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

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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 Stat5f/f Ncr1-iCreTg animals. Stat5f/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 interferon (IFN)γ 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 1), 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 spleen10 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 (γc) 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
STAT5B fulfill largely redundant roles, although STAT5B has been implicated in NK-cell development. 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. 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−/− 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 Stat5ΔN/ΔN mice). The residual Stat5ΔN proteins bind DNA and activate the transcription of some but not all target genes. Furthermore, Stat5ΔN proteins are constitutively active even in the absence of cytokines. Therefore, the interpretation of phenotypes obtained with Stat5ΔN/ΔN 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 Stat5ΔN animals are viable.22 Stat5ΔN mice (henceforth Stat5ΔN 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. Within the T-lymphoid lineage, Stat5 is mainly required for the expansion of CD8+ T cells and T-cell receptor γδ (TCRγδ) lymphocytes. 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.23

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 Stat5ΔN 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 mouse Ncr1 gene via homologous recombination in Escherichia coli. 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 into a bacterial artificial chromosome (BAC; RP23-267N11; purchased from Children’s Hospital Oakland Research Institute, Oakland, CA) harboring the mouse Ncr1 gene. Correctly recombined BACs were transiently electroporated into a bacterial artificial chromosome (BAC; RP23-267N11; purchased from Children’s Hospital Oakland Research Institute, Oakland, CA) harboring the mouse Ncr1 gene. Correctly recombined BACs were transiently electroporated into a bacterial artificial chromosome (BAC; RP23-267N11; purchased from Children’s Hospital Oakland Research Institute, Oakland, CA) harboring the mouse Ncr1 gene.
Proliferation was measured by 3H-thymidine incorporation. NK-cell medium containing 10% fetal calf serum (FCS), NK-cell purification, cell suspensions were incubated with anti-DX5–conjugated goat anti–rabbit immunoglobulin G (Santa Cruz Biotechnology) at 4°C overnight under agitation and counterstained with phycoerythrin-CD16/CD32 (2.4G2; BD Pharmingen) was added to avoid nonspecific effects of Fc receptors. NK-cell purification, expansion, and function were generously donated by Wilfried Ellmeier (Medical University, Vienna, Austria). For flow cytometry, single-cell suspensions were prepared from mice obtained from eBioscience. PBS57-loaded and -unloaded CD1d tetramers were intraperitoneally injected with 1 mg of BrdU (in 100 μL PBS) and after 12 hours, the incorporation of BrdU in 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 3H-thymidine incorporation. Four mice per genotype were pooled. Data are representative of 2 independent experiments. (A) Simplified scheme of NCRI expression in NK-cell development. (B–E) Flow cytometry of NK1.1-iCreTg mice and littermate controls. Dot plots indicate the percentage of (B) gated Lin−CD3− NK cells (NK1.1−DX5−) in the periphery, and (D) expression of Stat5 in BM, (C) gated CD3+ NK cells (NK1.1+DX5−) stained with CD27 and CD11b antibodies (n = 4 per genotype). Data are representative of at least 3 independent experiments. (E) In vitro 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). (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 3H-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 6, NK cells were seeded in triplicate in 96-well plates. After 12 hours, proliferation was measured by standard 3H-thymidine incorporation. Four mice per genotype were pooled. Data are representative of 2 independent experiments. (H) Cytotoxicity of IL2-expanded splenic NK cells purified from indicated genotypes. At day 6, NK cells were seeded in triplicate in 96-well plates. After 12 hours, proliferation was measured by standard 3H-thymidine incorporation. Four mice per genotype were pooled. Data are representative of 2 independent experiments. (I) Cytotoxicity of IL2-expanded splenic NK cells purified from indicated genotypes. At day 6, NK cells were seeded in triplicate in 96-well plates. After 12 hours, proliferation was measured by standard 3H-thymidine incorporation. Four mice per genotype were pooled. Data are representative of 2 independent experiments.
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 × 10^9 B16F10 and subcutaneously with MC38, and monitored daily for disease onset. Tissues were isolated, abundant upstream and downstream flanking DNA. By homology-directed 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^+^-iCreTg–transgenic mice to produce eGFP^+^-iCreTg-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 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, it is also known to be expressed in peripheral NK-like TCRγδ cells. 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 TCRβ 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 (TCRβ^−NK1.1^−Nkp46^−; Figure 1G). We therefore concluded that Cre recombination in Ncr1-iCreTg mice is restricted to NK cells.

**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^+^-iCreTg–transgenic mice to produce eGFP^+^-iCreTg-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 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, it is also known to be expressed in peripheral NK-like TCRγδ cells. 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 TCRβ 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 (TCRβ^−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...
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.13 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 stages14 (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) lиммат cells (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<sub>low</sub>/CD27<sub>low</sub> to CD11b<sub>high</sub>/CD27<sub>high</sub> to CD11b<sub>low</sub>/CD27<sub>high</sub> to CD11b<sub>high</sub>/CD27<sub>low</sub> is thought to reflect the development 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 lиммат cells. 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-Rae1<sub>y</sub> cells expressing the NKG2D ligand Rae-1.35 These 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.

**Figure 4. Loss of STAT5 is incompatible with NK-cell viability.** (A) PCR genotyping of MACS-purified splenic NK cells of *Stat5<sup>f/f</sup>* and *Stat5<sup>f/y</sup>* *Ncr1-iCreTg* mice. Four mice per genotype were pooled. (B) Real-time PCR analysis of *Stat5α* and *Stat5β* mRNA levels of FACS-sorted splenic CD3<sup>-</sup>DX5<sup>-</sup> NK cells. Ten mice per genotype were pooled. (C) Histograms show percentage and mean fluorescence intensity of various NK-cell markers on CD3<sup>-</sup>NKP46<sup>+</sup> 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<sup>-</sup>DX5<sup>-</sup> cells. Four mice per genotype were pooled. (E) MACS-purified splenic NK cells from *Stat5<sup>f/y</sup>* mice were cultured under IL2 (4 mice per genotype were pooled) and infected with Ad/Cre-GFP (indicated as *Stat5<sup>f/y</sup>*) or mock-infected (indicated as *Stat5<sup>f/f</sup>*) Cells that received the empty vector tolerated the expression of Ad/GFP, whereas those that had received Ad/Cre-GFP expressed the Cre recombine 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.
levels for STAT5α and STAT5β 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 Stat5f/f mice, making it unlikely that these remaining NK cells are immature or NK-like cells as described in IL15−/− and IL15Rx−/− 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 with their controls suggesting that the “escapers” were dominantly expressed of Ad/GFP (indicated as Stat5f/f), NK cells that had received Ad/Cre-GFP had a disadvantage and declined (indicated as Stat5f/f; 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 viability. 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 Stat5f/f and Stat5f/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 NKP5 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 Stat5f/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 Stat5f/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<sup>f/f</sup> Ncr1<sup>CreTg</sup> mice. (A–D) B16F10 cells were injected intravenously into (A) WT, WT depleted of CD8<sup>+</sup> cells, and WT depleted of NK1.1<sup>+</sup> cells and (C) Stat5<sup>f/f</sup> and Stat5<sup>f/f</sup> Ncr1<sup>CreTg</sup> 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 AxioImager 21, 10× objective, NA 0.25; air; camera: Pixelink Color, 1600 × 1200; software: PixelINK Capture 3.0. (E-F) MC38 cells were injected subcutaneously into (E) WT, WT depleted of CD8<sup>+</sup> cells, and WT depleted of NK1.1<sup>+</sup> cells and (F) Stat5<sup>f/f</sup> and Ncr1<sup>CreTg</sup> Stat5<sup>f/f</sup> mice, and after 17 days, tumor weights were analyzed. (G) Histograms showing CD44<sup>+</sup> or CD25<sup>+</sup> 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.

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,<sup>42</sup> whereas IL7 was shown to play a critical role in thymic NK-cell development.<sup>43</sup> The fact that IL15<sup>−/−</sup> and IL15Ra<sup>−/−</sup> mice are largely devoid of peripheral NK cells<sup>46,37</sup> suggests that IL15 is a cytokine that is dominant in regulating NK-cell development and homeostasis. The transfer of IL15Ra<sup>−/−</sup> bone marrow into WT recipients resulted in the rescue of NK cells with greatly reduced expression patterns of the Ly49 receptor repertoire.<sup>44</sup> In fact, NK cells do not need self-expression of IL15Ra, but do require IL15Ra expression by accessory cells in their environment that deliver their signals via trans-presentation.<sup>45</sup> The role of STAT5 in this cytokine-signaling network remained to be determined.

Deletion of Stat5 in a Ncr1<sup>−/−</sup>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<sup>CreTg</sup> 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<sup>fl/fl</sup> Ncr1<i>-iCreTg</i> animals represent “escapers” because they display unaltered mRNA levels for Stat5<sub>a/b</sub> when quantitatively analyzed by real-time PCR. This scenario is also supported by our in vitro observation of mature NK cells purified from Stat5<sup>fl/fl</sup> spleens, in which adenosvirally mediated Cre expression was incompatible with survival. Whereas Stat5<sup>fl/fl</sup>-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. STAT5 has also been shown to regulate important anti-apoptotic genes such as Mcl-1, Bcl-x<sub>L</sub>, and Bcl-2. 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.

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<sup>fl/fl</sup> Ncr1<i>-iCreTg</i> 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 Stat5<sub>a/b</sub> 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. The developmental block that we observed in Stat5<sup>fl/fl</sup> Ncr1<i>-iCreTg</i> mice imitates the phenotype of E4BP4<sup>−/−</sup> 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<sup>fl/fl</sup> Ncr1<i>-iCreTg</i> mice. It is not manageable to evaluate the cytolytic capacity of Stat5-deficient NK cells because the few NK cells present in Stat5<sup>fl/fl</sup> Ncr1<i>-iCreTg</i> 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 Stat5<sub>b−/−</sub> mice in response to IL2 and IL15. 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<sup>fl/fl</sup> Ncr1<i>-iCreTg</i> mice did not affect the tumor surveillance of MC38 cells, which is attributed to CD8<sup>+</sup> 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. The constitutive activation of STAT5 is prominent in hematological malignancies, where it may exert a key role in maintenance of the malignant state. 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. 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. 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.

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Authorship


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References


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

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