STAT5 is a key regulator in NK cells and acts as molecular switch from tumor surveillance to tumor promotion

Running title: NK cells as tumor promoters

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Abstract

Natural Killer (NK) cells are tightly regulated by the JAK-STAT signaling pathway and cannot survive in the absence of STAT5. We now report that STAT5-deficient NK cells can be rescued by overexpression of Bcl-2. Our experiments define STAT5 as a master regulator of NK-cell proliferation and lytic functions. While NK cells are generally responsible for killing tumor cells, the rescued STAT5-deficient NK cells promote tumor formation by producing enhanced levels of the angiogenic factor VEGF-A. The importance of VEGF-A produced by NK cells was verified by experiments with a conditional knock-out of Vegf-A in NK cells. We show that STAT5 normally represses the transcription of VEGF-A in NK cells, both in the mouse and in the human. The findings reveal that STAT5-directed therapies may have negative effects: in addition to impairing NK cell-mediated tumor surveillance they may even promote tumor growth by enhancing angiogenesis.

Statement of Significance

The importance of the immune system in effective cancer treatment is widely recognized. We show that the new signal-interceptors targeting the JAK-STAT5 pathway may have dangerous side effects that must be taken into account in clinical trials: inhibiting JAK/Stat5 has the potential to promote tumor growth by enhancing NK cell-mediated angiogenesis.
Introduction

Natural Killer (NK) cells are innate lymphocytes that develop from a common lymphoid progenitor in the bone marrow (1). They represent the first line of defense against infected, stressed and malignant cells. Recent evidence has assigned distinct features and functions to tissue-specific NK cells (2). NK cells have organ-specific properties such as distinct profiles of receptor expression or cytokine production (3). Uterine NK cells secrete high levels of VEGF-A and are involved in placental vascularization. The physiological functions of other organ-specific NK-cell subsets are less well understood (4).

All aspects of NK-cell development are regulated by cytokines, their downstream signaling pathways and transcriptional regulators. These include key cytokines such as IL-2, IL-12, IL-15, IL-18 and IL-21 (5), most of which signal via the common γ chain (5) and activate the JAK-STAT pathway (6). JAK kinases (JAK1-3 and TYK2) bind to cytokine receptors and are activated by ligand/receptor binding. The activated kinase phosphorylates STAT transcription factors (STAT1-STAT6) (6,7).

Consistent with its function as the major STAT protein downstream of IL-7, IL-2 and IL-15, STAT5 is absolutely essential for conventional NK-cell development and survival; Stat5Δ/ΔNcr1-iCreTg mice lack NK cells (8). It is also important for lymphoid cell development (9): STAT5 is constitutively active in a plethora of lymphoid malignancies (10). Recent studies have described somatic Stat5b mutations as active drivers of lymphoid malignancies (11,12) and considerable efforts are under way to develop STAT5 inhibitors. Such inhibitors will not only target malignant hematopoietic cells but will also have severe consequences for immune functions that depend on the activation of STAT5 by cytokines. A precise understanding of the detailed function of STAT5 in immune cells is required to predict the potential side effects of a STAT5-directed therapy.
We now provide the first evidence that STAT5 is more than a master regulator of NK cell survival, proliferation and cytotoxicity: it also inhibits NK cell-mediated tumor angiogenesis by directly suppressing the transcription of the angiogenic factor VEGF-A. This finding forces us to reconsider the potential consequences of STAT5-directed therapies for NK-cell surveilled tumors.

**Results**

**Overexpression of Bcl-2 rescues survival of STAT5-deficient NK cells**

STAT5 regulates the anti-apoptotic genes Bcl-xl, Bcl-2 and Mcl-1 in various cell types (13–17). The lack of expression of these genes would explain the almost complete absence of mature NK cells in Stat5^Δ/Δ^Ncr1-iCre^Tg^ mice. We investigated the level of the transcripts of these genes in NK cells derived from heterozygotic Stat5^Δ/+^Ncr1-iCre^Tg^ mice. We found about 50% of the wild-type number of mature natural killer cells in the periphery of Stat5^Δ/+^Ncr1-iCre^Tg^ mice (Figure 1A), indicative of a gene dosage effect. The reduced levels of Stat5a and Stat5b are paralleled by decreased levels of Bcl-xl, Bcl-2 and Mcl-1 (Figure S1A+B). To investigate the association between STAT5 levels, the expression of anti-apoptotic genes and NK-cell survival we crossed Stat5^Δ/Δ^Ncr1-iCre^Tg^ mice - which display a drastic reduction in NKp46^+^ NK cell numbers (8) - to vav-bcl-2 mice. Expression of the Bcl-2 transgene rescues STAT5-deficient NK cells; the numbers of CD3^+^NKp46^+^ cells in the spleen, lymph nodes, liver and lung of Stat5^Δ/Δ^Ncr1-iCre^Tg^-vav-bcl-2 mice are comparable to those in Stat5^0/0^ animals, although they did not reach the levels of vav-bcl-2 transgenic animals. The overexpression of Bcl-2 also leads to an increased number of NK cells in the bone marrow in both Stat5^Δ/Δ^Ncr1-iCre^Tg^-vav-bcl-2 and vav-bcl-2 mice (Figure 1B+ S1C).
Q-PCR analysis of IL-2-cultured Stat5Δ/ΔNcr1-ιCreTg-vav-bcl-2 showed drastically reduced levels of both Stat5 isoforms in Stat5Δ/ΔNcr1-ιCreTg-vav-bcl-2 NK cells. The residual STAT5 most likely reflects non-deleting NK cells and “recent deleters”, which retain some STAT5 due to the long half-life of the protein (8) (Figure 1C). PCR confirmed that the Stat5 gene had been deleted in Stat5Δ/ΔNcr1-ιCreTg-vav-bcl-2 NK cells (Figure S1D). We also found a consistent reduction of Stat5a and Stat5b levels in vav-bcl-2 NK cells (Figure 1C) which most likely results from a decreased expression of cytokine receptors in these mice (data not shown). To confirm the rescue of STAT5-deficient NK cells by Bcl-2, we generated Stat5fl/flMx1-Cre-vav-bcl-2 mice, which allow Stat5 to be deleted conditionally by treatment with Poly (I:C). In contrast to Stat5Δ/ΔNcr1-ιCreTg mice, where deletion only affects late NK-cell stages, Stat5fl/flMx1-Cre animals delete Stat5 in all NK cells irrespective of their developmental stage. In line with our previous observations, deletion of STAT5 in Stat5Δ/ΔMx1-Cre mice resulted in the lack of splenic NK cells, whereas Stat5Δ/ΔMx1-Cre-vav-bcl-2 animals had comparable numbers of NK cells to Stat5fl/fl mice (Figure S1E). Deletion of STAT5 and the levels of Bcl-2 were verified by qPCR and Western blotting (Figure S1F-H). The results confirmed that Bcl-2 expression enables cells to survive the deletion of STAT5.

**STAT5 regulates NK cell maturation**

NK cell maturation takes place in distinct stages, which can be distinguished by the expression of CD27 and CD11b. NK cells acquire CD27 before they express CD11b. CD27+CD11b+ NK cells represent cytotoxic NK cells, which cease to express CD27 to become CD27−CD11b+ (18,19). Loss of one allele of Stat5 results in reduced numbers of CD27+CD11b+ NK cells, paralleled by an increase of immature CD27−CD11b− and CD27+CD11b− NK cells (Figure 1D+E). The absence of both Stat5 alleles in Stat5Δ/ΔNcr1-ιCreTg-vav-bcl-2 mice is associated with the appearance of
highly immature NK cells (CD27^{-}CD11b^{-}) (Figure 1D). A similar pattern was observed in vav-bcl-2 mice, as Bcl-2 expression stabilizes the immature NK-cell stage (20). NK-cell maturation can be induced by type 1 interferon in vav-bcl-2 mice, which is blocked by the absence of Stat5 in Stat5^{Δ/Δ}Mx1-Cre-vav-bcl-2 mice (Figure 1E). Thus STAT5 is involved in NK-cell maturation; NK cells overexpressing Bcl-2 do not mature but can be stimulated to do so. The impaired NK-cell maturation in Stat5^{Δ/+}Ncr1-iCre^{Tg}, vav-bcl-2 and Stat5^{Δ/Δ}Ncr1-iCre^{Tg}-vav-bcl-2 mice is paralleled by significantly decreased expression of the transcription factor Id2 (Figure 2A, S2A). It is also characterized by reduced mRNA and protein levels of the T-box transcription factor family members T-bet and Eomes (Figure 2A, S2B-E), although the results with T-bet did not consistently reach statistical significance (Figure S2B-E).

**STAT5 regulates NK cell proliferation and the lytic machinery**

STAT5 regulates genes involved in cell-cycle control, including c-myc and D-type cyclins (21,22). To investigate the requirement for STAT5 in NK cell proliferation, we analysed growth curves of purified NK cells maintained in IL-2. While Stat5^{β/β} and vav-bcl-2 NK cells proliferate with comparable kinetics, Stat5^{Δ/Δ}Ncr1-iCre^{Tg}-vav-bcl-2 NK cells have a pronounced growth disadvantage (Figure 2B). The reduced proliferative ability of STAT5-deficient NK cells was confirmed in vivo using BrdU incorporation (Figure 2C). Stat5^{Δ/Δ}Ncr1-iCre^{Tg}-vav-bcl-2 NK cells displayed reduced expression of cyclin D2 (CcnD2) and c-Myc (Myc), prominent regulators of growth and cell-cycle control (Figure 2D). In contrast to proliferation, apoptosis did not appear to be affected by the absence of Stat5 in Stat5^{Δ/Δ}Ncr1-iCre^{Tg}-vav-bcl-2 NK cells, as revealed by AnnexinV/7-AAD staining (Figure S2F).

NK cells produce high levels of perforin, granzymes and IFN-γ (23). To test whether STAT5 is involved in the regulation of NK-cell cytotoxicity we analysed mRNA and protein levels of
Perforin (Prf1), Granzyme A/B (Grzma/b) and IFN-γ (Ifng). Stat5\(^{Δ/Δ}\)Ncr1-iCre\(^{Tg}\)-vav-bcl-2 NK cells consistently showed a significant reduction in the levels of all effector molecules (Figure 2E, Figure S3A-C). In line with the reduced STAT5 activation found in non-activated ex vivo-derived vav-bcl-2 NK cells we found a decrease in levels of the effector molecules (Figure 2E and S3A). In contrast, no differences were observed upon activation in interferon-treated cells (Figure S3B+C). In vitro cytotoxicity assays confirmed the physiological significance of these observations: NK cells with diminished levels of STAT5 cannot efficiently lyse target cells (Figure 2F). Supporting evidence was provided by adoptive transfer of IL-2-expanded NK cells into Rag2\(^{-/-}\)gc\(^{-/-}\) mice: in the absence of STAT5 the NK cells were unable to suppress the growth of RMA-S cells (Figure S3D).

**Loss of STAT5 in NK cells is associated with a tumor-promoting effect in vivo**

To study NK cell-mediated tumor surveillance in vivo, we injected 5x10\(^4\) B16F10 melanoma cells into the tail vein of Stat5\(^{fl/fl}\), Stat5\(^{Δ/Δ}\)Ncr1-iCre\(^{Tg}\), Stat5\(^{Δ/Δ}\)Ncr1-iCre\(^{Tg}\)-vav-bcl-2 and vav-bcl-2 mice. Survival (Figure 3A) and tumor nodules in the lung (Figure 3B) were analysed in two independent experiments. Despite the presence of normal numbers of NK cells in Stat5\(^{Δ/Δ}\)Ncr1-iCre\(^{Tg}\)-vav-bcl-2 mice (Figure 1B), no efficient rejection of the melanoma cells was observed: the onset of disease in Stat5\(^{Δ/Δ}\)Ncr1-iCre\(^{Tg}\)-vav-bcl-2 mice was as accelerated as in Stat5\(^{Δ/Δ}\)Ncr1-iCre\(^{Tg}\) mice (Figure 3A). The majority of vav-bcl-2 mice were able to reject the B16F10 cells efficiently, possibly related to the higher numbers of NK cells (Figure 1B). To analyse tumor burden in the lung, mice were again injected with 5x10\(^4\) B16F10 tumor cells and sacrificed after 23 days. Numbers of tumor nodules in Stat5\(^{Δ/Δ}\)Ncr1-iCre\(^{Tg}\)-vav-bcl-2 mice were similar to those in Stat5\(^{Δ/Δ}\)Ncr1-iCre\(^{Tg}\) mice (Figure 3B). The presence of NK cells expressing high levels of Bcl-2 but lacking STAT5 was not sufficient to control tumor growth (Figure 3A+B).
NK cells also play a prominent role in the surveillance of hematopoietic tumors (24–26). We subcutaneously (s.c.) injected the hematopoietic cell line RMA-S and v-abl transformed cells into Stat5\(^{Δ/Δ}\)Ncr1\(-\)iCre\(^{Tg}\)-vav-bcl-2 mice. Both experiments had to be terminated after about a week (day 6 for v-abl and day 9 for RMA-S cells) as tumors developed extremely rapidly in Stat5\(^{Δ/Δ}\)Ncr1\(-\)iCre\(^{Tg}\)-vav-bcl-2 mice. The accelerated tumor growth in Stat5\(^{Δ/Δ}\)Ncr1\(-\)iCre\(^{Tg}\)-vav-bcl-2 mice gave rise to a high tumor mass, while Stat5\(^{Δ/Δ}\)Ncr1\(-\)iCre\(^{Tg}\) mice had small tumors at the same time point (Figure 3C-E for v-abl, S4A+B for RMA-S). We reasoned that at this early tumor stage the residual “non-deleting” NK cells in Stat5\(^{Δ/Δ}\)Ncr1\(-\)iCre\(^{Tg}\) mice were able to control tumor growth. Importantly, we did see significant differences between Stat5\(^{fl/fl}\) and Stat5\(^{Δ/Δ}\)Ncr1\(-\)iCre\(^{Tg}\) mice under challenge with a higher number of cells when the tumor burden was examined at later stages, confirming the critical role of NK cells (Figure S4C). Despite the presence of equal numbers of NK cells in Stat5\(^{Δ/Δ}\)Ncr1\(-\)iCre\(^{Tg}\)-vav-bcl-2 and Stat5\(^{fl/fl}\) mice, a tumor-promoting effect was only observed in the presence of STAT5-deficient NK cells; the effect could be reversed by depletion of NK cells (Figure 3F, S4D).

This result suggests that NK cells that survive the loss of STAT5 are able to accelerate tumor formation and that STAT5 acts as a molecular switch in NK cells, regulating tumor suppression and tumor promotion. Irrespective of the ability of NK cells to recognize and eradicate tumor cells, lowering STAT5 levels in NK cells will exacerbate tumor growth. We tested this interpretation in a tumor model that is not subject to immune surveillance by NK cells (25,27). Upon s.c. injection, RMA cells induced tumor development to a comparable extent and with similar kinetics in Stat5\(^{Δ/Δ}\)Ncr1\(-\)iCre\(^{Tg}\), Stat5\(^{fl/fl}\) and vav-bcl-2 animals. Tumor growth was significantly accelerated in Stat5\(^{Δ/Δ}\)Ncr1\(-\)iCre\(^{Tg}\)-vav-bcl-2 mice (Figure 4A+B). To verify that the tumor-promoting potential resides within the Stat5-deficient NK cells, we depleted NK cells
using an anti-NK1.1 antibody in Stat5ΔΔNcr1-iCreTg-vav-bcl-2 mice and controls. NK-cell depletion abrogated the tumor-promoting potential and Stat5ΔΔNcr1-iCreTg-vav-bcl-2 animals had a tumor burden comparable to that of Stat5fl/fl mice (Figure 4C). This clearly demonstrates that the NK cells themselves are responsible for the enhanced tumor progression.

**NK cells secrete VEGF-A and promote endothelial cell growth**

The development of subcutaneous tumors relies on the formation of new blood vessels (28). CD31 is a marker for angiogenesis and we found significantly more CD31+ cells in tumors derived from Stat5ΔΔNcr1-iCreTg-vav-bcl-2 mice (Figure S4E). VEGF-A plays a key part in tumor angiogenesis. We detected VEGF-A expression in NK cells under basal conditions. Experiments with IL-2-expanded NK cells showed that Stat5ΔΔNcr1-iCreTg-vav-bcl-2 NK cells have significantly enhanced levels of Vegf-A mRNA compared to Stat5fl/fl and vav-bcl-2 cells (Figure 4D). Comparable results were obtained in Stat5ΔΔMx1-Cre-vav-bcl-2 NK cells: levels of Vegf-A mRNA were significantly increased upon Stat5 deletion (Figure S5A). To test the physiological significance of VEGF-A produced by NK cells we embedded endothelial cells in a 3D collagen matrix and enriched it with the supernatant of in vitro-expanded NK cells. After 24 hours we observed basal sprouting with the supernatant of Stat5fl/fl and vav-bcl-2 NK cells in parallel with basal VEGF-A production, whereas the supernatant of Stat5ΔΔNcr1-iCreTg-vav-bcl-2 NK cells drastically increased the average sprout number as well as the cumulative sprouting length (Figure 4E-F). A neutralizing VEGF-A antibody prevented the increased sprouting (Figure S5B). The pro-angiogenic effect was confirmed in assays with aortic rings embedded in 3D collagen matrices. Supernatants of Stat5fl/fl and vav-bcl-2 NK cells induced a certain level of sprouting but addition of supernatant from Stat5ΔΔNcr1-iCreTg-vav-bcl-2-derived NK cells caused significantly enhanced sprouting (Figure S5C). The supernatants of NK cell cultures thus
contain pro-angiogenic factors, which are produced in higher concentrations in the absence of STAT5.

**NK cell-derived VEGF-A exerts tumor promoting functions in vivo**

We have shown that NK cells produce angiogenic factors that trigger endothelial cell sprouting in vitro and that the tumor-promoting effect observed in Stat5Δ/ΔNcr1-iCreTg-vav-bcl-2 mice correlates with increased tumor angiogenesis. To test the in vivo significance of our findings we crossed Vegf-Aβ/βfl mice to Ncr1-iCreTg mice to produce Vegf-AΔ/ΔNcr1-iCreTg mice, in which the deletion of Vegf-A is restricted to NKp46+ NK cells. The numbers of NK cells in the periphery (spleen, blood, lymph nodes) were unaffected despite the deletion of Vegf-A in splenic ex vivo-derived NKp46+ NK cells (Figure S6A+B). We found no consistent or significant changes in NK-cell proliferation or maturation (Figure S6C+D). To investigate the role of NK cell-derived VEGF-A in tumor development, we injected v-abl+ tumor cells s.c. into the flanks of the mice. Vegf-AΔ/ΔNcr1-iCreTg mice showed a significantly reduced tumor burden (Figure 5A-C). The reduction in tumor angiogenesis was confirmed immune-histochemically: tumors of Vegf-AΔ/ΔNcr1-iCreTg mice had significantly fewer CD31+ cells (Figure 5D). VEGF-A deficient NK cells were not able to efficiently induce sprouting of endothelial spheroids (Figure S6E). The reduced tumor burden was abolished by depletion of NK cells (Figure S6F+G).

To confirm the pro-tumorigenic potential of NK cell-derived VEGF-A, we injected RMA-S cells s.c. into the flanks of mice (Figure S6H). In addition we used a retrovirally induced leukemia model that triggers a slowly and oligoclonally evolving disease that closely reflects the situation in human patients. We injected Vegf-Aβ/βfl and Vegf-AΔ/ΔNcr1-iCreTg newborn mice with a replication-incompetent ecotropic retrovirus encoding v-abl. Vegf-AΔ/ΔNcr1-iCreTg mice
displayed a significantly increased disease latency and survived longer than $Vegf-A^{b/b}$ controls (Figure 5E). Consistent with the results in Figure 3A and B, $Vegf-A^{b/b}$ and $Vegf-A^{A/A}Ncr1-iCreTg$ mice showed no differences in tumor burden in the B16F10 melanoma model (Figure S6I). This probably stems from the fact that B16F10 melanoma forms multiple small nodules in the lung that require less angiogenesis. In addition the lung is well oxygenized, reducing the need for angiogenesis in evolving small tumors.

Myeloid cells are a source of VEGF-A in the tumor microenvironment (29,30). We found that both myeloid cells and NK cells infiltrate $v-abl^{+}$ tumors in comparable numbers between all genotypes (Figure 5F, S7A-C). Although both NK cells and myeloid cells are present around blood vessels, the results of experiments with VEGF-A-deficient NK cells show the in vivo significance of NK cell-derived VEGF-A for hematopoietic tumors.

**STAT5 regulates VEGF-A production in mice and human**

We have shown that loss or absence of STAT5 is associated with increased levels of VEGF-A. To investigate how STAT5 interferes with the production of VEGF-A we stimulated ex vivo-derived NK cells with IL-2 or IL-2+IL-15. Both cytokines strongly activate STAT5, accompanied by a decrease in $Vegf-A$ mRNA (Figure 6A). STAT5 ChIP analysis of primary NK cells after stimulation showed an increase in STAT5 binding to the $Vegf-A$ promoter (Figure 6B).

To investigate which cytokines affect STAT5 activation in NK cells, we stimulated IL-2-expanded WT NK cells with IL-6, IL-12, IL-15, IL-18, IL-21, IL-23 or IFN-β. While IL-6 and IL-23 do not alter STAT5 activation, IL-10, IL-12, IL-18, IL-21 and IFN-β lead to a clear downregulation of STAT5-Y694 phosphorylation (Figure 6C). In line with the decreased STAT5 activation, there is a clear increase in $Vegf-A$ mRNA levels in NK cells after stimulation with IL-
10, IL-12, IL-18, IL-21 or IFN-β (Figure S8A). STAT5 occurs in two isoforms, STAT5A and STAT5B, which are encoded by adjacent genes and share at least 90% homology. They are generally considered to have highly redundant functions (31) but using NK cells purified from Stat5a and Stat5b knockout mice we found that the amount of Vegf-A mRNA was increased only in the absence of STAT5B (Figure 6D).

Human peripheral blood NK cells are also able to produce VEGF-A, with immature CD16−CD56bright NK cells secreting higher levels than mature CD16+CD56+ NK cells (4). We FACS-sorted both NK-cell populations from healthy human donors (n=8) and analysed the levels of STAT5 and VEGF-A. We found that CD16CD56bright NK cells contain high levels of VEGF-A, accompanied by low levels of STAT5A, B and the downstream target BCL-2 when compared to CD16+CD56+ NK cells (Figure 6E, Figure S8B). Within the CD16CD56bright NK cell population we detected an inverse correlation between VEGF-A and STAT5B, the relevant STAT5 isoform regulating VEGF-A (Pearson Correlation: R=0.689, p=0.003 comparing STAT5A and VEGF-A and R=-0.547, p=0.028 comparing STAT5B and VEGF-A, Figure S8C). The data were confirmed by a scatter plot and a microarray expression analysis comparing resting and IL-2-activated human NK cells (Figure 6F, Figure S8D). Upon stimulation with IL-2, human NK cells upregulate STAT5 target genes such as EOMES, CCND2 and CISH, whereas the expression of VEGF-A is reduced (Figure 6F, Figure S8D).

A number of inhibitors of STAT5 and its upstream kinases is currently the subject of pre-clinical investigations and clinical trials. Treatment of murine IL-2-expanded NK cells with a pre-clinical STAT5 inhibitor caused a significant upregulation of Vegf-A expression and a clear downregulation of the STAT5 target genes Bcl-2, Mcl-1 and Cish (Figure S8E). Treatment of mouse NK cells with Ruxolitinib, an inhibitor of the upstream kinases JAK1/2/3, significantly
increased the levels of Vegf-A mRNA (Figure 7A). Treatment of FACS-sorted CD3⁻CD56⁺
PBMCs with 0.5 µM Ruxolitinib was sufficient to prevent activation of STAT5, as indicated by
STAT5-Y694 phosphorylation (Figure 7B). After a three-hour treatment with the inhibitor,
human peripheral blood NK cells expressed significantly higher levels of VEGF-A (Figure 7C),
suggesting that Ruxolitinib not only inhibits STAT5 signaling but also stimulates the secretion of
pro-angiogenic factors. This finding is of direct relevance to patients. The effect is observed in vivo: Ruxolitinib treatment of Vegf-Afl/fl mice subcutaneously injected with v-abl⁺ tumor cells
gave rise to an increased tumor burden that was not observed in Vegf-AΔ/ΔNcr1-iCreTg mice
(Figure 7D+E). This experiment confirms that Ruxolitinib accelerates tumor progression by
enhancing NK-cell-derived VEGF-A expression.

Discussion

We report that STAT5-deficient NK cells are rescued by overexpression of the anti-apoptotic
gene Bcl-2. This finding enabled us to investigate the functions of STAT5 in NK cells. STAT5 is
a key regulator of NK-cell maturation, proliferation and cytotoxicity. Not only does STAT5
regulate the transcription of genes that have been identified as targets in other cell types (Myc,
Ccnd2 or Prf1) (32), but also it is involved in the regulation of the transcription factors Eomes
and Id2. Vav-bcl-2 mice show reduced basal levels of STAT5 in non-activated NK cells, together
with an impairment of NK-cell maturation and reduced levels of effector molecules, while NK
cell proliferation and cytotoxicity are largely unimpaired. Stimulation with cytokines causes
maturation, consistent with the idea that the delay in maturation is related to a decreased level of
cytokine receptors in non-activated bcl-2-transgenic NK cells (data not shown). Vav-bcl-2 mice
are comparable to wild-type mice in many key functions, such as NK-cell tumor surveillance. In contrast, $\text{Stat}^{\Delta/\Delta}\text{Ncr1-iCre^{Tg-vav-bcl-2}}$ mice that lack STAT5 in NK cells show severely hampered NK-cell function.

The most striking finding is that NK cells promote tumor formation in the absence of STAT5. We show that wild-type cytotoxic NK cells secrete significant levels of Vegf-A and that transcription of Vegf-A is suppressed by STAT5, especially by STAT5B. Loss of STAT5 causes increased production of Vegf-A in two mouse models. The increased levels of Vegf-A promote tumor formation. Experiments with conditional knockout mice have confirmed the significance of the pro-tumorigenic role of NK cell-derived Vegf-A: loss of NK cell-derived Vegf-A decreases tumor burden while increasing disease latency.

Immune cells such as tumor-associated macrophages promote tumor angiogenesis and lymphangiogenesis (29,30). However, tumor-infiltrating NKp46$^+$ NK cells are also found in the surroundings of blood vessels and VEGF-A produced by NK cells enhances the formation of lymphoid tumors. Infiltrating immature NK cells that secrete VEGF-A have been detected in patients with non-small cell lung cancer, breast and colon tumors (33,34). The presence of VEGF-A-producing NK cells is associated with a poor prognosis, showing that NK-cell-derived VEGF-A plays a part in human disease. Which cells are primarily responsible for producing VEGF-A in a particular context presumably depends on the type and stage of the tumor. It is currently unclear whether tumor-associated NK cells and macrophages interact to promote tumor growth and angiogenesis. It will be of great interest to test whether NK cells contribute to the polarization of macrophages.
Depending on the context, NK cells may have either cytotoxic or angiogenic functions. We propose that STAT5 regulates the switch between the two effects. There is ample evidence that tumor-infiltrating NK cells have low cytotoxic potential. It has been speculated that treatment with IL-2 and IL-15 re-activates non-cytotoxic NK cells (35). Our data support the idea: these cytokines signal via STAT5 and may help to revert tumor-infiltrating but exhausted NK cells into killers that produce perforin and IFN-γ. Decidual NK cells have previously been shown to possess two types of activity: they normally produce significant amounts of VEGF-A but stimulation with IL-15 switches them to cytotoxic cells (36). We find that cytokines present in the tumor microenvironment, such as IL-10, IL-12, IL-18, IL-21 and IFN-β, decrease the activity of STAT5 (reflected by reduced phosphorylation of STAT5-Y694) while enhancing expression of VEGF-A. These results are consistent with the notion that cytokine-induced STAT5 regulation acts to convert NK cells from cytotoxic killers to tumor promoters.

The development of STAT5 inhibitors is currently at the preclinical stage. To the best of our knowledge, no specific STAT5 inhibitor has entered clinical trials. Nevertheless, several inhibitors of the upstream JAK kinases have been approved by the FDA, e.g. for the treatment of rheumatoid arthritis, and are currently being tested in a wide variety of clinical studies. In patients with advanced myelofibrosis, the JAK1/2/3 inhibitor Ruxolitinib is of clinical benefit. It reduces the tumor burden associated with a reduction in the levels of cytokines including VEGF-A, which is most likely produced by the tumor cells themselves (37). Previous work has shown that long-term Ruxolitinib treatment impairs the maturation and cytolytic functions of NK cells. MPN patients treated with the inhibitor suffer from recurrent infections (38). We now extend these findings by showing that Ruxolitinib significantly increases the amount of VEGF-A in NK cells. Ruxolitinib treatment of mice injected with v-abl+ lymphoma cells caused a significantly
enhanced tumor burden that depends on NK cell-derived VEGF-A. This NK-cell mediated effect is presumably important in cases of minimal residual disease (MRD) controlled by NK cells or in other situations where limited tumor burden is controlled by the immune system, as described in the EEE model (39). Under these circumstances NK cells keep the tumor in check and there is a delicate balance between tumor and immune cells. The balance can be disrupted by the presence of factors that suppress STAT5, such as cytokines or JAK-STAT inhibitors.

We conclude that treatment with inhibitors of STAT5 or its upstream kinase not only hampers the cytotoxic function of NK cells but might also induce Vegf-A expression, thereby worsening the prognosis. NK cells have also been shown to inhibit angiogenesis by secreting IFN-γ (40). The reduced release of IFN-γ in STAT5-deficient NK cells may contribute to angiogenesis, although the molecular details remain to be elucidated. It is becoming clear that NK cells are not mere killing machines but complex players with tightly controlled effector responses. These need to be thoroughly investigated before we can predict with confidence the outcome of STAT5-directed therapy. In the meantime, it is important to monitor very closely the tumor development in patients undergoing clinical trials of JAK/STAT inhibitors, with complementary anti-VEGF therapy indicated if tumor growth is accelerated.

**Methods**

**Mice**

Mice were bred on C57BL/6 background and maintained at the University of Veterinary Medicine Vienna under pathogen-free conditions. Following mice were studied: Vegf-A^{β/β}, Vegf-$A^{Δ/Δ}$Ncr1-iCre^{Tg}, Stat5^{β/β}Mx1-Cre (inducible Cre expression after type 1 interferon response, e.g. after Poly(I:C) injection), Stat5^{β/β}Mx1-Cre-vav-bcl-2, Stat5^{Δ/Δ}Ncr1-iCre^{Tg}, Stat5^{Δ/Δ}Ncr1-iCre^{Tg},
Stat5Δ^{Ncr1-iCre}vav-bcl-2, Stat5^{0/+}, Stat5^{0/β} and vav-bcl-2 littermates (8,41–43). B10;B6-Rag2^{2m1Fwa II2rg^m1Wjl} mice (Taconic) were used for adoptive transfer experiments.

All experiments were carried out with age-matched 6-12 week old mice and were approved by the institutional animal care committee and review board conform to Austrian law (license 66.009/0019-II/10b/2010 14.1.10,68.205/0218-II/3b/2012 and BMWFW-68.205/0103-WF/V/3b/2015). Stat5α/− and Stat5β/− (44,45) were backcrossed with C57BL/6 once and then littermates were crossed for this study.

**Cell culture**

Splenic NK cells were isolated using the MACS positive selection kit (DX5, Miltenyi) and cultured with 5000U/ml rhIL-2 (Proleukin®, Roche). NK cell stimulations were performed in the presence of 5000U/ml rhIL-2 ± 5ng/ml rmIL-12 (R&D), 50ng/ml rmIL-15 (PeproTech), 100U/ml rmIFN-β (Sigma), 5ng/ml rmIL-10 (R&D), 100ng/ml rmIL-21 (Immunotools), 50ng/ml rmIL-23 (R&D), 100ng/ml rmIL-18 (R&D), 50ng/ml rmIL-6 (R&D), 50/1µM Ruxolitinib (Chemietek) or 4 hours with a small-molecule STAT5-Inhibitor (46) and DMSO was used as vehicle control.

RMA and RMA-S cell lines were kindly provided by A.Cerwenka and authenticated by flow cytometry. V- abl+c cell lines were generated in the laboratory of Prof. Veronika Sexl. All lines were tested for the presence of mycoplasma by PCR and surface marker expression by flow cytometry every 6 months (last authentication: November 2015).
**Spheroid sprouting assays**

Murine endothelial cells were suspended in 80% endothelial cell growth medium (RPMI containing 10% FCS mixed 1:1 with Endothelial Cell Growth Medium MV (PromoCell) and 20% methylcellulose (Sigma) and seeded as drops (800 cells/100μl) in nonadherent dishes. The dishes were incubated upside down as hanging drops for 24 hours. Under these conditions, all suspended cells contribute to the formation of a single spheroid per drop of defined size and cell number. Spheroids were harvested and seeded in a collagen matrix and supernatant of IL-2 expanded NK cells or 30ng/ml mVegf-A (PeproTech) was added. 2μg/ml LEAF™ purified anti-mouse VEGF-A Antibody (2G11-2A05, Biolegend) was added for the neutralization experiments. After 24-hours incubation, spheroids were scanned and photographed with an Olympus IX71 microscope using cellSens Dimension Software (Olympus). The sprout number and the cumulative length of the sprouts from each spheroid were calculated with ImageJ software.

**Poly(I:C) treatment**

Mice were treated three times by i.p. injection of 200μg Poly(I:C) (Invivogen) during 14 days. 7 days after the last injection mice were analysed. Activation of the Cre recombinase and deletion of Stat5 results in a global Stat5 deletion in Stat5^{0/b}Mx1-Cre and Stat5^{0/b}Mx1-Cre-vav-bcl-2 including the entire hematopoietic compartment and is denoted as Stat5^{Δ/Δ}Mx1-Cre and Stat5^{Δ/Δ}Mx1-Cre-vav-bcl-2.

**NK cell cytotoxicity**

*In vitro* cytotoxicity assays were performed as previously published (25,47).

**NK cell depletion**
Mice were treated three times by i.p. injection of 100 µg PK136 (anti-NK1.1) antibody before s.c. injection of tumor cells and three times during the tumor progression. Successful deletion of CD3⁻NK1.1⁺NKp46⁺ NK cells was checked in blood of treated animals by flow cytometry.

**In vivo tumor challenge**

In the B16F10 melanoma model, mice were injected i.v. with 5x10⁴ B16F10 cells. After 23 days lungs were analysed. For survival assays, mice were sacrificed at first signs of dyspnea and health detractions.

In the v-abl, RMA and RMA-S tumor model, 5x10⁵-5x10⁶ tumor cells were injected s.c. into the flanks of the mice and the health status was controlled daily. After 6-12 days mice were sacrificed and tumor weight was determined. Tumor diameters were measured with a caliper and the tumor volume in mm³ was calculated by the formula: Volume = (width)² x length/2.

In the A-MuLV model newborn mice were injected with 100 µL of replication- incompetent ecotropic retrovirus encoding for v-abl by s.c. injection as described previously (48). Mice were checked daily for disease onset.
**Drug treatment of mice**

Ruxolitinib (Chemietek) was dissolved to make a stock solution in DMSO and further diluted for oral gavage in PBS containing 0.5% methylcellulose (w/v) and 0.1% Tween 80. Mice were treated continuously once a day after tumor cell injection by oral gavage with 95mg/kg Ruxolitinib.

**Antibodies and flow cytometry**

The following antibodies (clones) were purchased from BD Biosciences: CD3ε(145-2C11), CD3(UCHT1), CD56(B159), CD16(3G8), CD11b(M1/70), CD27(LG.3A10), IFN-γ(XMG1.2) or purchased from eBioscience/Affymetrix: CD3ε(17A2, 145-2C11), CD49b(DX5), NK1.1(PK136), NKp46(29A1.4), Granzyme B(NGZB), T-bet(4B10), Eomes(Dan11mag) and Perforin (eBioOMAK-D). Granzyme A(3G8.5) was purchased from Santa-Cruz Biotechnology. Liver and lung were perfused and liver lymphocytes were obtained by a Percoll (GE Healthcare) gradient. Lung tissue was Collagenase/DNaseI (Sigma) digested prior to FACS staining. Bones were smashed with a mortar and filtered through a nylon mesh to obtain single cell suspension. Whole blood and splenocytes were depleted of erythrocytes and anti-CD16/CD32 (eBioscience) was added to all samples prior to staining. Intracellular stainings were performed with the Foxp3/Transcription factor staining buffer set (eBioscience) after application of a fixable viability dye (eBioscience). BrdU (Sigma) was injected i.p. 16 hours prior to analysis and incorporation was checked using an anti-BrdU antibody (BU20A, eBioscience) and DNase1 (Sigma) digestion according to eBioscience’s protocol. Apoptosis stainings with IL-2 expanded NK cells were performed with Annexin V Apoptosis Detection Kit (eBioscience).
All samples were recorded on a FACS Canto II flow cytometer or sorted on a FACS Aria III (BD Biosciences) and analysed with BD FACS Diva software version 6.1.2 and 7.0.

**Isolation of human peripheral blood NK cells**

Blood of healthy donors was diluted 1:2 with 1xPBS and lymphocytes were isolated using LymphoPrep™ (Axis Shield). CD3⁻CD56⁺, CD3⁻CD16⁻CD56bright and CD3⁻CD16⁺CD56⁺ cells were sorted on a FACS AriaIII (BD) and RNA was isolated by RNeasy Micro Kit (Qiagen).

**ChIP**

MACS-purified and IL-2 cultured NK cells (10⁷) were either left untreated or stimulated with IL-15 for 30 minutes followed by cross-linking using 1% formaldehyde for 10 min at 37 °C. The reaction was stopped by the addition of 0.5M glycine for 5 min. Cell nuclei were prepared and lysed in 1ml of lysis buffer on 4°C o/n. Chromatin was sheared by sonication yielding chromatin fragments between 200-500bp and diluted 2.5-fold in ChIP dilution buffer. IPs were performed at 4°C o/n with an anti-STAT5 antibody (C-17, sc-835, Santa Cruz Biotechnology). Chromatin was pre-cleared using 25µl salmon sperm DNA/protein A-agarose beads and incubated with the antibody o/n. Immune complexes were collected with 25µl beads for three hours and washed with RIPA buffer, high salt buffer, LiCl buffer and TE buffer. Samples were eluted twice in elution buffer (2% SDS, 10mM DTT and 100mM NaHCO₃). DNA cross-linking was reversed by heating at 65 °C o/n followed by proteinase K digestion. DNA was extracted with phenol-chloroform, precipitated in isopropanol and resuspended in TE Buffer. The predicted binding site was obtained using Ensemble for the sequence of the Vegf-A promoter and blasting for the potential STAT binding motif TTC(N)₂⁻⁴GAA. Obtained DNA fragments were analysed by qPCR using the following primer pairs: Vegf-A: Fw:5’-GCATGCATGTGTGTGTGT-3’ and 20
Rev:5’- GGCAGGGACGTATGAGGATA-3’, Cish: Fw:5’-CGCGCTGCTATTGGCCCTCCC-3’ and Rev:5’-GTCTGGGGCCCTGAGCAGTG-3’, CD19<sub>down</sub>: Fw:5’-CCCTCTTCATTCGTTTTCCA-3’ and Rev:5’-CCAGGAAGAATTTGAGAAAAATCA-3’. N-fold enrichment was calculated relative to a negative region downstream of the CD19 gene (CD19<sub>down</sub>).

**Histology**

Tumors were either paraformaldehyde-fixed and paraffin-embedded or OCT frozen. Sections (3μm) were stained with Hematoxylin/Eosin according to standard histological procedures. CD31 (cell signaling #9654S) stainings were performed as previously reported (49). The stainings were scanned and photographed with an Olympus IX71 or a Nikon Eclipse E1000 microscope using cellSens Dimension (Olympus) or NIS-elements (Nikon) software. Stainings of CD31 and NKp46 (29A1.4, Biologend) and CD31 and F4/80 (CI:A3-1, ABD Serotec) were performed with frozen tumor sections as previously reported (50) and scanned with a LSM 5 Exciter (Zeiss) or a Nikon Eclipse E1000 microscope. Tumors were photographed and counted at random areas by three individual researchers in a blinded manner.

**Microarray**

Data were extracted from ArrayExpress dataset E-GEOD-8059 and E-GEOD-50838 (Affymetrix Human Gene 1.1 ST Arrays). The heatmap was generated using ClustVis software (1). FDR, false discovery rate.

**Statistical analysis**

Student’s t-test, one-way ANOVA, Tukey’s post hoc test and Mantel-Cox log-rank tests (survival curves) tests were performed using GraphPad Prism® Software version 5.04 and 6.02 (San Diego California USA). For multiple comparison of survival curves, Bonferroni- correction was applied. Statistical analysis is indicated for each experiment specifically (* p < 0.05; ** p < 0.01; *** p < 0.001).

**Author Contributions**


**Acknowledgements**

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References


Figure Legends

Figure 1: Overexpression of *Bcl-2* suffices to rescue NK cells in the absence of STAT5 and reveals a role of STAT5 in NK cell maturation. (A) Splenocytes of *Stat5^fl/+^* and *Stat5^Δ/+^Ncr1-iCre^Tg^* mice were stained for CD3 and NKp46 and analysed by flow cytometry for the percentage of CD3^-NKp46^+ NK cells gated on lymphocytes. Bar graphs depict mean±SEM (n=13 per genotype). Unpaired t-test was used for statistical analysis. (B) Total NK cell number of splenic CD3^-NKp46^+ NK cells of *Stat5^fl/fl^*, *Stat5^Δ/Δ^Ncr1-iCre^Tg^*, *vav-bcl-2* and *Stat5^Δ/Δ^Ncr1-iCre^Tg^-vav-bcl-2* mice. Splenocytes were analysed by flow cytometry and total cell numbers were calculated. One representative out of 5 independent experiments with n≥5 per genotype is shown. Bar graphs depict mean±SEM, Tukey’s post-hoc test was applied for statistical analysis. (C) RNA was isolated of MACS-purified and IL-2 cultured *Stat5^fl/fl^*, *Stat5^Δ/Δ^Ncr1-iCre^Tg^-vav-bcl-2* and *vav-bcl-2* NK cells and transcribed into cDNA used for qPCR expression analysis of *Stat5a* and *Stat5b*. Data represent means±SEM of two independent experiments. Expression levels were calculated relative to the housekeeping gene *Rplp0* and all values were normalized to *Stat5^fl/fl^* NK cells. Tukey’s post-hoc test was applied for statistical analysis. (D+E) For the analysis of NK cell maturation stages, *Stat5^fl/fl^*, *Stat5^Δ/Δ^Ncr1-iCre^Tg^*, *Stat5^Δ/Δ^Ncr1-iCre^Tg^-vav-bcl-2*, *Stat5^Δ/Δ^Mx1-Cre*, *Stat5^Δ/Δ^Mx1-Cre-vav-bcl-2* and *vav-bcl-2* splenic CD3^-NKp46^+ NK cells were analysed for CD27 and CD11b expression by flow cytometry. Data are representative for at least two independent experiments with n≥7 per genotype (n=3 for *Stat5^Δ/Δ^Mx1-Cre*). Numbers represent mean±SEM. Tukey’s post-hoc test was applied for statistical analysis of each maturation stage. *Stat5^Δ/Δ^Mx1-Cre* and *Stat5^Δ/Δ^Mx1-Cre-vav-bcl-2* and respective controls were Poly(I:C) treated (resulting in a type I interferon response) to induce Cre activation.
Figure 2: STAT5-deficient NK cells possess an aberrant transcription factor expression, hampered proliferation and an impaired lytic machinery. (A) RNA was isolated of MACS-purified and IL-2 cultured Stat5\(^{0/0}\), Stat5\(^{+/+}\)Ncr1-iCre\(^{Tg}\), Stat5\(^{+/+}\)Ncr1-iCre\(^{Tg}\)-vav-bcl-2 and vav-bcl-2 NK cells and transcribed into cDNA for qPCR expression analysis of Id2, Tbx21 and Eomes. Data represent means±SEM of two independent experiments. Expression levels were calculated relative to the housekeeping gene Rplp0 and all values were normalized to Stat5\(^{0/0}\) NK cells. Tukey’s post-hoc test was applied for statistical analysis. (B) MACS-purified Stat5\(^{0/0}\), Stat5\(^{+/+}\)Ncr1-iCre\(^{Tg}\)-vav-bcl-2 and vav-bcl-2 NK cells were IL-2 cultured and living cells were counted every day for growth curve analysis. One representative experiment out of three with similar outcome is shown. Tukey’s post-hoc test was applied for statistical analysis of each time point. (C) BrdU was injected i.p. into mice of the respective genotypes. After 16 hours, splenic cells were isolated and stained for CD3\(^{+}\)NKp46\(^{+}\), fixed, permeabilized, treated with DNase1, stained with anti-BrdU and analysed by flow cytometry. Data represent means±SEM (n≥4 per genotype) Tukey’s post-hoc test was applied for statistical analysis. (D) RNA was isolated of MACS-purified and IL-2 cultured Stat5\(^{0/0}\), Stat5\(^{+/+}\)Ncr1-iCre\(^{Tg}\)-vav-bcl-2 and vav-bcl-2 NK cells and transcribed into cDNA used for qPCR expression analysis of Ccnd2 and Myc. Data represent means±SEM of two independent experiments. Expression levels were calculated relative to the housekeeping gene Rplp0 and all values were normalized to Stat5\(^{0/0}\) NK cells. Tukey’s post-hoc test was applied for statistical analysis. (E) RNA was isolated of MACS-purified and IL-2 cultured Stat5\(^{0/0}\), Stat5\(^{+/+}\)Ncr1-iCre\(^{Tg}\)-vav-bcl-2 and vav-bcl-2 NK cells and transcribed into cDNA for qPCR expression analysis of Prf1, Grzma, Grzmb and Ifng. Data represent means±SEM of two independent experiments. Expression levels were calculated relative to the housekeeping gene Rplp0 and all values were normalized to Stat5\(^{0/0}\) NK cells. Tukey’s post-hoc test was applied for statistical analysis. (F) In vitro cytotoxicity assay of IL-2-expanded primary
NK cells and RMA-S (left panel) or YAC-1 (right panel) target cell lines. The effector:target (E:T) cell ratios ranged from 1:1 to 10:1 and after 4 hours incubation on 37°C the lysis of the targets cells was analysed by flow cytometry. Symbols represent means and error bars indicate SEM of triplicates. Tukey’s post-hoc test was applied for statistical analysis of each E:T ratio. Data are representative of at least two independent experiments with similar outcome.

**Figure 3: STAT5-deficient NK cells show reduced cytolytic capacity.** (A+B) \(5 \times 10^4\) B16F10 melanoma cells were injected i.v. into Stat5\(^{fl/fl}\), Stat5\(^{Δ/Δ}\)Ncr1\(-i\text{Cre}^\text{Tg}\), Stat5\(^{Δ/Δ}\)Ncr1\(-i\text{Cre}^\text{Tg}\)-\text{vav-bcl-2} mice and \text{vav-bcl-2} (n≥6 per genotype). (A) Mice were sacrificed at first signs of paralysis and health detractions. For statistical analysis a Bonferroni-corrected log rank test was applied. (B) The number of tumor nodules in the lung was assessed after 23 days. Tukey’s post-hoc test was applied for statistical analysis. (C+D) \(10^6\) \(v\text{-abl}^+\) leukemic cells were injected s.c. in the flanks of Stat5\(^{fl/fl}\), Stat5\(^{Δ/Δ}\)Ncr1\(-i\text{Cre}^\text{Tg}\), \text{vav-bcl-2} and Stat5\(^{Δ/Δ}\)Ncr1\(-i\text{Cre}^\text{Tg}\)-\text{vav-bcl-2} mice. Data represent means±SEM of three independent experiments (n≥18 per genotype). After 6 days the tumor weight was determined and documented by (C) photographs representing one out of three independent experiments with similar outcome. (D) Statistics represent means±SEM of two independent experiments. Tukey’s post-hoc test was applied for statistical analysis. (E) Tumor growth curve of \(v\text{-abl}^+\) s.c. tumors in Stat5\(^{fl/fl}\), Stat5\(^{Δ/Δ}\)Ncr1\(-i\text{Cre}^\text{Tg}\), \text{vav-bcl-2} and Stat5\(^{Δ/Δ}\)Ncr1\(-i\text{Cre}^\text{Tg}\)-\text{vav-bcl-2} mice. Tukey’s post-hoc test was applied for statistical analysis of each time point. (F) NK cells were depleted twice before and after s.c. injections of \(10^6\) \(v\text{-abl}\) cells. After 10 days the tumor weight of Stat5\(^{fl/fl}\), Stat5\(^{Δ/Δ}\)Ncr1\(-i\text{Cre}^\text{Tg}\), Stat5\(^{Δ/Δ}\)Ncr1\(-i\text{Cre}^\text{Tg}\)-\text{vav-bcl-2} and \text{vav-bcl-2} mice (n≥4 per genotype) was determined. There is no significant increase in the tumor burden of NK cell depleted Stat5\(^{fl/fl}\) and \text{vav-bcl-2} mice as the experiment was terminated at an early time point. Tukey’s post-hoc test was applied for statistical analysis.
Figure 4: Stat5\textsuperscript{Δ/Δ}Ncr1-iCre\textsuperscript{Tg}-vav-bcl-2 NK cells have the potential to trigger tumor promotion and show increased production of VEGF-A. (A+B) 5x10\textsuperscript{5} RMA cells were injected s.c. in the flanks of the mice (n\geq12 per genotype). After 14 days the tumor weight was determined and documented by (A) photographs representing one out of two independent experiments with similar outcome. (B) Statistics represent means±SEM of two independent experiments. Tukey’s post-hoc test was applied for statistical analysis. (C) NK cells were depleted twice before and after s.c. injections of 5x10\textsuperscript{5} RMA cells. After 14 days the tumor weight of Stat5\textsuperscript{fl/fl} and Stat5\textsuperscript{Δ/Δ}Ncr1-iCre\textsuperscript{Tg}-vav-bcl-2 mice (n\geq4 per genotype) was determined. Tukey’s post-hoc test was applied for statistical analysis. (D) RNA was isolated of MACS-purified and IL-2 cultured Stat5\textsuperscript{fl/fl}, Stat5\textsuperscript{Δ/Δ}Ncr1-iCre\textsuperscript{Tg}-vav-bcl-2 and vav-bcl-2 NK cells and transcribed into cDNA for qPCR expression analysis of Vegf-A. Data represent means±SEM of two independent experiments. Expression levels were calculated relative to the housekeeping gene Rplp0 and all values were normalized to Stat5\textsuperscript{fl/fl} NK cells. Tukey’s post-hoc test was applied for statistical analysis. (E+F) Spheroids of endothelial cells were embedded into a collagen matrix and IL-2 media (control), supernantant of IL-2 cultured Stat5\textsuperscript{fl/fl}, Stat5\textsuperscript{Δ/Δ}Ncr1-iCre\textsuperscript{Tg}-vav-bcl-2 and vav-bcl-2 NK cells or mVEGF-A (positive control) were added and sprouting was documented after 24 hours. (E) Representative pictures of one experiment out of two (with similar outcome) are depicted. (F) Average sprout number and cumulative sprouting length was quantified by ImageJ software. Tukey’s post-hoc test was applied for statistical analysis.

Figure 5: NK cell-derived VEGF-A promotes tumorigenesis. (A+B) 10\textsuperscript{6} v-abl\textsuperscript{+} cells were injected s.c. in the flanks of the Vegf-A\textsuperscript{fl/fl} and Vegf-A\textsuperscript{Δ/Δ}Ncr1-iCre\textsuperscript{Tg} mice. After 12 days tumor weight was determined and documented by (A) photographs representing one out of three
independent experiments with similar outcome. (B) Statistics represent means±SEM of three independent experiments (n≥27 per genotype). Unpaired t-test was applied for statistical analysis. (C) Tumor growth curve of v-abl+ s.c. tumors in Vegf-A^fl/fl and Vegf-A^Δ/Δ Ncr1-iCre^Tg mice. Unpaired t-test was applied for statistical analysis of the indicated time points. (D) CD31 staining of v-abl+ tumors (random representative areas) derived from Vegf-A^fl/fl and Vegf-A^Δ/Δ Ncr1-iCre^Tg mice. Number of CD31+ cells per specific field of different tumor sections were counted by two independent researchers in a blinded manner. Unpaired t-test was applied for statistical analysis. (E) Newborn Vegf-A^fl/fl (n=8) and Vegf-A^Δ/Δ Ncr1-iCre^Tg (n=10) mice were injected s.c. with a replication-incompetent ecotropic retrovirus encoding for v-abl. Mice were sacrificed at first signs of paralysis and health detractions. Mantel-Cox log-rank tests was applied for statistical analysis. (F) CD31 (green) and NKp46 (red) co-stainings of v-abl+ tumors (random representative areas) derived from Vegf-A^fl/fl and Vegf-A^Δ/Δ Ncr1-iCre^Tg mice. Number of CD31+ and NKp46+ cells of different tumor sections were counted by two independent researchers in a blinded manner. Number of CD31+ cells per specific field was significantly reduced in tumors derived from Vegf-A^Δ/Δ Ncr1-iCre^Tg mice, while the number of infiltrating NK cells was unchanged. NK cell/CD31+ cell ratio is shown. Unpaired t-test was applied for statistical analysis.

**Figure 6: STAT5B suppresses VEGF-A production by directly binding to its promoter.**

(A) Ex vivo-derived MACS-purified and sorted NK cells were stimulated for 2 hours with IL-2 or IL-2+ IL-15 (2 or 4 hours). RNA was isolated and transcribed into cDNA used for qPCR analysis of Vegf-A mRNA levels. Data represent means±SEM of two independent experiments. Expression levels were calculated relative to the housekeeping gene Rplp0 and all values were normalized to stimulation with IL-2 (2 hours) highlighting the relative decrease. Tukey’s post-hoc test was applied for statistical analysis. (B) Primary IL-2 cultured NK cells were stimulated
for 30 minutes with IL-2 or IL-2+IL-15. The reaction was stopped by addition of formaldehyde. ChIP was performed using an anti-STAT5 antibody and n-fold-enrichment of Vegf-A or Cish (positive control) was calculated relative to the expression of a negative region (“CD19 down”). Statistics represent means±SEM of three independent experiments and tukey’s post-hoc test was applied for statistical analysis. (C) MACS-purified and IL-2 expanded C57BL/6 WT NK cells were stimulated with the respective cytokines for 60 and 200 minutes. Stimulated NK cells were harvested and protein lysates were used for western blot to detect pStat5-Y694. ß-Actin was used as loading control. (D) Stat5 WT (+/+), Stat5a-/- and Stat5b-/- NK cells were MACS-purified and IL-2 expanded for 5 days. RNA was isolated and transcribed into cDNA used for qPCR analysis of Vegf-A mRNA levels. Data represent means±SEM of two independent experiments. Expression levels were calculated relative to the housekeeping gene Rplp0 and all values were normalized to Stat5 WT (+/+) NK cells. Tukey’s post-hoc test was applied for statistical analysis. (E) Lymphocytes of healthy human blood donors (n=8) were enriched and CD16-CD56 bright and CD16+CD56+ NK cells were sorted. RNA was isolated and transcribed into into cDNA for qPCR analysis of human STAT5A, STAT5B, VEGF-A and BCL2 mRNA levels. Expression levels were calculated relative to the housekeeping gene Rplp0 and all values were normalized to CD16+CD56+ cells. Unpaired t-test was used for statistical analysis showing that CD16+CD56+ cells have higher expression levels of STAT5A and STAT5B but lower expression of VEGF-A compared to CD16+CD56 bright cells. (F) Scatter plot showing expression microarray data of resting and IL-2 stimulated human NK cells. Expression values of STAT5A, STAT5B, VEGF-A, EOMES, CCND2 and CISH are highlighted. Microarray data were obtained from ArrayExpress database (E-GEOD-8059).
Figure 7: The upstream kinase inhibitor increases NK cell-derived Vegf-A expression and enhances tumor promotion in vivo.

(A) IL-2 expanded murine NK cells were treated with 0.5µM Ruxolitinib or a vehicle control (DMSO) for 3 hours and stimulated in the last hour additional with IL-2. NK cell-derived Vegf-A mRNA production was analysed by qPCR and calculated relative to the house keeping gene Rplp0. Data represent means±SEM of two independent experiments. Expression levels were normalized to the vehicle control. Unpaired t-test was applied for statistical analysis (B) Human blood NK cells were sorted (CD3−CD56+ n=4) and treated with 0.5µM, 1µM Ruxolitinib or a vehicle control (DMSO) for 3 hours and stimulated in the last hour with 500U IL-2. pSTAT5-Y694 as well as STAT5A/B was detected by western blotting. (C) Human blood NK cells were sorted (CD3−CD56+ n=4) and treated with 0.5µM Ruxolitinib or a vehicle control (DMSO) for 3 hours and stimulated in the last hour with 500U IL-2. NK-cell derived VEGF-A mRNA production was analysed by qPCR and calculated relative to the house keeping gene RPLP0. Expression levels were normalized to the vehicle control. Unpaired t-test was applied for statistical analysis. (D) 10^6 v-abl^+ cells were injected s.c. in the flanks of the Vegf-A^{fl/fl} and Vegf-A^{Δ/Δ}\text{Ncr1-}\text{iCreTg} mice. After tumor cell injection mice were treated once a day with Ruxolitinib or a vehicle control by oral gavage. After 8 days tumor weight was determined and documented by photographs (left). Statistics (right) represent means±SEM (n≥8 per genotype). Tukey’s post-hoc test was applied for statistical analysis. (E) Tumor growth curve of v-abl^+ s.c. tumors injected into Vegf-A^{fl/fl} and Vegf-A^{Δ/Δ}\text{Ncr1-}\text{iCreTg} mice treated with Ruxolitinib or a vehicle control. Tukey’s post-hoc test was applied for statistical analysis of each individual time point.
Figure 2

A

![Graph A](image1)

B

![Graph B](image2)

C

![Graph C](image3)

D

![Graph D](image4)

E

![Graph E](image5)

F

![Graph F](image6)
Figure 4

A

Stat5^{fl/fl}
Stat5^{A/A}Ncr1-iCre^{Tg}
vav-bcl-2
Stat5^{A/A}Ncr1-iCre^{Tg}-vav-bcl-2

1 cm

B

RMA

tumor weight rel. to body weight

C

RMA ± NK cell depletion

tumor weight rel. to body weight

D

Vegf-A

rel. expression

E

IL-2
Stat5^{fl/fl}
Stat5^{A/A}Ncr1-iCre^{Tg}-vav-bcl-2
vav-bcl-2
mVEGF-A

F

sprout number

average number of sprouts

n-fold increase in cumulative sprouting length per spheroid

IL-2 media only
Stat5^{fl/fl}
Stat5^{A/A}Ncr1-iCre^{Tg}-vav-bcl-2
vav-bcl-2
mVEGF-A
Figure 7

A. **Vegf-A**

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B. **Control vs. Ruxolitinib**

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C. **Vegf-A**

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D. Tumor samples

- **Vegf-A^+/+** + Vehicle
- **Vegf-A^+/+** + Ruxolitinib
- **Vegf-A^+/+** + Vehicle
- **Vegf-A^+/+** + Ruxolitinib

**Ruxolitinib treatment**

- **Vegf-A^+/+** + Vehicle
- **Vegf-A^+/+** + Ruxolitinib
- **Vegf-A^+/+** + Vehicle
- **Vegf-A^+/+** + Ruxolitinib

E. **Tumor growth**

- **Vegf-A^+/+** + Vehicle
- **Vegf-A^+/+** + Ruxolitinib
- **Vegf-A^+/+** + Vehicle
- **Vegf-A^+/+** + Ruxolitinib

Graph showing tumor growth over days.