as revealed by crossing *Ncr1*-iCreTg mice to the *eGFP*-LSLTg reporter strain. *Ncr1*-iCreTg mice were further used to study NK cell–specific functions of *Stat5* (*signal transducers and activators of transcription 5*) by generating *Stat5*$^{f/f}$ *Ncr1*-iCreTg animals. *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice were largely devoid of NK cells in peripheral lymphoid organs. In the bone marrow, NK-cell maturation was abrogated at the NK cell–precursor stage. Moreover, we found that in vitro deletion of *Stat5* in interleukin 2–expanded NK cells was incompatible with NK-cell viability. In vivo assays confirmed the complete abrogation of NK cell–mediated tumor control against B16F10-melanoma cells. In contrast, T cell–mediated tumor surveillance against MC38-adenocarcinoma cells was undisturbed. In summary, the results of our study show that STAT5 has a cell-intrinsic role in NK-cell development and that *Ncr1*-iCreTg mice are a powerful novel tool with which to study NK-cell development, biology, and function. (*Blood*. 2011;117(5):1565-1573)

Introduction

Natural killer (NK) cells are members of the innate immune system and represent a third lineage of lymphoid cells distinct from T and B lymphocytes. NK cells were initially discovered through their ability to spontaneously lyse tumor cells.[1] The importance of NK cells in tumor surveillance has been shown in vitro and in vivo in different mouse models.[2,3] Moreover, NK cells recognize and eliminate cells infected by certain viruses or parasites,[4,5] and produce and secrete cytokines such as interferonγ (IFNγ) and tumor necrosis factor (TNF), which stimulate the adaptive and innate immune responses.[6] Therefore, NK cells exert an important function in orchestrating the interplay of innate and adaptive immunity.

In adult mice, NK-cell differentiation takes place mainly in the bone marrow.[7] The earliest NK-cell precursors (NKPs) are characterized by the expression of the interleukin 2 (IL2) and IL15 receptor common β subunit, also known as CD122, and the absence of NK-lineage markers such as the NK1.1, DX5, and Ly49 receptors.[8] This cell type gives rise to immature NK (iNK) cells, which are positive for NK1.1, negative for DX5, and display reduced expression of certain Ly49 receptors. Further differentiation comprises mature NK (mNK) cells expressing NK1.1, DX5, and Ly49 receptors. Mature NK cells may leave the bone marrow and migrate to secondary lymphoid organs, lung, liver, and gut. Recently, IL22-producing lymphoid cells in the intestinal lamina propria have been characterized that are positive for NCRI (natural cytotoxicity receptor I), NKG2D, and NK1.1 and express the orphan transcription factor RORyt.[9] However, NKPs and iNKs are not uniquely restricted to the bone marrow because they have been found at other sites such as the spleen[10] and lymph nodes.[11] It has therefore been suggested that multiple sites may support NK-cell differentiation or, alternatively, that NKPs and iNKs from the bone marrow have access to the circulation. As NK cells mature, they sequentially acquire their characteristic NK cell–receptor repertoire.[12] NCRI, also known as NKp46, becomes expressed during the early iNK-cell stage and remains constitutively expressed.[13,14] The ligand(s) of this receptor has only been partially characterized.[15,16] NCRI is involved in the control of influenza infection by recognizing the viral hemagglutinin protein,[13] and most recently has been identified as factor modulating disease progression in type 1 diabetes.[17] The developmental pathways generating NK-cell diversity, including the transcriptional machinery behind them, are not well understood and remain elusive.

The differentiation and homeostasis of lymphocytes are regulated by cytokines such as the interleukins IL2, IL4, IL7, IL15, and IL21. All of these cytokines require the common γ chain (γ_c) and activate major signaling pathways, such as that involving the Janus family of tyrosine kinases (JAKs) and signal transducers and activators of transcription (STATs), thereby contributing to the biological effects of lymphoid cells.[18] The JAKs are stably associated with the cytokine receptor and induce the activation of STATs upon receptor stimulation, with STAT5 being predominantly activated by IL2, IL7, and IL15.[19] STAT5 consists of 2 highly homologous isoforms, STAT5A and STAT5B, which are encoded by separate genes. In the lymphoid system, STAT5A and

Submitted June 18, 2010; accepted November 18, 2010. Prepublished online as *Blood* First Edition paper, December 2, 2010; DOI 10.1182/blood-2010-06-291633.

The online version of this article contains a data supplement.

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*E.C. and V.S. contributed equally to this study.

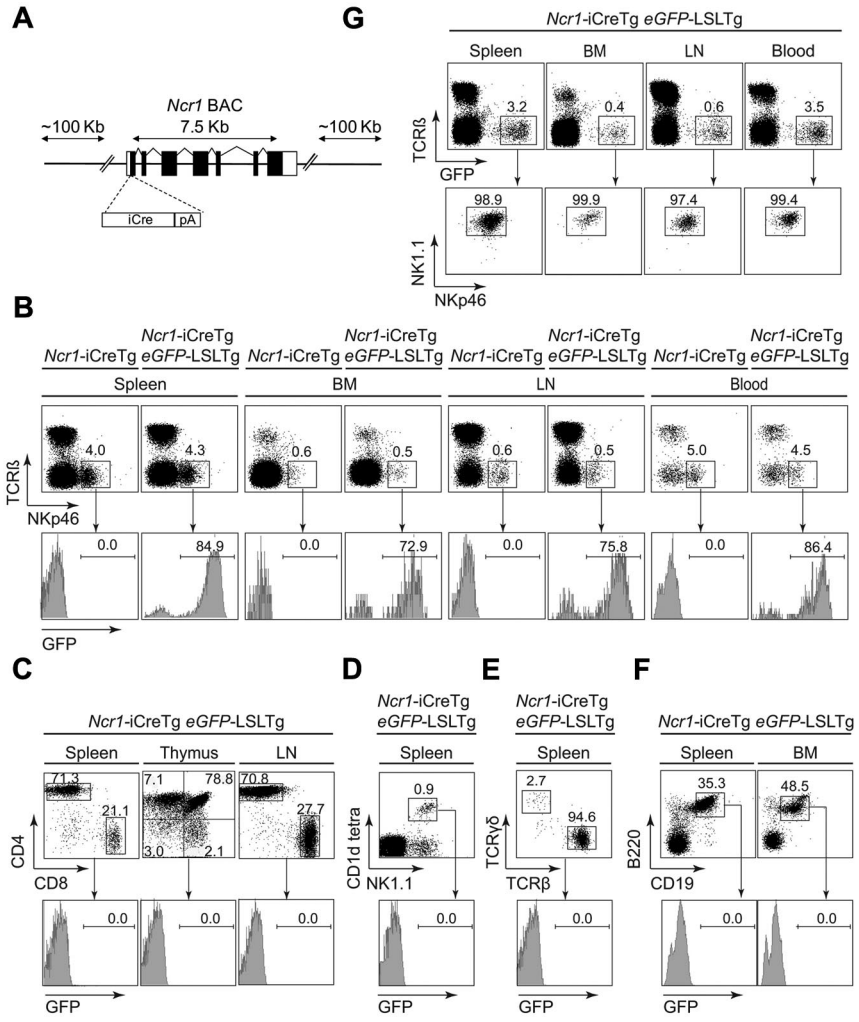

Figure 1. Generation and characterization of *Ncr1*-iCreTg mice. (A) Schematic illustration of the modified *Ncr1* BAC. iCre cDNA was inserted by homologous recombination into the exon containing the translation initiation codon of a BAC harboring the mouse *Ncr1* gene. (B) Efficiency of Cre-mediated *eGFP* expression verified via flow cytometry of *Ncr1*-iCreTg *eGFP*-LSLTg double-transgenic mice and littermate controls. Excision of a stop cassette flanked by loxP sites leads to *eGFP* expression that can be analyzed by flow cytometry. Numbers adjacent to outlined areas indicate the percentage of NK cells ($TCR\beta^-NKp46^+$) of various lymphoid organs. Histograms show the percentage of *eGFP* expression of gated NK cells. (C-F) Flow cytometry of *Ncr1*-iCreTg *eGFP*-LSLTg double-transgenic mice and their littermate controls showing the absence of *eGFP* expression in hematopoietic cell lineages other than NK cells. Dot plots indicate the percentages of (C) gated $CD3^+CD4^+CD8^+$ cells, (D) gated $CD3^-$ $NK1.1^+CD1d$ $tetra^+$ cells, (E) gated $CD3^+$ $TCR\gamma\delta^+$ $TCR\beta^+$ cells, and (F) $B220^+CD19^+$ cells. Histograms show the percentage of *eGFP* expression. (G) Almost all NK cells express *eGFP*. Approximately 99% of gated $TCR\beta^-eGFP^+$ cells are NK cells ($NK1.1^+NKp46^+$; $n \geq 4$ per genotype). Data are representative of at least 3 independent experiments. BM, bone marrow; LN, lymph nodes.

STAT5B fulfill largely redundant roles, although STAT5B has been implicated in NK-cell development.[20] In *Stat5b*$^{-/-}$ mice, NK-cell numbers were found to be reduced to 50%, and whole splenocyte cultures showed a reduced cytolytic capacity in response to IL2 and IL15 that was attributed to the NK-cell compartment.[21]

The generation of mice deficient for both *Stat5a* and *Stat5b* (*Stat5a/b*) verified their important role in lymphoid development and homeostasis. The first *Stat5a/b*-knockout mouse expressed N-terminally truncated proteins at various expression levels, depending on the tissue type, and led to a viable phenotype (now referred to as *Stat5*$^{\Delta N/\Delta N}$ mice). The residual STAT5$^{\Delta N}$ proteins bind DNA and activate the transcription of some but not all target genes.[20] Furthermore, STAT5$^{\Delta N}$ proteins are constitutively active even in the absence of cytokines. Therefore, the interpretation of phenotypes obtained with *Stat5*$^{\Delta N/\Delta N}$ mice is complex and requires revalidation in a model completely devoid of STAT5 proteins. This can be most obviously seen by the fact that mice lacking complete *Stat5a/b* die perinatally, whereas *Stat5*$^{\Delta N/\Delta N}$ animals are viable.[22] *Stat5a/b*$^{f/f}$ mice (henceforth *Stat5*$^{f/f}$ mice) crossed to B and T cell–specific Cre lines revealed multiple and complex functions of STAT5A/B (henceforth STAT5). In B cells, STAT5 mediates survival downstream of IL7 and is involved in immunoglobulin rearrangement and pre-B–cell expansion.[23] Within the T-lymphoid lineage, STAT5 is mainly required for the expansion of $CD8^+$ T cells and T-cell receptor γδ (TCRγδ) lymphocytes.[24] The key role of STAT5 in lymphoid cells is also underlined by the fact that STAT5 is constitutively active in many lymphoid malignancies, which may even critically depend on its presence.[25]

In this study, we describe the successful generation of a mouse model that allows for the first time conditional mutagenesis specifically in NK cells. Our work reveals a critical role for the transcription factor STAT5 in NK-cell development and survival. In *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice, NK cells are nearly completely absent, causing the virtual abrogation of NK cell–mediated tumor surveillance without affecting T cell–controlled immune surveillance.

Methods

Generation of *Ncr1*-iCreTg transgenic mice

Codon-improved Cre (iCre) recombinase was inserted into a bacterial artificial chromosome (BAC; RP23-267N11; purchased from Children's Hospital Oakland Research Institute, Oakland, CA) harboring the *Ncr1* gene via homologous recombination in *Escherichia coli*.[26] Briefly, a cassette containing iCre recombinase, an artificial intron, a bovine growth hormone polyadenylation signal, and an ampicillin-resistance gene flanked by FRT (Flp recombinase target) sites was recombined into the first exon of the *Ncr1* gene. Correctly recombined BACs were transiently electroporated with a plasmid expressing the Flp recombinase to delete the ampicillin gene. The BACs were verified by Southern blot and sequencing. BAC DNA

was digested with *Not*I, purified using a Sepharose CL4b column, and injected into the pro-nuclei of C57BL/6N oocytes. Three of 4 *Tg(Ncr1-iCre)264-267* founders expressed the transgene, and Tg(*Ncr1*-iCre)265 was selected for detailed analysis. Genotyping of Tg(*Ncr1*-iCre)265 mice (henceforth *Ncr1*-iCreTg) was performed using the following primers: 5′GACCATGATGCTGGGTTTGGCCCAGATG and 5′ATGCGGTGGGCTCTATGGCTTCTG yielding a 500 bp polymerase chain reaction (PCR) product.

Mice

Animals were 4-12 weeks old and maintained at the Biomedical Research Institute, Medical University Vienna, University of Veterinary Medicine Vienna. *Stat5*$^{f/f}$ and *eGFP*^{LSL} mice were described previously.[22,27] All animal experiments were approved by the Federal Ministry for Science and Research.

Antibodies and flow cytometric analysis

The following antibodies were purchased from BD Biosciences: NK-1.1 (PK136), CD49b (HMα2), CD3e (145-2C11), CD3 (17A2), CD4 (RM4-5), CD8a (53-6.7), CD45R/B220, CD19, γδ TCR (GL3), TCRβ (H57-597), CD122 (TM-beta 1), Ly6G/Ly6C (RB6-8CM), CD3e (145-2C11), TER119, CD45R/B220(RA3-6B2), CD27 (LG3A10), CD11b (M1/70), CD44 (IM7), Ly49A (A1), NKp46 (29A1.4), Ly49 D (4E5), KLRG1 (2F1), and CD25 (Pc61). NKp46 (29A1.4), Ly49H (3D10), and NKG2D (CX5) were obtained from eBioscience. PBS57-loaded and -unloaded CD1d tetramers were generously donated by Wilfried Ellmeier (Medical University, Vienna, Austria). For flow cytometry, single-cell suspensions were prepared and splenocytes were depleted of red blood cells. Purified rat anti–mouse CD16/CD32 (2.4G2; BD Pharmingen) was added to avoid nonspecific binding. For intracellular staining, cells were fixed with 2% paraformaldehyde at room temperature for 10 minutes, and incubated with ice-cold methanol for 20 minutes at −20°C. Fc receptors were blocked, and cells were incubated with STAT5 antibody (C17; Santa Cruz Biotechnology) at 4°C overnight under agitation and counterstained with phycoerythrin-conjugated goat anti–rabbit immunoglobulin G (Santa Cruz Biotechnology). For in vivo bromodeoxyuridine (BrdU)–incorporation assays, mice were intraperitoneally injected with 1 mg of BrdU (in 100 μL). After 12 hours, splenocytes were isolated, stained, fixed, permeabilized, and treated with DNase. Analysis of BrdU incorporation was performed using the BrdU Flow Kit (BD Pharmingen). Stained samples were analyzed using FACSCantoII and FACSDiva software Version 6.1.2 (BD Biosciences).

NK-cell purification, expansion, and function

NK cells were purified and expanded as described previously.[28] Briefly, single-cell suspensions were prepared from ≥ 4 spleens/genotype. For NK-cell purification, cell suspensions were incubated with anti-DX5–coated MACS beads (Miltenyi Biotec) and purified on a MACS separator (Miltenyi Biotec). NK cells were expanded for 6-12 days in RPMI 1640 medium containing 10% fetal calf serum (FCS), β-2ME, L-glutamine, and penicillin/streptomycin supplemented with rhIL2 (henceforth IL2; 5000 U/mL). The purity as assessed by flow cytometry was > 90%-95%. For cell sorting, splenocytes were depleted of red blood cells, incubated with anti–CD3 and anti–DX5, and sorted into a CD3$^-$DX5$^+$ population. For in vitro proliferation assays, purified NK cells at day 6 were seeded in flat-bottom, 96-well plates (1×10^5 cells/well) under IL2 (5000 U/mL). Proliferation was measured by ^{3}[H]-thymidine incorporation. NK-cell cytotoxicity was analyzed by flow cytometry, as described previously.[29] Briefly, 1×10^4 target cells/well were incubated with 5μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) for 6 minutes at 4°C in the dark. Expanded NK cells at day 10 were coincubated with CFSE-labeled YAC-1, RMA, and RMA-Rae1γ targets at the indicated E:T ratios for 4 hours at 37°C and placed on ice. Propidium iodide (Sigma-Aldrich) was added to each well immediately before flow cytometry. The percentage of specific lysis was determined as described previously.[29] To determine the effects of *Stat5* deletion in primary NK cells, *Stat5*$^{f/f}$ NK cells were purified, expanded for 3 days under IL2, and infected with adenovirus expressing either green fluorescent protein (GFP) or Cre-GFP (kindly provided by Wolfgang Mikulits, Medical University, Vienna, Austria) and 7 μg/mL of polybrene. GFP expression of CD3$^-$DX5$^+$ cells was tracked via flow cytometry. The day of the highest GFP expression was defined as day 0.

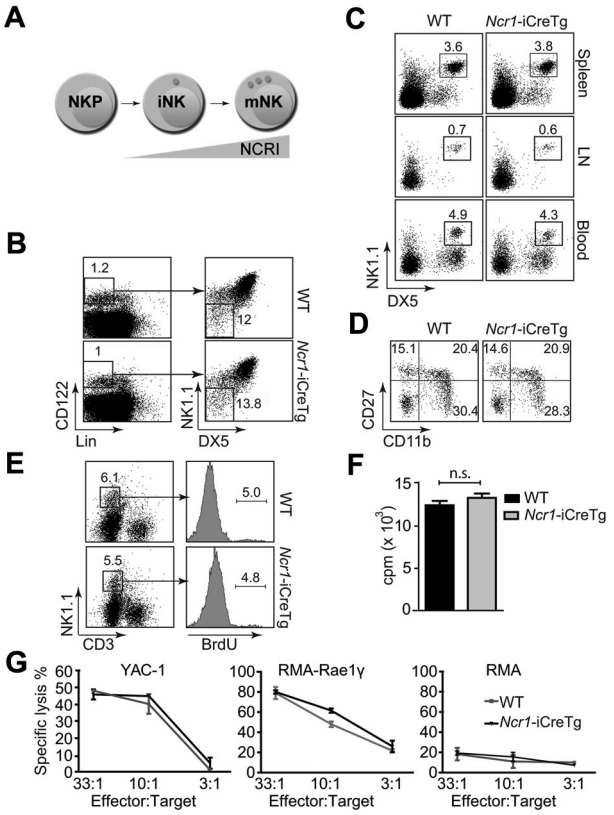

Figure 2. Development and function is not altered in Cre-expressing NK cells. Neither the development nor the proliferation or cytotoxicity of NK cells was influenced by Cre recombinase. (A) Simplified scheme of NCRI expression in NK-cell development. (B-E) Flow cytometry of *Ncr1*-iCreTg mice and littermate controls. Dot plots indicate the percentage of (B) gated Lin$^-$CD122$^+$ DX5$^-$NK1.1$^-$ NKPs in the BM, (C) gated CD3$^-$ NK cells (NK1.1$^+$DX5$^+$) in the periphery, and (D) expression of maturation markers of splenic NK cells gated as TCRβ$^-$NK1.1$^+$ stained with CD27 and CD11b antibodies (n ≥ 4 per genotype). Data are representative of at least 3 independent experiments. (E) In vivo proliferation of splenic NK cells. Mice were injected intraperitoneally with BrdU and after 12 hours, the incorporation of BrdU in splenic NK cells was analyzed. Numbers adjacent to outlined areas in the dot plot indicate the percentage of CD3$^-$NK1.1$^+$ cells. Histograms show the percentage of BrdU-positive cells (n ≥ 5 per genotype). Data are representative of 2 independent experiments. (F) In vitro proliferation of IL2-expanded NK cells purified from the spleens of mice of the indicated genotypes. At day 6, NK cells were seeded in triplicate in 96-well plates. After 12 hours, proliferation was measured by standard ^{3}[H]-thymidine incorporation. Four mice per genotype were pooled. Data are representative of 2 independent experiments. (G) Cytotoxicity of IL2-expanded splenic NK cells purified from indicated genotypes. At day 10, NK cells were coincubated in triplicate with CFSE-labeled YAC-1, RMA-Rae1γ, and RMA targets at the indicated E:T ratios. Five mice per genotype were pooled. Data are representative of 2 independent experiments.

T-cell stimulation

MACS-sorted (Miltenyi Biotec) T cells (5×10^5 cells/well) were stimulated with 1 μg/mL of plate-bound anti–CD3ε (145-2C11; BD Biosciences) and 2 μg/mL of anti–CD28 (37.51; BD Biosciences) on 48-well plates in the presence of 100 U/mL of IL2 for 48 hours. T cells were harvested after 5 days and analyzed by flow cytometry.

Cell lines and tumor models

B16F10 and MC38 cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated FCS, 100 U/mL of penicillin-streptomycin, 2mM L-glutamine, and 5μM β-mercaptoethanol.

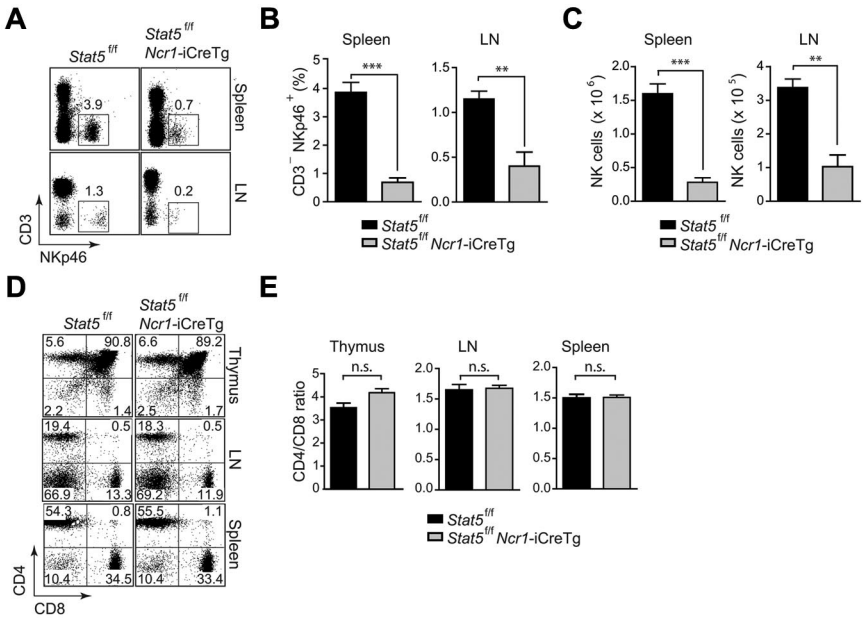

Figure 3. NK cells are severely reduced in *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice. Deletion of *Stat5* results in severely diminished NK-cell numbers. (A,D) Flow cytometry of *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice and littermate controls. Dot plots indicate the percentage of (A) peripheral NK cells ($CD3^-NKp46^+$) and (D) CD4 and CD8 expression on cells from various lymphoid organs. Cells were gated on total thymocytes (top panel), $CD3^+$ lymph node cells (middle panel), and $CD3^+$ splenocytes (bottom panel). (B) Bar graphs show the percentage of peripheral NK cells ($CD3^-NKp46^+$). (C) Bar graphs show the total splenic (left) and lymph node (right) NK-cell numbers. (E) Bar graphs show the CD4-to-CD8 ratio of total thymocytes (left panel), $CD3^+$ lymph node cells (middle panel), and $CD3^+$ splenocytes (right panel). (A,D) Data are representative of 3 independent experiments ($n \geq 4$ per genotype). (B,C,E) $n \geq 5$ per genotype.

YAC-1, RMA, and RMA-Rae1γ cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/mL of penicillin-streptomycin, 2mM L-glutamine, and 5μM β-mercaptoethanol. Mice were injected intravenously with 1×10^6 B16F10 and subcutaneously with MC38, and monitored daily for disease onset. Tissues were isolated, weighed, fixed in 3.7% formaldehyde, and analyzed. For $NK1.1^+$ and $CD8^+$ T-cell depletion, 100 μg of anti–NK1.1 antibody and anti–CD8 antibody was injected twice a week starting 2 days before beginning the experiment. Anti–NK1.1 antibody and anti–CD8 antibody were purified from the PK136-cell and 53-6.72-cell supernatants, respectively. The deletion was confirmed by flow cytometry of splenocytes.

Real-time PCR

Total RNA was isolated using TRI reagent (Sigma-Aldrich) according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis using the GeneAmp RNA PCR kit (Roche) and for the real-time PCR reaction performed on an Eppendorf RealPlex cycler using Taq DNA polymerase (5Prime) and SYBR Green. All experiments were performed in triplicate. The following primers were used: *Stat5a* Forward: CAGATCAAGCAAGTGGTCCC3′, *Stat5a* Reverse: TCGAGACTGTC-CATGGGCC, *Stat5b* Forward: GGCAGGGTCAGTAACGGAAG, *Stat5b* Reverse: GGCTCTGCAAAGGCGTTGTC. Samples were normalized to GAPDH expression; *Gapdh* Forward: TCTCCTCTGACTTCAACAGCG, *Gapdh* Reverse: ACCACCCTGTTGCTGTAGCC

Statistics

Statistical analysis was performed using Student *t* test except for the results shown in Figure 5B and C, for which a Mann Whitney U test was used (**$P = .0014$). The P values were defined as follows: *$P < .05$; **$P < .01$; and ***$P < .001$. Data are expressed as mean ± SEM and were analyzed with Prism software Version 5.03 (GraphPad).

Results

Generation and characterization of *Ncr1*-iCreTg mice

To restrict Cre recombinase expression to NK cells, we generated transgenic mice expressing Cre recombinase under the control of the *Ncr1* promoter (schematically illustrated in Figure 1A). We used a BAC clone containing the entire *Ncr1* gene, along with abundant upstream and downstream flanking DNA. By homologous recombination in bacteria, the coding part of the first exon of *Ncr1* was replaced by the iCre expression cassette. To test the functionality and lineage specificity, *Ncr1*-iCreTg–transgenic mice were crossed to the *eGFP*^{LSL}-reporter mouse line that expresses enhanced GFP (*eGFP*) upon Cre-mediated excision of a loxP-flanked stop cassette (see also supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).[27] As depicted in Figure 1B, *eGFP* expression in $TCR\beta^-NKp46^+$ NK cells varied between 70% and 90% in individual *Ncr1*-iCreTg *eGFP*-LSLTg double-transgenic mice. On average, we observed 81.0% ± 2.42% $eGFP^+$ NK cells in the spleen, 71.9% ± 1.56% in the bone marrow, 74.0% ± 2.41% in the lymph nodes, and 80.0% ± 2.75% in the blood (summarized in supplemental Figure 2). No *eGFP* expression was detected in T-lymphoid cells, as analyzed by CD3, CD4, and CD8 staining (Figure 1C). Despite the general agreement that NCR1 is constitutively and selectively expressed in NK cells,[30] it is also known to be expressed in peripheral NK-like TCRγδ cells.[31] Therefore, to unequivocally define *eGFP* expression in these cells, we stained splenocytes from *Ncr1*-iCreTg *eGFP*-LSLTg mice for NK1.1 and CD1d tetramer and for TCRαβ and TCRγδ. As depicted in Figure 1D and E, we failed to observe any *eGFP* expression in NKT and TCRγδ cells. Similarly, we failed to detect $eGFP^+$ B-lymphoid cells (Figure 1F). In addition, we confirmed the exclusive *Ncr1*-dependent Cre recombination by staining of splenocytes with TCRβ, NK1.1, and NKp46. As expected, $eGFP^+$ cells were restricted to the NK-cell compartment ($TCR\beta^-NK1.1^+NKp46^+$; Figure 1G). We therefore concluded that Cre recombination in *Ncr1*-iCreTg mice is restricted to NK cells.

Cre expression does not alter NK-cell development and function

Although Cre recombinase has been intensively used to induce genomic recombination, Cre expression has been reported to be toxic for some eukaryotic cells.[32,33] This may be related to chromosomal rearrangements caused by recombination between cryptic "pseudo-loxP" sites naturally occurring within the genome. Alternatively, integration of the transgene may disrupt the genes important for organ and/or cell development. Therefore, we next

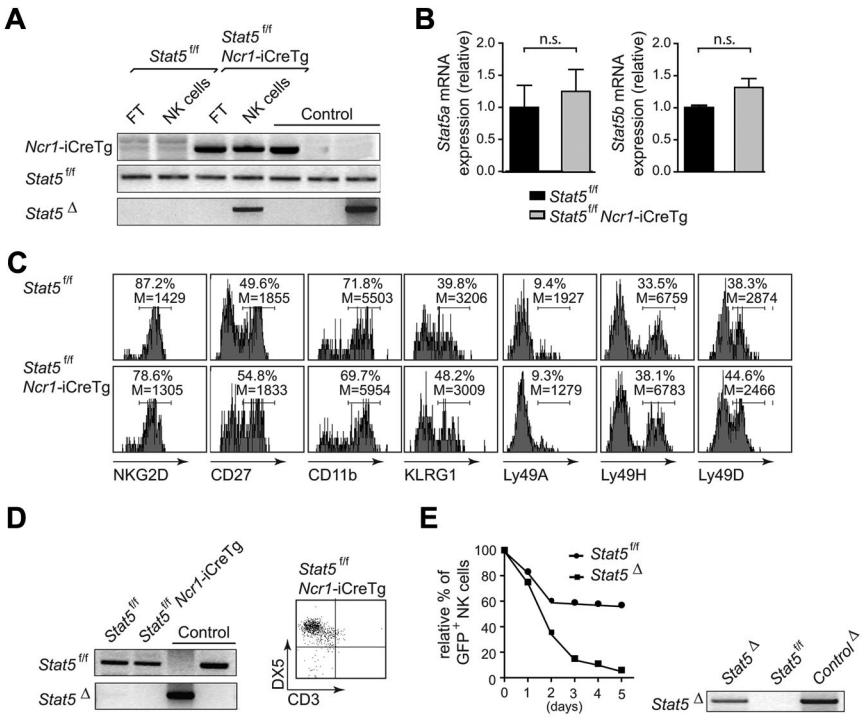

Figure 4. Loss of STAT5 is incompatible with NK-cell viability. (A) PCR genotyping of MACS-purified splenic NK cells of *Stat5*$^{f/f}$ and *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice. Four mice per genotype were pooled. (B) Real-time PCR analysis of *Stat5a* and *Stat5b* mRNA levels of FACS-sorted splenic CD3$^-$DX5$^+$ NK cells. Ten mice per genotype were pooled. (C) Histograms show percentage and mean fluorescence intensity of various NK-cell markers on CD3$^-$NKP46$^+$ splenocytes of the indicated genotypes. (D) MACS-purified splenic NK cells were cultured under IL2. After 10 days of culture, only those cells that expressed *Stat5* expanded, as indicated by the lack of a *Stat5* deletion band on PCR analysis (left panel). Flow cytometry confirmed the NK-cell nature of these cells (right panel). Dot plot indicates CD3$^-$DX5$^+$ cells. Four mice per genotype were pooled. (E) MACS-purified splenic NK cells from *Stat5*$^{f/f}$ mice were cultured under IL2 (4 mice per genotype were pooled) and infected with Ad/Cre-GFP (indicated as *Stat5*$^{\Delta}$) or mock-infected (indicated as *Stat5*$^{f/f}$). Those cells that received the empty vector tolerated the expression of Ad/GFP, whereas those that had received Ad/Cre-GFP expressed the Cre recombinase and declined (left panel). PCR genotyping of the cells confirmed the deletion of *Stat5* in Ad/Cre-GFP–infected NK cells (right panel). Data are representative of at least 2 independent experiments. FT, flow-through.

studied the effects of Cre expression per se on NK-cell development and function. *Ncr1*-iCreTg mice were born at the expected Mendelian ratio without any visible alterations in organ morphology or overt pathology (data not shown). NK cells develop mainly in the bone marrow. Basically, 3 major developmental stages can be distinguished by their NK1.1 and DX5 expression.[7] NKPs at the first developmental stage are negative for NK1.1 and DX5, iNKs express NK1.1 but not DX5, and mNKs are positive for both NK1.1 and DX5. At the iNK cell stage, *Ncr1* is expressed and remains expressed throughout all stages[14] (schematically illustrated in Figure 2A). No changes in NKPs (Lin$^-$CD122$^+$NK1.1$^-$DX5$^-$) in the bone marrow of *Ncr1*-iCreTg mice were detected compared with wild-type (WT) littermate controls (Figure 2B; absolute cell numbers are shown in supplemental Figure 3A). Moreover, peripheral mature NK cells were present at equal levels in the spleen, lymph nodes, and blood, as depicted in Figure 2C (absolute cell numbers are provided in supplemental Figure 3B). No difference in the mean fluorescence intensity of NKp46 was detected (supplemental Figure 4). Mature NK cells in the spleen are further subdivided into functionally distinct subsets depending on CD27 and CD11b expression. A 4-stage process from CD11b^{low}CD27^{low} to CD11b^{low}CD27^{high} to CD11b^{high}CD27^{high} to CD11b^{high}CD27^{low} is thought to reflect the developmental program associated with a progressive acquisition of NK-cell effector functions.[34] Again, analysis of the surface markers CD27 and CD11b revealed an unaltered NK-cell maturation in *Ncr1*-iCreTg mice (Figure 2D). Similarly, in vivo NK-cell proliferation in the spleen using BrdU incorporation and in vitro proliferation of IL2-cultured NK cells was unaffected (Figure 2E-F). Finally, given that lysis of target cells is a major NK-cell function, we performed in vitro cytotoxicity assays using MACS-purified, IL2-expanded splenic NK cells derived from *Ncr1*-iCreTg mice and their littermate controls. Figure 2G summarizes our efforts. YAC-1 cells that express low levels of major histocompatibility complex I (MHC I) were used as target cells, as well as RMA-Rae1γ cells expressing the NKG2D ligand Rae-1.[35] RMA cells served as a negative control because they are not efficiently lysed by syngeneic NK cells. In summary, all results were similar irrespective of the target cells used. No differences in the killing activity of *Ncr1*-iCreTg and their littermate controls were detectable. Overall, these experiments suggest that Cre expression in NK cells impairs neither NK-cell development nor NK-cell function.

NK cells are severely reduced in *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice

The transcription factor STAT5 is an important regulator of B- and T-lymphoid–cell development and function.[23,24] To investigate the role of STAT5 in NK cells, we crossed *Stat5*$^{f/f}$ mice to *Ncr1*-iCreTg animals. *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice were born at the expected Mendelian ratio (data not shown). Hematopoietic organs displayed no gross abnormalities; weight and cellularity of spleens and cellularity of the thymi and bone marrow were unaltered in *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice compared with *Stat5*$^{f/f}$ littermate controls (data not shown and supplemental Figure 5). Analysis of CD3$^-$NKp46$^+$ cells in the spleens and lymph nodes revealed a severe reduction of NK cells in *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice. The NK-cell population was almost entirely absent (Figure 3A-B). Figure 3C shows the total NK-cell number in the spleens and lymph nodes. In contrast, no changes in the T-cell compartment were observed. The numbers of CD4$^+$ and CD8$^+$ T lymphocytes and CD4/CD8 ratios were unaltered in thymi and secondary lymphoid organs (Figure 3D-E).

The few remaining purified NK cells from *Stat5*$^{f/f}$ *Ncr1*-iCreTg spleens showed a PCR band indicative for the deletion of *Stat5* that was present in neither the *Stat5*$^{f/f}$ fraction nor in the *Stat5*$^{f/f}$ *Ncr1*-iCreTg NK cell–depleted flow-through. However, we also detected a PCR band for the floxed allele, indicating an incomplete deletion (Figure 4A). We concluded that the remaining NK cells represent a mixture of (1) NK cells that have not deleted the floxed allele (so-called "escapers") and (2) NK cells that had just deleted the floxed allele, thus showing a deletion band. These "just deleters" would still possess STAT5 at the protein level, enabling their survival. The finding that there were comparable mRNA

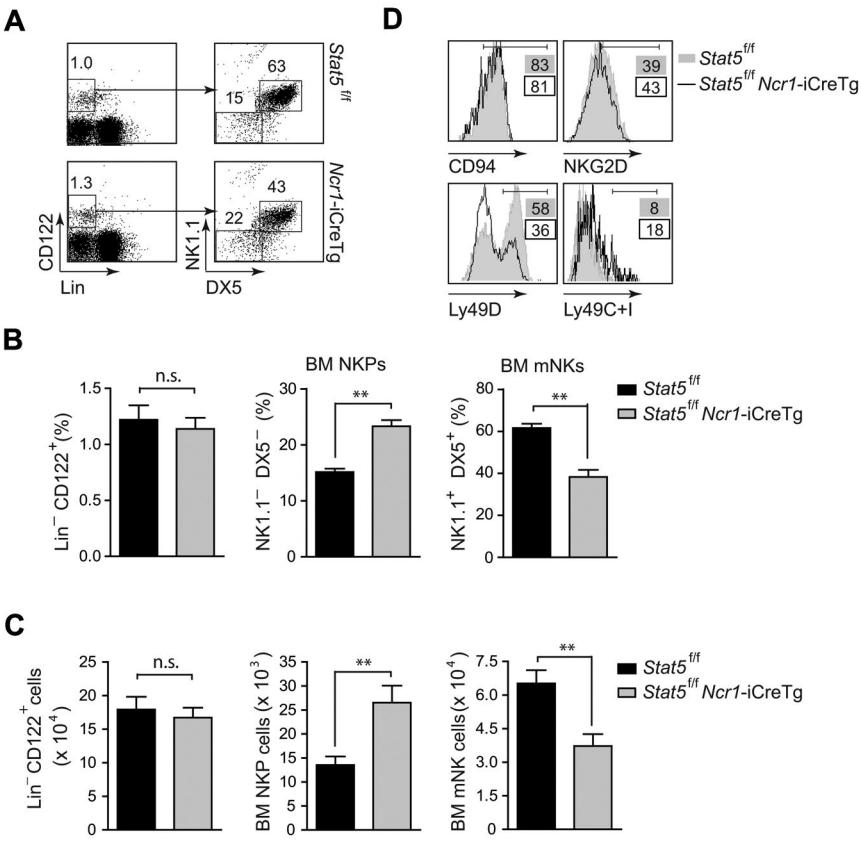

Figure 5. STAT5 is required for early NK-cell development in the bone marrow. (A) Flow cytometry of *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice and littermate controls. Representative dot plots indicate the percentage of gated Lin$^-$CD122$^+$ NKPs (DX5$^-$NK1.1$^-$) and gated Lin$^-$CD122$^+$ mNKs (DX5$^+$NK1.1$^+$) cells. (B) Bar graphs show the percentage of Lin$^-$CD122$^+$ cells, NKPs (DX5$^-$ NK1.1$^-$, gated on Lin$^-$CD122$^+$), and mNKs (DX5$^+$ NK1.1$^+$, gated on Lin$^-$CD122$^+$) in the bone marrow (n = 8 STAT5$^{f/f}$; n = 7 STAT5$^{f/f}$ *Ncr1*-iCreTg, $^{**}P = .0014$, Mann Whitney U test). (C) Bar graphs show absolute cell number of Lin$^-$CD122$^+$ cells, NKPs, and mNKs in the bone marrow (n = 8 STAT5$^{f/f}$; n = 7 STAT5$^{f/f}$ *Ncr1*-iCreTg, $^{**}P = .0014$, Mann Whitney U test). (D) Histograms show the expression of indicated differentiation markers as percentages. (A-D) Data are representative of at least 3 independent experiments. (D) n ≥ 4 per genotype.

levels for *STAT5a* and *STAT5b* in the remaining NK cells compared with their controls suggests that the "escapers" were dominantly present in this mixture (Figure 4B). A comprehensive FACS analysis revealed an expression pattern of NK-cell markers comparable with that of the controls derived from *Stat5*$^{f/f}$ mice, making it unlikely that these remaining NK cells are immature or NK-like cells as described in IL15$^{-/-}$ and IL15R$\alpha^{-/-}$ mice,[36,37] although we cannot entirely exclude this possibility (Figure 4C). After 10 days in culture under IL2, the few purified remaining NK cells were reanalyzed by FACS and PCR. At this time point, a deletion band could no longer be detected. All viable cells expressed *Stat5* and showed a band indicative only for the floxed allele (Figure 4D left panel). The NK-cell nature of these cells was confirmed by their CD3$^-$DX5$^+$ surface expression (Figure 4D right panel). We reasoned that the absence of STAT5 is incompatible with NK-cell viability.

To substantiate these findings, we purified and cultivated NK cells derived from *Stat5*$^{f/f}$ animals. After 3 days of expansion under IL2, the cells were infected with an adenovirus expressing Cre recombinase (Ad/Cre-GFP) or mock-infected (Ad/GFP). Figure 4E summarizes our efforts. Whereas the NK cells tolerated the expression of Ad/GFP (indicated as Stat5$^{f/f}$), NK cells that had received Ad/Cre-GFP had a disadvantage and declined (indicated as Stat5$^{\Delta}$; Figure 4E left panel). The deletion of *Stat5* was confirmed by PCR analysis (Figure 4E right panel). In summary, these observations led us to conclude that STAT5 is required for NK-cell survival.

STAT5 is required for NK-cell development from the NKP to the iNK cell stage

Our data so far indicated that the lack of STAT5 does not allow the survival of splenic NK cells and that STAT5 is indispensable for the viability of NK cells. We next investigated whether the lack of STAT5 would affect NK-cell differentiation in the bone marrow. As depicted in Figure 5A and B, flow cytometric analysis of *Stat5*$^{f/f}$ and *Stat5*$^{f/f}$ *Ncr1*-iCreTg bone-marrow cells confirmed a significant decrease of mNK cells. In contrast, the *Ncr1*-iCreTg–dependent deletion of *Stat5* was accompanied by an increase in NKP numbers. This increase in NKPs points to a developmental block occurring at the very first stage of NK-cell development, whereas no differences in Lin$^-$CD122$^+$ precursor cells were detected. Absolute cell numbers are shown in Figure 5C. Flow cytometric analysis of several NK-cell markers unveiled lower levels of the activatory receptor Ly49D, whereas the levels of the inhibitory receptors Ly49C and Ly49I were higher. In contrast, the levels of CD94 and NKG2D were comparable between the 2 genotypes. This finding reflects the immature nature of *Stat5*$^{f/f}$ *Ncr1*-iCreTg–derived NK cells in the bone marrow (Figure 5D).

Severe impairment of NK cell–dependent, but not T cell–dependent, tumor surveillance in *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice

NK cells are well known for their tumor-suppressive role.[38,39] To investigate whether the *Ncr1*-iCreTg–dependent deletion of *Stat5* is of functional consequence and affects NK cell–mediated anti-tumor activity, we made use of B16F10 melanoma cells.[40] B16F10 cells display low MHC class I levels, indicating a role for NK cells in tumor clearance (supplemental Figure 6). To verify that these cells are indeed exclusively under the tumor surveillance of NK cells, we injected the tumor cell line intravenously into WT mice. These mice were subsequently depleted for either NK cells or cytotoxic T cells using antibodies directed against NK1.1 and CD8, respectively. Twenty-one days

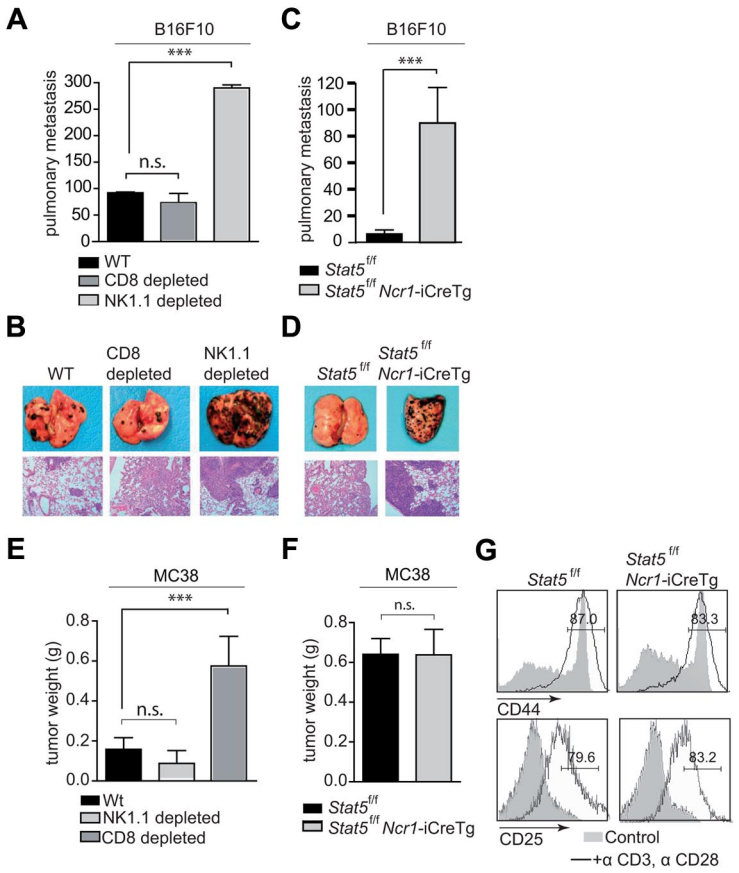

Figure 6. Tumor surveillance of NK cell–controlled tumors is missing in *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice. (A-D) B16F10 cells were injected intravenously into (A) WT, WT depleted of CD8$^+$ cells, and WT depleted of NK1.1$^+$ cells and (C) *Stat5*$^{f/f}$ and *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice. Numbers of metastatic infiltrates per lung were counted under the binocular microscope after (A) 21 days and (C) 12 days. (B,D) One representative example of an infiltrated lung of the indicated genotype is shown. Top panel: photographs, digital camera, Canon EOS 300D. Bottom panel: H&E-stained histological lung sections; magnification, 100× Zeiss Axiolmager 21, 10× objective, NA 0.25, air; camera: Pixelink Color, 1600 × 1200; software: PixelNK Capture 3.0. (E-F) MC38 cells were injected subcutaneously into (E) WT, WT depleted of CD8$^+$cells, and WT depleted of NK1.1$^+$ cells and (F) *Stat5*$^{f/f}$ and *Ncr1*-iCreTg *Stat5*$^{f/f}$ mice, and after 17 days, tumor weights were analyzed. (G) Histograms showing CD44 (top panel) and CD25 (bottom panel) expression on in vitro–activated T cells from the indicated genotypes. CD8$^+$ T cells were cultured under IL2 and stimulated with plate-bound anti–CD3 + anti–CD28 antibodies. Cells were gated on CD3$^+$CD8$^+$ populations. Open histograms indicate the percentage of CD44$^+$ or CD25$^+$ T cells. Gray histograms indicate unstimulated T cells. (A, C, E, F) n ≥ 5 per genotype. (G) Five mice per genotype were pooled. Data are representative of 3 independent experiments.

later, the experiment was terminated and lung metastases were counted. As depicted in Figure 6A and B, depletion of NK1.1$^+$ cells significantly enhanced the formation of tumor nodules in the lung. In contrast, no differences were observed when CD8$^+$ T cells were depleted compared with the WT. Therefore, we next challenged *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice and control mice with B16F10 cells. After 12 days, the experiment was terminated. *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice showed a profound cell infiltration in the lungs. In contrast, only few infiltrating tumor cells were found in the control *Stat5*$^{f/f}$ animals (Figure 6C-D). These data revealed the strongly impaired NK cell–dependent tumor surveillance in *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice. We wondered whether this severe defect in NK-cell development and function would affect T cell–mediated tumor surveillance, so we used MC38 adenocarcinoma cells to determine this.[41] MC38 tumor cells display high MHC class I levels, pointing to a cytotoxic T-lymphocyte–mediated target-cell recognition (supplemental Figure 6). Indeed, these cells were recognized and lysed by CD8$^+$ cytotoxic T cells, as verified by antibody-dependent depletion of either NK cells or CD8$^+$ T cells (Figure 6E). In this case, only the depletion of CD8$^+$ cells significantly enhanced tumor formation, whereas the repeated application of anti–NK1.1 antibody had no effect compared with the WT. When MC38 cells were subcutaneously injected into *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice and *Stat5*$^{f/f}$ littermate controls, we failed to detect any changes in tumor formation between the 2 genotypes (Figure 6F). Similarly, upon T-cell activation in vitro, we did not detect alterations in the expression of activation markers such as CD44 and CD25 on CD3$^+$CD8$^+$ T cells purified from *Stat5*$^{f/f}$ *Ncr1*-iCreTg versus *Stat5*$^{f/f}$ mice (Figure 6G).

Discussion

In this study, we describe the generation of a novel mouse line expressing Cre recombinase under the control of the *Ncr1* promoter. This mouse line expresses the Cre recombinase exclusively in NK cells without affecting other lymphoid compartments, allowing us to delineate the essential and cell-intrinsic role of the transcription factor STAT5 in NK cells.

STAT5 is an important component downstream of cytokines that regulate NK-cell biology, such as IL2, IL7, and IL15. IL2 has been implicated in the regulation of NK-cell activity,[42] whereas IL7 was shown to play a critical role in thymic NK-cell development.[43] The fact that IL15$^{-/-}$ and IL15Rα$^{-/-}$ mice are largely devoid of peripheral NK cells[36,37] suggests that IL15 is a cytokine that is dominant in regulating NK-cell development and homeostasis. The transfer of IL15Rα$^{-/-}$ bone marrow into WT recipients resulted in the rescue of NK cells with greatly reduced expression patterns of the Ly49 receptor repertoire.[44] In fact, NK cells do not need self-expression of IL15Rα, but do require IL15Rα expression by accessory cells in their environment that deliver their signals via trans-presentation.[45] The role of STAT5 in this cytokine-signaling network remained to be determined.

Deletion of *Stat5* in a *Ncr1*-dependent manner prevents normal NK-cell development in the bone marrow. We can unequivocally rule out any toxic effects of Cre recombinase itself because *Ncr1*-iCreTg mice display normal NK-cell development and unaltered NK-cell functions. The simplest explanation for this phenotype is that STAT5 represents a critical survival factor. The

Ncr1-induced expression of Cre recombinase and the subsequent deletion of *Stat5* coincide with the disappearance of the developing NK cells. NCR1 becomes expressed at the iNK-cell stage, when the NK cells start to be greatly reduced in number. The few NK cells present in *Stat5*$^{f/f}$ *Ncr1*-iCreTg animals represent "escapers" because they display unaltered mRNA levels for *Stat5a/b* when quantitatively analyzed by real-time PCR. This scenario is also supported by our in vitro observation of mature NK cells purified from *Stat5*$^{f/f}$ spleens, in which adenovirally mediated Cre expression was incompatible with survival. Whereas *Stat5*$^{f/f}$-derived NK cells tolerated expression of the empty vector, the expression of Cre recombinase led to the disappearance of the cells. Similar observations have been made in B-lymphoid cells, in which STAT5 regulates survival during B-cell development.[23] STAT5 has also been shown to regulate important anti-apoptotic genes such as *Mcl-1*, *Bcl-x*$_L$, and *Bcl-2*.[46] In addition, STAT5 is capable of interfering with and activating the phosphoinositide 3-kinase pathway via growth factor receptor–bound protein 2–associated binding protein 2 (GAB), another signaling pathway important for survival.[47]

A strong argument against this simplistic view restricting STAT5 to a role as the mediator of survival is the fact that significantly higher numbers of NKPs accumulate in the bone marrow of *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice. This accumulation is indicative of a developmental block at the NKP stage, and suggests that the decline in STAT5 expression at the transition to the iNK-cell stage is incompatible with further development. The hindered transition into the iNK-cell stage leads to an accumulation of NK cells at the NKP stage. A developmental block is also reflected by the immature nature of bone marrow NK cells, as indicated by the altered expression levels of the activatory receptor Ly49D and the inhibitory receptors Ly49C and Ly49I. The incompleteness of the phenotype, with a reduction but not a complete absence of iNK cells, most likely reflects the long half-life of the STAT5 protein. We have observed that the genomic deletion of *Stat5a/b* in B-lymphoid cells induces apoptosis only after 7 days due to the slow protein degradation (A. Hoelbl, unpublished data, January 2010).

The molecular mechanisms and transcriptional machinery guiding NK-cell development is largely unknown. Important insights have recently been made by showing the essential functions of the transcription factors ID2 and E4BP4.[48-50] The developmental block that we observed in *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice imitates the phenotype of E4BP4- and IL15R-deficient mice. It is therefore attractive to speculate that this axis, IL15/IL15R-STAT5-E4BP4, determines the fate of NKPs. Further studies are required to delineate the transcriptional network downstream of STAT5 that drives NK-cell development.

The lack of functional NK cells is also confirmed from our in vivo experiments using B16F10 melanoma cells. Tumor nodules in the lung evolve significantly faster in *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice. It is not manageable to evaluate the cytolytic capacity of *Stat5*-deficient NK cells because the few NK cells present in *Stat5*$^{f/f}$ *Ncr1*-iCreTg animals are "escapers" and do express STAT5. Therefore, it is difficult to relate our findings to the report describing a reduced cytolytic response in whole splenocytes derived from *Stat5b*$^{-/-}$ mice in response to IL2 and IL15.[21] These animals lack STAT5B in all cells involved, so any effects are therefore difficult to attribute to a single cell compartment. One might also envision that alterations in cytokine secretion or composition may contribute to these previous observations.

Despite the tight connection between the innate and adaptive arms of the immune system, the severe reduction of NK cells in *Stat5*$^{f/f}$ *Ncr1*-iCreTg mice did not affect the tumor surveillance of MC38 cells, which is attributed to CD8$^+$ T lymphocytes. Thus, in our experimental setting, the lack of NK cells did not influence the adaptive counterpart in tumor immune surveillance.

Intensive efforts are currently under way to develop inhibitors for STAT proteins to be used in clinical research. STAT3 and STAT5 are prime targets because both are implicated in tumor formation and maintenance and both have been shown to be potent proto-oncogenes.[51] The constitutive activation of STAT5 is prominent in hematological malignancies, where it may exert a key role in maintenance of the malignant state.[52,53] Blocking STAT5 activation in adult mice is surprisingly well tolerated; we recently observed that deleting *Stat5* in adult mice for a period of several weeks was only accompanied by minor side effects, including a decrease in B-cell numbers.[54] In these investigations, no information on NK cells was collected. However, based on the results of the present study, we anticipate that an inhibition of STAT5 would be associated with a strong decrease in NK-cell numbers and a severe reduction of NK cell–mediated tumor surveillance. This might be particularly problematic in some cases of leukemia. NK cells are capable of recognizing and eradicating leukemic cells very efficiently, as has been demonstrated in mice and humans.[55] They become particularly important for the clearance of residual disease because even after an effective and successful chemotherapy, some leukemic cells remain in the body and have to be cleared by the immune system. Thus, it will be important to evaluate which malignant diseases are subjected to NK cell–mediated tumor surveillance before employing inhibitors directed against STAT5.

Acknowledgments

We are grateful to F. Colucci, A. Jamieson, and T. Decker for support and discussions. We thank W. Mikultis for providing Adeno supernatants, W. Ellmeier and J. Raberger for CD1d tetramers, L. Hennighausen for the *Stat5*$^{f/f}$ mouse, and S. Kawamoto for the *eGFP*^{LSL} mouse. We thank the Cell Sorting Core Unit of the Medical University Vienna for expert cell sorting.

Financial support for this project was provided by grants from the Austrian Science Foundation (FWF-SFB 28), the Vienna Science and Technology Fund (WWTF-LS07-037), a GENAU grant (Austromouse II, III), and Biomodels Austria.

Authorship

Contribution: E.E., W.W., E.Z., O.S., E.C., and V.S. designed and performed research; E.E., W.W., E.C., and V.S. analyzed data; D.S., T.K., T.R., M.M., and E.C. provided vital new reagents and analytic tools; and E.E. and V.S. wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

The current affiliation for O.S. is Immunology Frontier Research Center (IFReC), Osaka University, Osaka, Japan.

Correspondence: Veronika Sexl, Institute of Pharmacology, Währingerstrasse 13A, A-1090 Vienna, Austria; e-mail: veronika.sexl@meduniwien.ac.at.

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