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DISSERTATION

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

angestrebter akademischer Grad

Doktor/in der Naturwissenschaften (Dr. rer.nat.)

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Wien, im August 2010

I would like to express my deeply gratitude to ...

... Prof. Veronika Sexl for proposing me exciting projects, for providing all the conditions required for the realization of my projects and for numerous constructive discussions during my PhD study. Without her guidance and persistent support my PhD would not have been possible

... Dr. Emilio Casanova for supervising me during the generation of the *Nerz* mouse and for always encouraging me throughout my whole PhD life

... Karo, Tiní, Wolfi and Ruth for all their support, emotionally and technically, valuable scientific discussions and for enumerating funny lunchtimes

... Lívi, Nina and Nadine for having been the best office mates I could wish for

... Dagmar for valuable inputs and fruitful discussions

... Julia and Shinya for always being there whenever I was in need

... Andrea, Angelika, Biene, Christina, EMP and Eva Z for a pleasant working atmosphere in the lab

... Bernhard for his deep love and for spending wonderful times together with me

... my parents for respecting my way of life

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SUMMARY

The importance of NK cells in innate immunity as well as for the adaptive immune response has long been characterized. Accordingly, NK cell effector functions have been well studied. In contrast, little is known about NK cell developmental pathways and the transcriptional machinery behind.

The transcription factor STAT5 is essential for regulating proliferation, differentiation and homeostasis of hematopoietic cells. This is also reflected by the fact that deletion of *Stat5* in mice causes embryonic lethality. Conditional knock out studies using B and T cell specific Cre lines revealed an essential role for STAT5 in T and B lymphoid development. However, the lack of a NK cell restricted Cre line has precluded the investigation of STAT5 in NK cells.

During my PhD study I have generated a transgenic mouse line that expresses the Cre recombinase specifically in NK cells. To do so, I used a BAC transgenic approach where the Cre recombinase was expressed under the control of the *Ncr1*^{iCre} promoter. I tested the functionality and lineage specificity by crossing *Ncr1*^{iCre} mice to the EGFP^{LSL} reporter mouse line. I verified that *Ncr1* dependent Cre recombination was restricted to the NK cell compartment. To exclude any toxic effects of the Cre recombinase expression *per se* in *Ncr1*^{iCre} mice, I performed a comprehensive analysis of NK cell development and function.

In order to investigate the role of STAT5 in NK cells, I crossed *Stat5*^{f/f} mice to *Ncr1*^{iCre} transgenic animals. *Stat5*^{f/f} *Ncr1*^{iCre} mice were largely devoid of NK cells in peripheral lymphoid organs. NK cell development was impaired in the bone marrow; NK cell maturation was blocked at the NK cell precursor stage. *In vitro* deletion of *Stat5* in IL-2 expanded NK cells was incompatible with NK cell viability reflecting our *in vivo* data. To investigate the functional consequences of the *Ncr1* dependent deletion of *Stat5*, I performed *in vivo* studies and monitored NK cell mediated antitumor activity. Whereas NK cell mediated tumor surveillance in *Stat5*^{f/f} *Ncr1*^{iCre} mice was strongly impaired, CD8⁺ T cell dependent tumor control was undisturbed. Thus the results obtained from my PhD study demonstrate a cell intrinsic role for STAT5 in NK cell development and reveals that *Ncr1* mice are a powerful novel tool to investigate NK cell development, biology and function.

ZUSAMMENFASSUNG

Aus bisher publizierten Studien wissen wir, dass Natürliche Killer (NK) Zellen eine wichtige Rolle im angeborenen als auch im erworbenen Immunsystem spielen. Im Gegensatz zu den sehr gut charakterisierten Effektorfunktionen dieser Zellen ist nur wenig über die molekularen Mechanismen der NK Zell Entwicklung bekannt. Um das therapeutische Potential dieser Zellen nützen zu können ist es allerdings unumgänglich, diesen Vorgang besser verstehen zu lernen.

Der Transkriptionsfaktor STAT5 ist essentiell für die Regulierung wichtiger zellulärer Funktionen wie Wachstum, Differenzierung und Homeostase. Das Fehlen dieses Gens führt zu embryonaler Letalität in Mäusen. Konditionelle Knockout Studien mit B und T Zell spezifischen Cre Mauslinien zeigen, dass STAT5 elementar für die Entwicklung dieser Zellen ist. In NK Zellen konnte die Rolle von STAT5 *in vivo* noch nicht erforscht werden, es gab bis dato keine NK Zell spezifische transgene Cre Maus Linie.

Während meiner Doktorarbeit generierte ich eine Mauslinie, die es ermöglicht, die Wichtigkeit eines Zielproteins spezifisch in NK Zellen zu untersuchen. Dafür wurde die Cre Rekombinase unter der Kontrolle des *Ncr1* Promotors exprimiert. Die Funktionalität und die Zellspezifität der Cre Rekombinase konnte in NK Zellen mithilfe einer GFP-STOPfloxSTOP Reportermauslinie gezeigt werden. Durch Verpaarung dieser *Ncr1*^{iCre} Linie mit STAT5 geflochten Mäusen konnte erstmals STAT5 spezifisch in NK Zellen deletiert werden. Das Fehlen von STAT5 in NK Zellen führte zu einer gestörten Reifung dieser Zellen im Knochenmark. Die Entwicklung zu reifen NK Zellen war blockiert und es kam zu einer Anhäufung von unreifen Vorläuferzellen. In peripheren lymphoiden Geweben waren kaum reife NK Zellen zu detektieren. Die *in vitro* Deletion von *Stat5* in NK Zellen bestätigte meine *in vivo* Ergebnisse. Um die funktionalen Konsequenzen dieser Deletion zu studieren, führte ich weitere *in vivo* Studien durch, wobei ich die NK-Zell vermittelte Tumorüberwachung studierte. Das Fehlen von STAT5 in NK Zellen führte zu einer stark verringerten Beseitigung von Tumorzellen durch NK Zellen, die Tumorüberwachung des angeborenen Immunsystems war nicht beeinträchtigt.

Die Resultate meiner Doktorarbeit zeigten eine zellintrinsische Rolle von STAT5 für die NK Zell Entwicklung und demonstrierten, dass die hier generierte *Ncr1* Mauslinie zukünftig sehr hilfreich sein wird, um NK Zell spezifische Funktionen zu studieren.

INTRODUCTION

1.1 Natural killer cells

Natural killer (NK) cells represent a lymphoid subset of immune cells that principally mediates innate immunity but additionally fulfills important roles in interacting with the adaptive immune response thereby defining immune responses (1).

NK cells attracted the attention of immunologists some thirty years ago by their ability to spontaneously lyse certain tumor cells *in vitro* without previous sensitization and so the term “Natural Killer” was coined by Eva Klein in 1975 (2). In 1984, Klas Kärre formulated the “missing self” hypothesis, where he proposed that NK cell activation is controlled by inhibitory receptors specific for self molecules called major histocompatibility complex (MHC) class I. His idea was a milestone in understanding how NK cells discriminate between self and aberrant cells (3, 4). The initial view has undergone some modifications and nowadays it is known that target cell recognition and NK cell activation is regulated by multiple germ-line encoded activating as well as inhibitory receptors with a delicate balance of positive and negative signals (5). With this heterogeneous arsenal of receptors, NK cells are not only capable of recognizing and eliminating tumor cells but also cells infected by certain viruses or parasites (6-8). Moreover, NK cells secrete a wide range of cytokines and chemokines such as interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-13 and RANTES, thereby orchestrating the interplay of the innate and the adaptive immune system (9). Given this important attributes, most reports in the last decade have concentrated on unraveling the mechanism of target cell recognition and signaling pathways leading to NK cell effector function. In contrast, molecular pathways guiding NK cell development remain largely unknown. Nevertheless, a detailed understanding how NK cells develop is essential to develop the therapeutic potential of NK cells.

1.2 NK cell development and differentiation

In adult mice, NK cell development takes mainly place in the bone marrow (10, 11) and progresses through three major stages defined by the expression of NK cell specific surface markers (12, 13).

Committed NK cell precursors (NKP) originate from multipotent hematopoietic cells and constitute the first developmental stage of NK cell development. NKP express the common β chain of the IL-2 and IL-15 receptors (CD122) and lack surface proteins associated with mature hematopoietic cells (referred to as lineage negative or Lin⁻). Moreover, NKP are negative for known NK cell markers such as NKR1C (CD161c) recognized by NK1.1 antibodies in C57Bl/6 and C57Bl/10 mouse strains, the α_2 integrin CD49b (defined by the DX5 mAb clone) and Ly49 receptors. However, this subset seems to be heterogeneous in that some cells express NKG2D and 2B4 and others not (14). Therefore, some reports suggest that true NKP might be NKG2D and 2B4 positive (only 10 % of NKP give rise to NK cells *in vitro*) (12, 15). Nevertheless, the function of NKG2D on this immature cell type remains to be determined since NKG2D is normally associated with cytotoxicity potential.

As NKP further differentiate into immature (i) NK cells, they progressively acquire NK1.1 and the natural cytotoxicity receptor (NCR) 1, an activating receptor expressed constitutively and exclusively by NK cells (16). NCR1 is involved in the recognition and lysis of various tumor (17, 18) and virus infected cells (19, 20). The ligands of this receptor are not well characterized but heparan-sulfate proteoglycans might be involved (21). Furthermore, receptors specific for MHC class I molecules like CD94 in combination with NKG2A/C/E and certain Ly49 receptors are expressed and tested in a poorly understood process called “NK cell education” to become self tolerant (22). Ly49 receptors are expressed in a monoallelic and variegated manner due to epigenetic regulation and to the use of a bidirectional switch promoter resulting in a stable expression of individual (up to five) Ly49 genes in only a subset of NK cells (23-25). These iNK cells are not cytotoxic *in vitro* towards the lymphoma cell line YAC-1 and do not secrete IFN γ (12).

As differentiation proceeds, the cells subsequently express CD49b and acquire their cytolytic potential and cytokine producing capacity. Upregulation of CD49b defines the mature (m) NK cell stage. This population is expanded in the bone marrow and further maturation is associated with the expression of two additional markers: CD11b (Mac1) and CD43 (sialophorin). Indeed,

increased expression of CD11b as well as CD43 correlates with functional maturity (26). The cells are now fully responsive, emigrate from the bone marrow to peripheral sites and recirculate through the blood. However, not only mNKs have access to the periphery; recent studies suggest that also NKPs and iNKs are found at peripheral sites such as the lymph nodes in human and the liver (26, 27) (**Fig.1**). Liver iNKs, for example, are CD11b^{lo}CD49b^{lo}, express CD94-NKG2 and lack Ly49 receptors. They are poor IFN γ producer but express TRAIL, a TNF family member, capable of inducing apoptosis in target cells via death domains containing TRAIL receptors. Takeda et al have shown that TRAIL⁺ liver iNKs can develop into TRAIL⁻ mNK cells after adoptive transfer experiments *in vivo* (28). These observations reinforce the idea that distinct tissue sites may support NK cell differentiation.

In the thymus, a population of NK cells has been described that display high levels of CD127 (the IL-7 receptor α -chain), low levels of CD11b and lack Ly49 receptors (29). Moreover, they show reduced lytic capacity but secrete abundant pro-inflammatory cytokines such as IFN γ , TNF and GM-CSF when re-stimulated *in vitro*. Thymic NK (tNK) cells preferentially home to the lymph nodes and fail to develop in athymic (*Foxn1*^{-/-}) mice but are barely present in the spleen, bone marrow and liver. Based on their lower cytotoxicity but increased capacity to produce cytokines, it has been suggested that they might play an important role in achieving immune regulatory functions at the thymus and the lymph nodes (30). Unlike conventional NK cells, development of tNK cells requires IL-7 and the transcription factor GATA3. A multipotent early thymic progenitor (ETP) has been described in the liver that compromises the most immature T cell progenitor and is able to differentiate into T cells as well as NK cells (31, 32). This finding is consistent with the idea that tNK cells develop *in situ* from a hematopoietic precursor which seed the thymus and differentiate by a developmental program distinct from that used in the bone marrow (1). Alternatively, NK cell precursors from the bone marrow might emigrate to the thymus where they receive tissue specific signals that induce their altered phenotype.

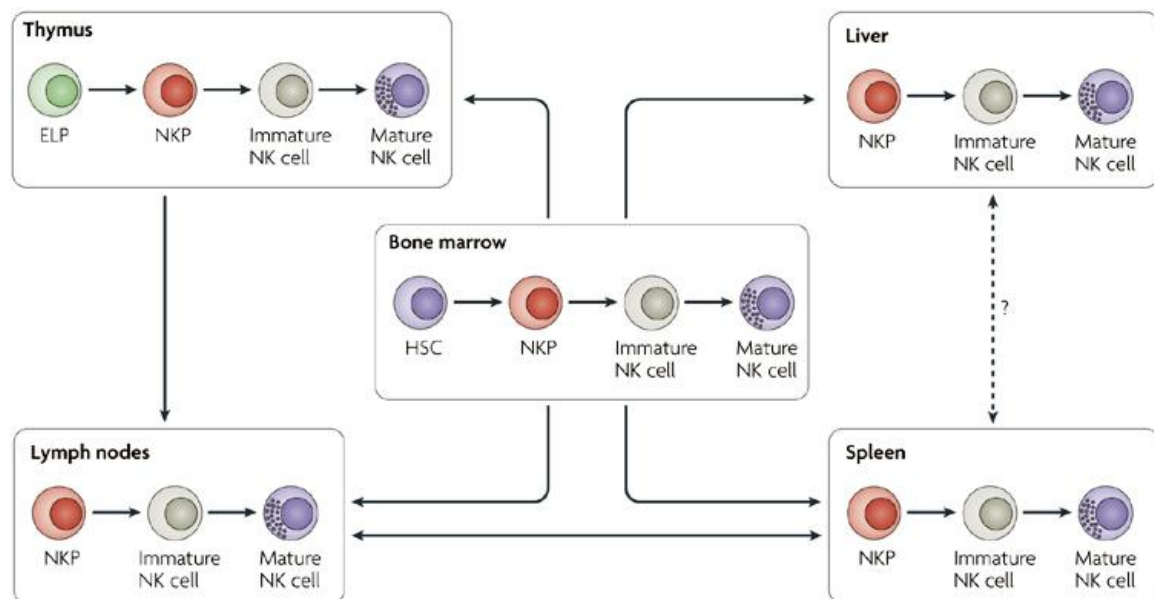


Figure 1. Multiple sites for NK cell development. (Taken from Di Santo 2007 in Nat. Rev. Immunol. (1))

Three distinct stages can be distinguished in the life of a NK cell. At the first stage, NKPs are generated from hematopoietic precursors and ELPs. Committed NKPs give rise to iNK cells at the second stage. This process involves the acquisition and fine tuning of molecules needed for target cell recognition. At the last stage, mNK cells acquire full functional competence. NK cell differentiation takes mainly place in the bone marrow but it occurs also at distinct peripheral tissue sites. It has been suggested that, in addition to mNK cells, NKPs and iNKs may recirculate between the different tissue sites. HSC: hematopoietic stem cell. ELP: early lymphoid precursor. NKP: NK cell precursor. iNK: immature NK cell. mNK: mature NK cell.

Diversity among NK cell

As the distinct tissue sites of differentiation already implicate, NK cells are not homogenous and a large body of evidence exists for specialist NK cell subsets. Different cell surface markers have been used to classify phenotypically and functionally distinct NK cell subsets. As described above, hepatic iNK cells are TRAIL⁺ and tNK cells express CD127. But not only developmental origins contribute to NK cell diversity. Mature NK cells can be distinguished on the basis of their CD11b and CD27 expression. CD11b^{lo}CD27^{hi} NK cells are primarily found in the bone marrow and show a high rate of homeostatic proliferation. CD11b^{hi}CD27^{hi} cells are present in the spleen, liver and lymph nodes and display potent effector function. In contrast, CD11b^{hi}CD27^{lo} cells are poorly cytotoxic and may represent senescent cells (26, 33). It has been proposed that NK cell maturation follows a 4-stage process that starts at a CD11b^{low}CD27^{lo} stage and proceed through CD11b^{lo}CD27^{hi} → CD11b^{hi}CD27^{hi} → CD11b^{hi}CD27^{lo} stages (34). Furthermore, during pregnancy, uterine NK (uNK) cells secrete vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) at significantly higher levels than their peripheral blood counterparts thereby

supporting the remodeling of the arterial systems that may encourage successful implantation and placental development (35, 36). Recently, two different subsets of NK cells in the uterus have been identified: CD3⁻CD122⁺ cells that are indistinguishable from peripheral NK cells and NCR1⁺NK1.1⁻DX5⁻NKG2D⁺ cells that express a unique receptor repertoire (37). However, the origin and the detailed function of uNK cells remains elusive.

More recently, a novel subset of NK cells secreting IL-22 (NK22) has been identified in the mucosa-associated lymphoid tissue (MALT)(38, 39). IL-22 belongs to the family of IL-10 related cytokines and protects the epithelial cell barrier against mucosal pathogens through the upregulation of anti-apoptotic and bactericidal proteins (40-42). Various studies have shown that IL-22 is an important mediator to protect against inflammatory diseases such as hepatitis, autoimmune myocarditis and inflammatory bowel disease (43-46). NK22 cells increase IL-22 production upon IL-23 stimulation, display a CD3⁻NKp46⁺CD127⁺ phenotype and variably express NK1.1 (30). NK22 express a transcription factor called ROR γ t that is known to be required for the development of lymphoid tissue inducer (LTi) cells (47). Mice lacking ROR γ t display significant decreased levels of NK22 cells and fail to develop lymph nodes and cryptopatches (48-50). It has been shown that Rag2^{-/-} mice infected with *C.rodentium* (which lack IL-22 produced from T cells) and depleted of NK cells showed accelerated mortality (48). Therefore, the idea arose that NK22 cell precursor may reside in secondary lymphoid tissues and could differentiate upon microbial-driven inflammation (30). Interestingly, a major subset of NK22 cells are NK1.1⁻ and do not require IL-15, unlike classical NK cells. This phenotype closely resembles LTi cells. LTi cells are found in the gut, depend on the transcription factor ID2 for their development and are mediators in the formation of gut-associated Peyer's patches and other secondary lymphoid organs. In contrast, a minor subset of NK22 cells is NK1.1⁺ and IL-15 dependent. It is currently under debate whether LTi-like and NK22 cells originate from the conventional NK cell lineage and represent two consecutive developmental stages or, alternatively, whether they derive from one precursor through different pathways (30).

One of the most unexpected recent findings is related to the discovery that some mNK cells can be functionally divided into a memory like NK cell subset. As mentioned above, NK cells have traditionally been considered as effector cells of the innate immunity. This is supported by the fact that NK cells (in contrast to T and B cells which belong to the adaptive immunity) do not express recombination-activating gene (RAG) proteins necessary for somatic rearrangement of V(D)J gene segments in order to achieve an almost unlimited number of highly diverse antigen

specific receptors. Another cardinal feature of adaptive immune cells is their immunological memory. However, it has recently been shown that NK cells might cross over this traditional boundary with their potential capacity to learn and remember. This subtype of KLRG1^{hi}CD43^{hi}CD27⁻ NK cells secretes more IFN γ compared to naïve mNKs and has a potent cytolytic capacity (14). O’Leary et al have shown in a provocative study that NK cells can mediate a hapten specific contact hypersensitivity (CHS) response, which is traditionally thought to be a classical delayed type hypersensitivity response conducted by T cells (51). Moreover, the adoptive transfer of NK cells from hapten sensitized mice into naïve animals led to a CHS reaction when recipients were challenged with the original hapten. The CHS response was specifically seen after the transfer of Ly49C/I⁺ liver NK cells. Another study has demonstrated that NK cells activated via the cytokines IL-12 and IL-18 display an NK cell intrinsic, enhanced IFN γ production upon re-stimulation (52). This effect persists for at least three weeks, which is rather long considered that the half life of an NK cell lies between one week and 17 days (53, 54). Additionally, it has been shown that Ly49H⁺ NK cells specifically expand after MCMV infection (55), persist at least two month thereafter and provide an even better protection against MCMV when transferred into newborn mice (56). Although no definitive phenotypic markers for NK cell memory were found, mature subsets of NK cells may exist that have previously been activated by antigens and remain functionally distinct from other mature subsets (30, 57).

1.3 Cytokines and signaling pathways required for NK cell development

Cytokines such as IL-2, IL-4, IL-7, IL-15 and IL-21 are soluble proteins that regulate proliferation, development and homeostasis of lymphocytes. These interleukins belong to the type I cytokine family and signal through receptors that all contain the common cytokine receptor γ chain (γ_c). The receptors for IL-4, IL-7, IL-9 and IL-21 are composed of two polypeptide chains whereas the IL-2 receptor (IL-2R) and the IL-15R are trimeric complexes that have unique α chains responsible for high affinity binding of its cytokine but share a common β chain (IL-2R β , CD122) (**Fig2**). Ligand binding results in the activation of the janus activated kinase (JAK)-signal transducer and activator of transcription (STAT) signaling cascade and other signaling pathways (58).

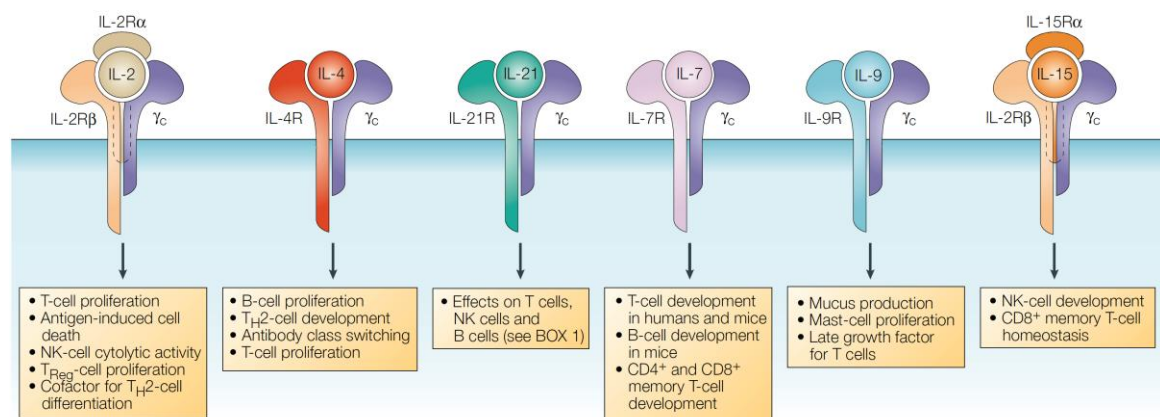


Figure 2. Cytokine receptors regulating proliferation, differentiation and homeostasis of lymphoid cells.

(Taken from Spolski 2005 in Nat. Rev. Immunol. (58))

These cytokine receptors are composed of either two (IL-4R, IL-21R, IL-7R, IL-9R) or three polypeptide chains (IL-2R, IL-15R). All of these receptors share a common cytokine receptor γ chain (γ_c). Each of these receptors harbors a unique component, for example the IL-2R contains a unique IL-2R α chain and the IL-4R contains a unique IL-4R subunit. In addition, the IL-2R and the IL-15R share a common β subunit called IL-2/IL15 β (IL-2R β chain, CD122). Mutations in the γ_c chain lead to a severe disease called X linked severe combined immunodeficiency (XSCID). These type I cytokine receptors activate different signaling cascades such as the JAK-STAT pathway. JAK: janus activated kinase. STAT: signal transducer and activator of transcription

The IL-2R is expressed on NK cells but also on several other immune cells such as T and B lymphocytes, neutrophils and monocytes and is produced by a subpopulation of activated CD4⁺ T cells. IL-2 has been described as T cell growth factor, promotes differentiation of T and B cells, plays a key role in activation induced T cell death and is now considered as a major regulator of T cell responses (59). IL-15 was initially identified through its capacity to stimulate proliferation of an IL-2 dependent cell line in the presence of anti-IL-2 antibodies (60, 61). The existing functional similarities can be in part explained because of the shared IL-2/IL-15R β and γ_c chains, (62, 63). However, a series of later studies have demonstrated that IL-2 and IL-15 exert quite different functions in the immune system and that IL-15 regulates a much broader spectrum of biological processes. This is also reflected by the fact that IL-15 is produced by a large variety of cell types including monocytes, macrophages, DCs, fibroblasts, epithelial cells of various origins and epidermal skin cells, among others (64).

Roles of IL-2 and IL-15 in NK cell biology

Studies have shown that IL-2 administration to bone marrow cultures leads to NK cell differentiation *in vitro* and allows NK cell expansion (59). However, NK cells are present in IL-2^{-/-}

mice (65), even though the NK cell activity was markedly reduced (66). In contrast, IL15^{-/-} mice are severely deficient of peripheral NK1.1⁺ cells (67). IL15Rα^{-/-} mice display a 3.5 fold reduction of CD3⁻DX5⁺ splenic NK cells and whole splenocytes show a severely reduced cytolytic capacity which was attributed to the NK cell compartment (68). Treatment of IL15^{-/-} mice with IL-15 leads to increased NK cell numbers and induces NK cell activity (67, 68). Mice with a targeted deletion of the IL-2/IL-15Rβ chain or γ_c are devoid of peripheral NK cells (69-71). In contrast, IL-7 and IL-4 appears to be neglectable for in NK cell development; IL7^{-/-} mice have increased NK cell levels (15, 72) and IL-4 deficient mice only exhibit a slight reduction of splenic NK cells (15). These studies clearly define IL-15 as the main cytokine regulating NK cell development. Interestingly, IL-15 does not determine NK cell lineage commitment as IL-15 dependent signals are not required for the development of NKPs (15). However, the exact role of IL-15 in later stages of NK cell differentiation, proliferation and maintenance has not been clearly established, although a role for the survival of NK cells has been proposed (73, 74). The few NK cells that develop in the absence of IL-15 exhibit differences in the Ly49 receptor repertoire and display reduced CD11b levels indicating a more immature phenotype. Residual NK cells in a Rag2^{-/-} IL15^{-/-} mouse model are capable of cytotoxicity and IFNγ production although to a lesser extent (15). These findings indicate that IL-15 is the main cytokine involved in NK cell development, survival and expansion but is less essential for NK cell effector function.

One interesting attribute of the IL-15/IL15R signaling complex was recently discovered. Despite conventional soluble cytokine delivery, it has been proposed that intracellular IL-15 binds to a high affinity IL-15 binding protein (e.g. IL15Rα in the endoplasmic reticulum (ER)) that is shuttled to the cell surface where it activates IL-15 signaling components on neighboring cells through a cell-cell interaction (75). This transpresentation extend IL-15 actions from autocrine or juxtacrine signaling to paracrine or endocrine signaling (64) and it allows a more direct and controlled delivery to responsive cells.

The JAK-STAT signaling pathway

As discussed above, cytokines are secreted proteins that mediate intercellular communication thereby regulating important biological functions such as development, proliferation and homeostasis. The binding of a cytokine to its respective receptor leads to the activation of various signaling cascades including the JAK-STAT pathway. In the canonical mode of the JAK-STAT signaling pathway, cytokine binding induces oligomerization of receptor subunits.

Subsequently, the JAK kinases, which are non covalently bound to the receptor, are brought into close proximity, allowing transphosphorylation on tyrosine residues thereby releasing their intrinsic catalytic activity. JAKs then phosphorylate tyrosine moieties in the cytoplasmic region of the clustered receptors. These regions are recognized by Src homology-2 (SH2) domains of monomeric STAT proteins, which become attached to the receptors. The STAT proteins are then phosphorylated by JAKs, dimerize via their SH2 domains, dissociate from the receptor and translocate to the nucleus where they activate gene transcription (**Fig.3**).

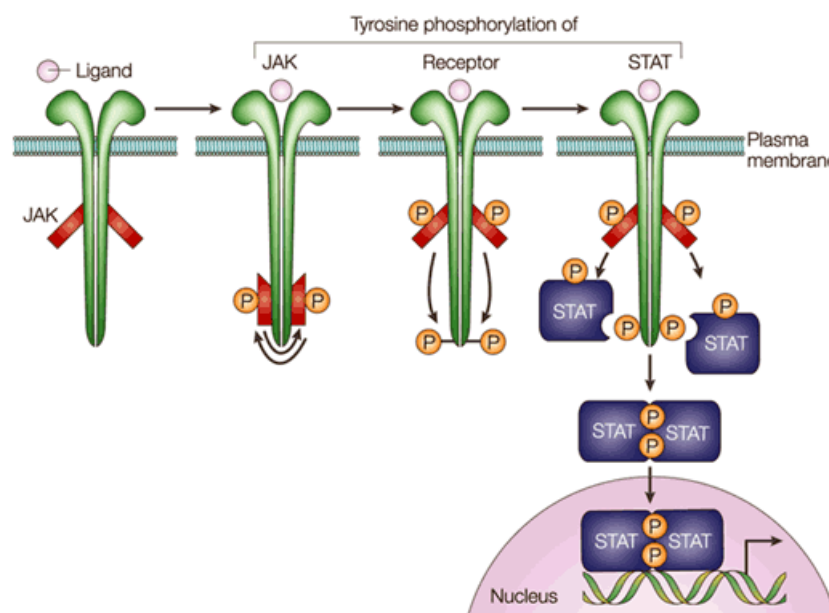


Figure 3. The canonical JAK-STAT signaling pathway. (Taken from Levy 2002 in Nat. Rev. Mol. Cell Biol.) (76)

Following ligand binding to its cognate receptor, receptor associated JAKs are activated. Specific tyrosine moieties on the receptor are subsequently phosphorylated by JAKs thereby creating SH2 docking sites for monomeric STAT proteins. STATs are tyrosinophosphorylated (around residue 700 of their amino acid sequence) by JAKs, dimerize via their SH2 domains, dissociate from the receptor and translocate as dimers into the nucleus where they bind to gene promoters and activate transcription.

A non canonical JAK-STAT pathway has been originally identified in *Drosophila* but recent findings indicate its occurrence also in mammals. It has been shown that a portion of the unphosphorylated STAT pool is located in the nucleus on heterochromatin associated with heterochromatin protein (HP) 1. Upon phosphorylation, STAT dissociates from heterochromatin, which in turn leads to the displacement of HP1 and to heterochromatin destabilization (77). It has been shown that JAK-STAT activation triggers major histocompatibility complex (MHC)

remodeling leading to chromosomal decondensation before transcriptional activation (78). Another study describes IFN γ chromatin remodeling upon JAK-STAT activation during T helper cell differentiation (79). Unphosphorylated STAT proteins have been reported in the nucleus, however, their subnuclear localization relative to heterochromatin has not been clarified yet (80-83). Furthermore, it has been shown that unphosphorylated STATs are constantly shuttling between the cytoplasm and the nucleus (81, 82) and it has been demonstrated that unphosphorylated STAT proteins influences gene transcription by mechanism different from those used by phosphorylated STATs (84). Since JAK nuclear translocation has previously been reported (85) it remains elusive whether JAKs enter the nucleus to phosphorylate STAT proteins or, whether the redistribution of unphosphorylated nuclear STAT proteins follows from a changed equilibrium between nuclear and cytoplasmic (or phosphorylated and unphosphorylated) STATs due to JAK activation (77).

Characteristic features of JAKs and STATs

As described previously, JAKs are tyrosine kinases that are preassociated with membrane bound cytokine receptors as most of these receptors do not possess any kinase activity. Until now, four mammalian JAKs have been identified: JAK1, JAK2, JAK3 and Tyk2. Each JAK molecule contains a conserved kinase domain and a catalytically inactive pseudokinase domain (therefore the name janus kinase from the roman two-faced god, it refers to the idea that JAKs have two-faces consisting of the kinase and pseudokinase domains). The pseudokinase is assumed to regulate the kinase domain. The mammalian STAT family has seven members: STAT1, STAT2, STAT3, STAT4, STAT5A and STAT5B and STAT6. STATs are highly homologous in several regions including a SH2 domain involved in activation and dimerization of STAT molecules, a DNA binding domain, and a transactivation domain located at the C-terminus. The N-terminus is thought to be required for the regulation of STAT activity, such as tyrosine phosphorylation and tetramer formation (**Fig.4**).

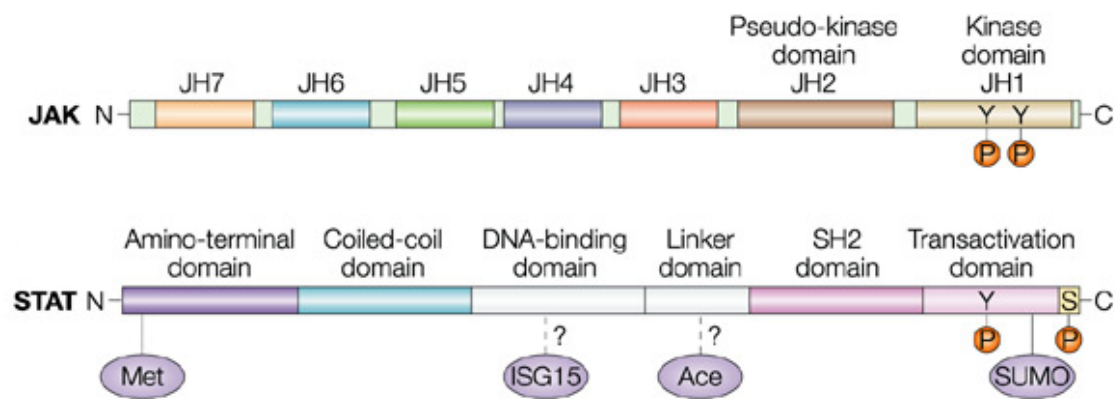


Figure 4. Schematic domain structure of JAKS and STATs. (Taken from Shuai 2003 in Nat. Rev. Immunol. (86))

The domains JH1-JH7 are based on sequence similarity of the four identified JAKs. The JH1 is a functional catalytic domain with two tyrosine residues which are phosphorylated upon ligand binding. Phosphorylation results in conformational changes that facilitate substrate binding. JH2 is the pseudokinase domain probably involved in the regulation of the JAK activity and it might be a potential docking site for STATs. The domain JH3 shares homology with SH2 and JH4-JH7 is known as FERM domains necessary for localizing the protein to the plasma membrane. STATs contain a transactivation domain (TAD) regulating transcription at their C-terminus and alternatively spliced isoforms of STAT1, STAT3 and STAT4 lacking this domain display attenuated transcriptional activity. Serine phosphorylation promotes and enhances gene expression of selected genes and TAD phosphorylation is involved in regulation and crosstalk of different receptors by binding of other transcription factors and coactivators. The activation of STATs can also be regulated by other protein modifications such as methylation (MET), sumoylation (SUMO), ISGylation (ISG15) and acetylation (Ace).

Genetic knockout studies have shown that the loss of JAK kinases leads to severe defects and in the case of JAK2 to embryonic lethality. The JAK-STAT signaling pathway must therefore be tightly regulated. A great deal of work has revealed that JAK-STAT signaling can be regulated at many stages by different mechanisms (86). SOCS proteins are one of the key regulators, normally expressed at low levels in unstimulated cells, which become rapidly induced upon cytokine activation. SOCS proteins are capable of binding directly to tyrosine phosphorylated JAKs leading to inhibition of JAK activity (87-89). Another SOCS member called CIS binds directly to receptor docking sites of STATs thereby competing with STAT proteins. Finally, SOCS proteins are involved in the ubiquitin proteasome pathway mediating the degradation of JAKs and other signaling proteins. Other key regulators are various protein tyrosine phosphatases (PTPs) involved in the negative regulation of the JAK-STAT pathway in the cytoplasm as well as in the nucleus (86). Distinct protein modifications (ubiquitylation and ISGylation) and the cross talk between different JAK-STAT pathways and other signaling cascades provide additional levels of regulation.

Given the fact that approximately more than 25 cytokines signal via a limited panel of JAKs and STATs (and via other signaling pathways leading e.g. to the activation of MAPKs not discussed here), one major unresolved question is how cytokines achieve their specificity. Cytokine signaling could theoretically be made specific by each cytokine binding to its own specific receptor. It turn more and more out, however, that cytokine receptors share particular receptor chains such as the IL2/IL5 β chain or the γ_c , which would limit specificity. Genetic studies have shown that different cells express distinct receptor combinations. Therefore, a single cell may integrate signals from various receptors and different classes of receptors use preferentially a certain JAK combination (65). Hematopoietic cell development and proliferation is for example mediated by cytokine receptors using JAK2, γ_c receptors use JAK1 and JAK3 whereas other receptors use only JAK1 (**Fig. 5**).

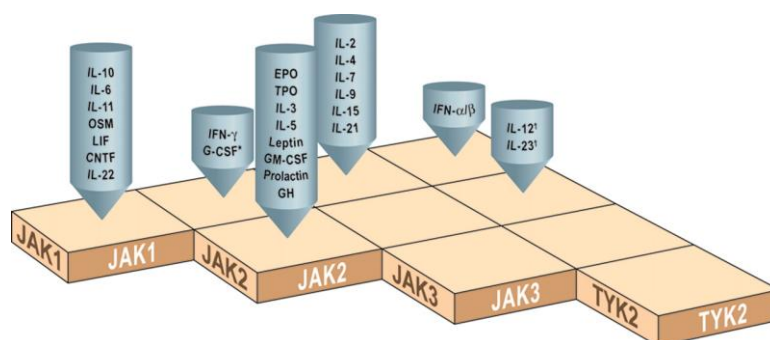


Figure 5. Different combinations of JAKs are used by distinct cytokines. (Taken from Murray 2007 in J Immunol. (90))

Shown are various JAK combinations responding to different cytokines. Distinct cell types and tissues express different receptor combinations unique to the microenvironment. Therefore, a single cell might integrate signals from diverse cytokine receptors. The cytokines IL-2 and IL-15 signal through JAK1 and JAK3 by using the γ_c chain, specificity is mainly reached by the unique alpha chain of the IL2 and IL15 cytokine receptor. Subsequently, STAT proteins are activated and induce a particular set of target genes.

The biology of STAT5

STAT5 consists of two highly homologous isoforms, STAT5A and STAT5B, encoded by two juxtaposed genes. Their transcriptional starts are within 10kb of each other located on chromosome 11 in mice (91). STAT5A and STAT5B cannot entirely compensate for each other and cell preferential transcriptional patterns have emerged (92). STAT5A $^{-/-}$ mice display impaired mammary development and differentiation during pregnancy (93) and T cells from these mice show decreased proliferation secondary to diminished IL-2R α chain expression (94). In contrast,

STAT5B^{-/-} mice resulted in stunted body growth (95) and decreased NK cell numbers (96). The first attempt to create a STAT5A/B knock out mouse resulted in hypomorphic N-terminally truncated STAT5A and STAT5B proteins (referred to as STAT5^{ΔN}) with different expression levels depending on the tissue type (97). Immunological defects of these mice were not as severe as had been expected from mutations resulting in the abortion of the JAK-STAT signaling pathway induced by a number of cytokines. It turned out that residual STAT5^{ΔN} proteins are still able to form dimers (but not tetramers), bind DNA and activate transcription of some target genes even without cytokine stimulation. The development of T and B cells are only minimally affected (98, 99), although peripheral T cells fail to proliferate in response to cytokine signaling and regulatory T cells are diminished in STAT5^{ΔN/ΔN} (100). The interpretation of the obtained phenotype is complex and requires revalidation in a mouse model completely devoid of STAT5 proteins. To solve this problem, conditional mice that carry a 110 kb *Stat5a/b* locus flanked by loxP sites have been generated permitting Cre recombinase based deletion of both STAT5 genes (101). Mice doubly deficient in STAT5A/B proteins are infertile and ≥95 % of the pups die perinatal.

Stat5A and STA5B proteins have essential and largely redundant roles in the development of immune cells and in orchestrating immunoregulation downstream of the IL-2, IL-7 and IL-15 cytokine receptor. STAT5A/B deficiency has a severe impact on the development of B and T cells (102-104). The conditional deletion of STAT5A/B in the T cell lineage results in the loss of cytotoxic T cells, impaired development of regulatory T cells and enhanced production of T helper 17 cells (105, 106).

Moreover, a variety of solid tumors, leukemias, and myeloproliferative disorders (MPDs) are characterized by constitutively active STAT5 proteins providing a survival and proliferation advantage for abnormal cells. STAT5 has been implicated in BCR-ABL induced chronic myeloid leukemia (CML) and in blast cells from patients with acute myeloid leukemia and acute lymphoid leukemia (91). It has been shown that STAT5 is absolutely required for the initial transformation and maintenance of BCR-ABL induced leukemia (107). Taken these findings together, STAT5 may represents a promising and attractive therapeutic target.

Transcription circuitry of the NK cell lineage

In contrast to the growing body of literature on the mechanisms controlling NK cell cytotoxicity, target cell recognition and proinflammatory cytokine production, much less is known about NK cell lineage specification and commitment. Several transcription factors have been characterized

to play distinct roles in developing NK cells, particularly from the iNK cell stage on, these include ETS-1 (108), PU.1 (109), ID2 (110), GATA-3 (111), and MEF (112). Very recently two research groups identified E4BP4 as critical transcription factor required for early NK cell development (113).

The ETs-1 family of transcription factors is composed of nearly 30 proteins that have a related winged helix-turn-helix DNA binding domain. It has been shown that *Ets-1*^{-/-} mice display a 3-fold reduction in NK cell numbers and have a defective cytolytic and cytokine production capacity (108). ETs-1 regulates CD122 in transient transfection assays implicating that this transcription factor promotes IL-15 responsiveness. IL-15 induces ERK-dependent phosphorylation of ETs-1, which in turn increases ETs-1 protein levels and transcriptional activation. Therefore, ETs-1 might be part of a positive feedback loop (114-116). In NKPs, ETs-1 mRNA is increased compared to CLPs and it has been speculated that ETs-1 might function in the initial activation of CD122 expression (23).

PU.1, another ETs family member, is involved in the development of multipotent lymphoid progenitors and is indispensable for B lymphocyte development (117). The deletion of PU.1 in mice leads to embryonic lethality. Fetal liver transplantation into Rag2^{-/-} γc^{-/-} mice showed that PU.1 is not required for mNK cells, even though the number of NKPs and mNKs is reduced (109). It remains elusive whether the diminished number of NKPs is due to an impaired development of multipotent lymphoid progenitors or whether PU.1 functions directly in NK cell lineage specification. Colucci et al further have shown that PU.1^{-/-} mNK cells fail to be activated by IL-2 or IL-12, display an altered Ly49 receptor repertoire and show reduced levels of IL-7Rα and *c-kit* indicating that PU.1 has additional roles in NK cell biology. Interestingly, these cells lyse target cells as efficiently as the wildtype control *in vitro* (109). PU.1 deficient mNK cells display increased ETs-1 mRNA implicating a compensating role for the loss of PU.1. MEF belongs to the ETs-family of proteins as well and mNK cells deficient of MEF have impaired effector functions (112).

T-BET and eomesodermin (EOMES) are T-box (TCACACCT) binding proteins implicated in NK cell development. T-BET is expressed in various immune cells and is known to be required for CD4⁺ T cell development. Mature NK cells from mice deficient for T-BET express *c-kit* and α_v integrin indicating an immature status. However, they also express high levels of CD69 and proliferate extensively suggesting a hyperactivated phenotype (118). T-BET and EOMES directly regulate the genes of IFNγ and CD122 and this combined function of EOMES and T-BET on the CD122

promoter regulates IL-15 responsiveness. It has been shown that these two transcription factors are necessary for the development of CD122^{hi} T-cells (119). Therefore, EOMES and T-BET might cooperate to regulate CD122 during NK cell development. Interestingly, heterozygosity of EOMES does neither effect NK cell development nor NK cell function. However, the additional loss of one or both alleles of T-BET revealed a synergistic loss of mature NK cells (119). It remains elusive, whether these T-box binding proteins are needed for specification of the NK cell lineage from a multipotent progenitor, or whether their functions lie in regulating CD122 expression and responsiveness to IL-15.

GATA3 has been shown to be essential for thymic NK cells (29). In contrast, GATA3 is expressed only at low levels in bone marrow NK cells and is needed for the development of mNK cells expressing CD43 and CD11b. Mature NK cells lacking GATA3 fail to home to the liver and are poor IFN γ producer but capable of target cell lysis (111). Interestingly, these NK cells show reduced levels of T-BET expression indicating a T-BET upstream function in the regulation of IFN γ transcription.

The inhibitor of differentiation (Id) proteins belong to the helix-loop-helix (HLH) family of transcription factors. Some members act as positive regulators, where they bind DNA either as homo- or heterodimers and regulate transcription. In contrast, negatively acting factors lack a DNA binding domain and associate with other members of the Id family, thereby preventing them from binding DNA or forming active heterodimers (120). ID2 is necessary for NKP development in the fetal thymus and for mNKs in the spleen (121, 122). This transcription factor is further expressed in bone marrow NKPs but, interestingly, is dispensable for the development of committed NKPs (110). However, *Id2*^{-/-} bone marrow NKPs showed increased ID3 levels, which might compensate for the loss of ID2. Furthermore, Boos and colleagues demonstrated a requirement for ID2 at the mNK cell stage in the bone marrow. In these cells, ID3 expression is markedly decreased (110). Id proteins are antagonists of E-box (CANNTG) binding (E) protein transcription factors and it has been suggested that the impaired NK cell development in the bone marrow is due to excessive E protein activity since loss of the E family member E2A restores mNK cells in *Id2*^{-/-} bone marrow (110). However, these mNK cells do not express CD11b and *Id2*^{-/-} *E2A*^{-/-} mNK cells fail to accumulate in the spleen or peripheral blood indicating that E2A may not be the only E protein target of ID2 in mNK cells (110). In summary, *Id2*^{-/-} mice have a 90 % reduction of mNK cells in the spleen but have no fewer NKPs or iNKs in the bone marrow. This

developmental block is later than that in the *IL15*^{-/-} mice, which display a much less iNK cell production.

Very recently, the E4-binding protein 4 (E4BP4) has been identified as the first transcription factor that is critically required for NK cell development. E4BP4 is a member of the basic region/leucine zipper (bZIP) transcription factor superfamily and plays an important role in IL-3 dependent pro-B cell survival (123). *E4bp4*^{-/-} mice show no gross abnormalities and have an undisturbed hematopoietic and lymphoid profile with the exception of NK cells. NKPs are normal but the levels of iNK cells and mNK cells are considerable lower. Moreover, CD3⁻NKp46⁺NK1.1⁺ NK cells are conspicuously absent from the bone marrow, spleen and blood (113). Gascoyne et al have further shown that CD8⁺ T cell responses are normal, but classical NK cell effector functions are not detectable, implicating a severe and selective NK cell deficiency (113). *E4bp4* is expressed in NKPs, is upregulated in iNKs and maintained in mNK cells. As described above, trans-presentation of IL-15 by membrane bound IL-15 receptor α chain represents the essential fuel that drives the differentiation from NKPs to iNK cells. The *E4bp4* expression pattern coincides with the IL-15 dependency of the different NK cell developmental stages. This suggests that E4BP4 is the critical transcription factor mediating the biological effects of IL-15. Interestingly, Gascoyne et al have shown that the expression of ID2 is much lower in E4BP4 deficient hematopoietic precursors and that E4BP4 induces the expression of ID2 as well as ectopic expression of ID2 partially restores NK cell development *in vivo*. Taken together, these data suggests ID2 as target of E4BP4 in early lymphoid precursors, which can then promote NK cell fate (124).

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AIM OF THE THESIS

The importance of NK cells in tumor surveillance has been shown previously *in vitro* as well as *in vivo*. Besides tumor cells, NK cells recognize and lyse cells infected by viruses or parasites. Moreover, NK cells secrete cytokines such as IL-13, IFN γ and TNF that stimulate the adaptive and the innate immune system. Thereby, NK cells accomplish an important role in orchestrating the interplay of the innate and adaptive immunity. A great deal of progress has been made in delineating NK cell effector functions, however, the molecular basis of NK cell development and its transcriptional machinery behind is not well understood and has been proposed as one of the most important problems to be addressed in NK cell biology. A better understanding of how NK cells develop into potent effector cells is a prerequisite for understanding their contribution to disease processes as well as for investigating their therapeutic potential. Deletion of key regulatory genes often results in severe phenotypes that interfere with the analysis of a particular cell type. Furthermore, due to redundancies within gene families, other members might compensate for the loss of target genes. Therefore, global deletion of a certain gene often limits the interpretation of an achieved phenotype. To overcome these problems, conditional gene inactivation with the loxP-Cre system has been widely used. However, until now, no NK cell specific Cre line has been reported.

To study NK cell specific functions of the transcription factor STAT5, we generated a mouse line that expresses the Cre recombinase specifically in NK cells. Therefore, we used a BAC transgenic approach where we expressed the Cre recombinase under the control of the *Ncr1* (*p46*) promoter. We verified the functionality and lineage specificity by crossing *Ncr1*^{iCre} mice with the EGFP^{LSL} reporter mouse line. To exclude any possible toxic effects of the Cre expression *per se*, we performed a comprehensive analysis of NK cell development and effector function *in vitro* and *in vivo* in *Ncr1*^{iCre} mice. Finally, we bred the *Ncr1*^{iCre} mice with *Stat5*^{f/f} mice to specifically delete Stat5 in NK cells and to elucidate the possible roles of STAT5 for NK cell development and function.

RESULTS

This section consists of 5 manuscripts. The results from the main project of my doctoral thesis are described in 3.1. Additionally, I contributed to several other studies during my PhD and the results from these projects are described in section 3.2, 3.3, 3.4 and 3.5.

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

Eva Eckelhart¹, Wolfgang Warsch¹, Eva-Maria Zebedin¹, Olivia Simma¹ ^a, Dagmar Stoiber², Thomas Kolbe³, Thomas Rüllicke³, Mathias Mueller³, Emilio Casanova² ^{*} and Veronika Sexl¹ ^{*}. Manuscript in revision (Blood)

3.2 Natural immunity enhances the activity of a DR5 agonistic antibody and carboplatin in the treatment of ovarian cancer.

Ahmed El-Gazzar¹, Paul Perco⁵, Eva Eckelhart², Mariam Anees¹, Veronika Sexl², Bernd Mayer⁵, Yanxin Liu⁶, Wolfgang Mikulits³, Reinhard Horvat⁴, Thomas Pangerl¹, Dexian Zheng⁶ and Michael Krainer¹. Manuscript published in Mol Cancer Ther 2010 Apr;9(4):1007-18

3.3 STAT5 is an important mediator of imatinib resistance in Abelson-induced leukemia.

Wolfgang Warsch^{*}, Karoline Kollmann^{*}, Eva Eckelhart^{*}, Sabine Fajmann^{*}, Sabine Cerny-Reiterer⁺, Andrea Hölbl^{*}, Karoline V. Gleixner⁺, Michael Dworzak[§], Christian Sillaber⁺, Gerda Egger[§], Peter Valent^{+,#}, Richard Moriggl[&] and Veronika Sexl^{*}. Manuscript in revision (Blood)

3.4 C-JUN promotes BCR-ABL induced lymphoid leukemia by inhibiting methylation of the 5' region of *Cdk6*.

Karoline Kollmann¹, Gerwin Heller², Rene Georg Ott¹, Ruth Scheicher¹, Eva Zebedin-Brandl¹, Olivia Simma¹, Wolfgang Warsch¹, Eva Eckelhart¹, Christine Schneckenleithner¹, Andrea Hoelbl¹, Sabine Zöchbauer-Müller², Marcos Malumbres and Veronika Sexl¹. Manuscript under review (Blood)

3.5 Leukemic challenge unmasks a requirement for PI3Kdelta in NK cell-mediated tumor surveillance.

Eva Zebedin¹, Olivia Simma¹, Christian Schuster¹, Eva Maria Putz¹, Sabine Fajmann¹, Wolfgang Warsch¹, **Eva Eckelhart**¹, Dagmar Stoiber², Eva Weisz², Johannes A. Schmid², Winfried F. Pickl³, Christian Baumgartner⁴, Peter Valent⁴, Roland P. Piekorz⁵, Michael Freissmuth¹, and Veronika Sexl¹. Manuscript published in Blood. 2008 Dec 1;112(12):4655-64

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

Manuscript in revision (Blood).

Eva Eckelhart¹, Wolfgang Warsch¹, Eva-Maria Zebedin¹, Olivia Simma^{1,a}, Dagmar Stoiber², Thomas Kolbe³, Thomas Rülcke³, Mathias Mueller³, Emilio Casanova^{2,*} and Veronika Sexl^{1,*}.

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ABSTRACT

We have 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^{iCre}* mice to the EGFP^{LSL} reporter strain. *Ncr1^{iCre}* mice were further used to study NK cell specific functions of *Stat5* by generating *Stat5^{f/f} Ncr1^{iCre}* animals. *Stat5^{f/f} Ncr1^{iCre}* 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 (NKP) stage. Moreover, we found that *in vitro* deletion of *Stat5* in IL-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, our study shows a cell intrinsic role for STAT5 for NK cell development and reveals that *Ncr1^{iCre}* mice are a powerful novel tool to study NK cell development, biology and function.

INTRODUCTION

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* as well as *in vivo* in different mouse models.^{2,3} Besides tumor cells, NK cells recognize and eliminate cells infected by certain viruses or parasites.⁴⁻⁶ In addition, NK cells produce and secrete cytokines such as interferon (IFN)- γ and tumor necrosis factor (TNF) that stimulate the adaptive and the innate immune response.^{7,8} Thereby, 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.⁹ The earliest NKPs are characterized by the expression of the IL-2 and IL-15 receptor common β subunit, also known as CD122, and the absence of NK lineage markers such as NK1.1, DX5 and Ly49 receptors.¹⁰ 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, IL-22 producing lymphoid cells in the intestinal lamina propria have been characterized which are positive for NCRI, NKG2D and NK1.1, and express the orphan transcription factor ROR γ t.¹¹ However, NKPs and iNKs are not uniquely restricted to the bone marrow since they have been found at other sites such as the spleen¹² and the lymph nodes.¹³ It has therefore been suggested either that multiple sites may support NK cell differentiation or, alternatively, 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.^{14,15} The natural cytotoxicity receptor I (NCRI), also known as NKp46, becomes expressed during the early iNK cell stage and remains constitutively expressed.^{16,17} The ligand(s) of this receptor are only partially characterized.^{18,19} NCRI is involved in the control of influenza infection by recognizing the viral hemagglutinin protein¹⁶ and has most recently been identified as factor modulating disease progression in type I diabetes.²⁰ Developmental pathways generating the NK cell diversity including the transcriptional machinery behind is not well understood and remains elusive.

The differentiation and homeostasis of lymphocytes are regulated by cytokines such as the interleukins IL-2, IL-4, IL-7, IL-15 and IL-21. All 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.²¹ The JAKs are stably associated with the cytokine receptor and induce the activation of STATs upon receptor stimulation with STAT5 being predominantly activated by IL-2, IL-7 and IL-15.²² STAT5 consists of two highly homologous isoforms, STAT5A and STAT5B, encoded by separate genes. In the lymphoid system, STAT5A/B fulfils largely redundant roles, although STAT5B has been implicated in NK cell development.²³ 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 IL-2 and IL-15 which was attributed to the NK cell compartment.²⁴

The generation of mice deficient for both, *Stat5a* and *Stat5b* (*Stat5a/b*), verified their important role for lymphoid development and homeostasis. The first *Stat5a/b* knock out 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 ^{ΔN} proteins bind DNA and activate transcription of some, but not all target genes²³. Furthermore, STAT5 ^{ΔN} 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 is most obviously seen in the fact that mice lacking complete *Stat5a/b* die perinatally whereas *Stat5* ^{$\Delta N/\Delta N$} animals are viable.²⁵ *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 IL-7 and is involved in immunoglobulin rearrangement and pre-B cell expansion.^{26,27} Within the T lymphoid lineage, STAT5 is mainly required for the expansion of CD8⁺ T cells and TCR $\gamma\delta$ lymphocytes.²⁸ 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.²⁹

In this study we describe the successful generation of a mouse model that allows for the first time the conditional mutagenesis specifically in NK cells. Our work reveals a critical role for the transcription factor STAT5 for NK cell development and survival. In *Stat5*^{f/f} *Ncr1*^{Cre} mice, NK cells

are nearly completely absent causing the virtual abrogation of NK cell mediated tumor surveillance without affecting T cell controlled immune surveillance.

RESULTS

Generation and characterization of *Ncr1*^{iCre} mice

In order to restrict Cre recombinase expression to NK cells, we generated transgenic mice expressing the 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*^{iCre} transgenic mice were crossed to the EGFP^{LSL} reporter mouse line that expresses enhanced green fluorescent protein (EGFP) upon Cre mediated excision of a loxP-flanked stop cassette.³¹ As depicted in Figure 1B, EGFP expression in CD3⁺DX5⁺ NK cells varied between 70% - 90% in individual *Ncr1*^{iCre} EGFP^{LSL} double transgenic mice. In average, we observed 85.33% ± 3.79% of EGFP⁺ NK cells in the spleen, 72.93% ± 2.61% in the bone marrow, 74.53% ± 5.06% in lymph nodes, 80.57% ± 2.98% in the blood (summarized in Figure S1). No EGFP expression was detected in T lymphoid cells as analyzed by CD3, CD4 and CD8 staining (Figure 1C). Despite the general agreement that NCRI is constitutively and selectively expressed in NK cells³⁴ it has been described to be expressed in peripheral NK-like TCRγδ cells.³⁵ Hence, to unequivocally define EGFP expression in those cells, we stained splenocytes from *Ncr1*^{iCre} EGFP mice for NK1.1 and CD1d tetramer, and TCRαβ and TCRγδ. As depicted in Figure 1D and Figure 1E, 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 constricted to the NK cell compartment (TCRβ⁻ NK1.1⁺ Nkp46⁺) (Figure 1G). We therefore concluded that Cre recombination in *Ncr1*^{iCre} mice is restricted to the 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.^{36,37} This was related to chromosomal rearrangements caused by recombination between cryptic “pseudo-loxP” sites naturally occurring within the genome. Alternatively, integration of the transgene might have disrupted genes important for organ and/or cell development. We therefore next studied the effects of Cre expression *per se* on NK cell development and function. *Ncr1*^{iCre} transgenic 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, three major developmental stages can be distinguished defined by their NK1.1 and DX5 expression.⁹ 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, NCRI is expressed and remains expressed throughout all stages¹⁷ (schematically illustrated in Figure 2A). No changes in NKPs (Lin⁻CD122⁺NK1.1⁻DX5⁻) in the bone marrow of *Ncr1*^{iCre} transgenic mice were detected compared to wild type littermate controls (Figure 2B). Also, peripheral mature NK cells were present at equal levels in spleen, lymph nodes and blood (Figure 2C). Mature NK cells in the spleen are further subdivided into functionally distinct subsets depending on CD27 and CD11b expression. A four-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.³⁸ Again, analysis of the surface marker CD27 and CD11b revealed an unaltered NK cell maturation in *Ncr1*^{iCre} mice (Figure 2D). Similarly, *in vivo* NK cell proliferation in the spleen using BrdU incorporation and *in vitro* proliferation of IL-2 cultured NK cells was unaffected (Figure 2E and 2F). Finally, given that lysis of target cells is a major NK cell function, we performed *in vitro* cytotoxicity assays using MACS purified IL-2-expanded splenic NK cells derived from *Ncr1*^{iCre} mice and their littermate controls. Figure 2G summarizes our efforts. YAC-1 cells that express low levels of MHC I were used as target cells as well as RMA-Rae1γ cells expressing the NKG2D ligand Rae-1.³⁹ RMA cells served as a negative control since 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*^{iCre} and their littermate controls were detectable. Overall, these experiments suggest that Cre expression in NK cells neither impairs NK cell development nor NK cell function.

NK cells are severely reduced in *Stat5*^{f/f} *Ncr1*^{iCre} mice

The transcription factor STAT5 is an important regulator of B and T lymphoid cell development and function.^{27,28} To investigate the role of STAT5 in NK cells, we crossed *Stat5*^{f/f} mice to *Ncr1*^{iCre} transgenic animals. *Stat5*^{f/f} *Ncr1*^{iCre} mice were born at the expected Mendelian ratio (data not shown). Hematopoietic organs displayed no gross abnormalities; weight and cellularity of spleen, cellularity of the thymus and bone marrow were unaltered in *Stat5*^{f/f} *Ncr1*^{iCre} mice compared to *Stat5*^{f/f} littermate controls (data not shown and Figure S2). Analysis of CD3⁺DX5⁺ cells in spleen and lymph nodes revealed a severe reduction of NK cells in *Stat5*^{f/f} *Ncr1*^{iCre} mice.

The NK cell population was almost entirely absent ($82\% \pm 7.1\%$ reduction in the spleen, $77.5\% \pm 4.7\%$ in the lymph nodes) (Figures 3A-B). Figure 3C shows the total NK cell number in the spleen. 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 thymus and secondary lymphoid organs (Figures 3D-E).

The few remaining MACS purified NK cells from *Stat5^{f/f} Ncr1^{iCre}* spleens showed a PCR band indicative for the deletion of *Stat5* which was neither present in the *Stat5^{f/f}* fraction nor in the NK cell depleted flow-through (Figure 4A). We noticed however that the *Stat5* deletion was incomplete since *Stat5a/b* mRNA levels of sorted splenic NK cells were not affected in *Stat5^{f/f} Ncr1^{iCre}* NK cells (Figure 4B). We reasoned that the absence of STAT5 is incompatible with NK cell viability. This was supported by the following observation: when we cultivated purified *Stat5^{f/f}* derived NK cells in IL-2, the cells expanded as expected (data not shown). In contrast, the *Stat5^{f/f} Ncr1^{iCre}* derived NK cells did not significantly expand (data not shown). After 6 days in culture, only few *Stat5^{f/f} Ncr1^{iCre}* derived NK cells were present. Importantly, these few remaining NK cells were all escapers and expressed STAT5 indicated by the lack of a *Stat5* deletion band, as analyzed by PCR (Figure 4C, left panel). The NK cell nature of these remaining cells was confirmed by their CD3DX5⁺ surface expression, depicted in Figure 4C, right panel. To substantiate these finding, we purified and cultivated NK cells derived from *Stat5^{f/f}* animals. After 3 days of expansion in IL-2, the cells were infected with an adenovirus expressing Cre recombinase (Ad/Cre-GFP) or mock infected (Ad/GFP). Figure 4D summarizes our efforts: Whereas the NK cells tolerated the expression of Ad/GFP, NK cells that had received Ad/Cre-GFP had a disadvantage and declined (Figure 4D, left panel). The deletion of *Stat5* was confirmed by PCR analysis (Figure 4D, right panel).

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-B, flow cytometric analysis of *Stat5^{f/f}* and *Stat5^{f/f} Ncr1^{iCre}* bone marrow cells confirmed a significant decrease of mNK cells. In contrast, the *Ncr1^{iCre}* dependent deletion of *Stat5* was accompanied by an increase in NKP numbers. This increase of NKPs points at a developmental block occurring at the very first stage of NK cell development. Moreover, flow cytometric analysis of several NK cell markers unveiled lower levels of the activatory receptor Ly49D

whereas the levels of the inhibitory receptors Ly49C+I was higher. In contrast, the levels of CD94 and NKG2D were comparable between the two genotypes. This finding reflects the immature nature of *Stat5^{f/f} Ncr1^{iCre}* derived NK cells in the bone marrow (Figure 5C).

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

NK cells are well known for their tumor suppressive role.^{40,41} To investigate whether the *Ncr1^{iCre}* dependent deletion of *Stat5* is of functional consequence and affects NK cell mediated antitumor activity, we made use of B16F10 melanoma cells. B16F10 cells display low MHC class I levels indicating a role for NK cells in tumor clearance (Figure S3). To verify that these cells are indeed exclusively under the tumor surveillance of NK cells, we injected the tumor cell line i.v. into wild type mice (WT). These mice were subsequently either depleted for NK cells or cytotoxic T cells by using antibodies directed against NK1.1 and CD8. 21 days thereafter, the experiment was terminated and lung metastases were counted. As depicted in Figure 6A-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 to the WT. Hence, we next challenged *Stat5^{f/f} Ncr1^{iCre}* mice and control animals with B16F10 cells. After 12 days the experiment was terminated. *Stat5^{f/f} Ncr1^{iCre}* 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^{iCre}* mice. We wondered, whether this severe defect in NK cell development and function would affect T cell mediated tumor surveillance. To test this, we used MC38 adenocarcinoma cells. MC38 tumor cells display high MHC class I levels pointing to a CTL mediated target cell recognition (Figure S3). Indeed, these cells are 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 to the WT. When MC38 cells were s.c. injected into *Stat5^{f/f} Ncr1^{iCre}* mice and *Stat5^{f/f}* littermate controls, we failed to detect any changes in tumor formation between the two 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^{iCre}* versus *Stat5^{f/f}* mice (Figure 6G).

MATERIALS AND METHODS

Generation of *Ncr1*^{iCre} transgenic mice

The iCre recombinase was inserted into a Bacterial Artificial Chromosome (BAC, RP23-267N11, purchased from the Children's Hospital Oakland Institute) harboring the *Ncr1* gene via homologous recombination in *E. coli* as previously described.³⁰ Briefly, a cassette containing iCre recombinase, an artificial intron, a bovine growth hormone polyadenylation signal and an ampicillin resistance gene flanked by FRT 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. Correctly recombined BACs were verified by southern blot and sequencing. BAC DNA was digested with *NotI*, purified using a sepharose CL4b column, injected into the pronuclei of C57BL/6 oocytes. Three out of four *Ncr1*^{iCre} founders expressed the transgene, one was selected for detailed analysis. Genotyping of *Ncr1*^{iCre} mice was performed using the following primers: 5'GACCATGATGCTGGGTTTGGCCCAGATG and 5'ATGCGGTGGGCTCTATGGCTTCTG yielding a 500-bp polymerase chain reaction (PCR) product.

Mice

All animals used were 4 to 12 weeks and maintained at the Biomedical Research Institute, Medical University of Vienna and at the University of Veterinary Medicine, Vienna. *Stat5*^{fl/fl} and EGFP^{LSL} mice were described previously.^{25,31} 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 Bioscience: PE-Cy7-anti-NK-1.1 (PK136), APC-anti-CD49b (HMA2), PerCP- and PE-anti-CD3e (145-2C11), FITC-anti-CD3 (17A2), FITC-anti-CD4 (RM4-5), APC-anti-CD8a (53-6.7), PerCP-Cy5.5-anti-CD45R/B220, APC-Cy7-anti-CD19, PE-anti- $\gamma\delta$ T-Cell Receptor (GL3), APC-anti-TCR β (H57-597), PE or FITC-anti-CD122 (TM-Beta 1), biotin-anti-Ly6G/Ly6C (RB6-8CM), biotin-anti-CD3e (145-2C11), biotin-anti-TER119, biotin-anti-CD45R/B220/RA3-6B2), PE-anti-CD27 (LG3A10), PerCP-Cy5.5-anti-CD11b (M1/70), PE-anti-CD44 (IM7) and APC-anti-CD25 (Pc61). PE-anti-NKp46 (29A1.4) was obtained from eBioscience. PBS57-loaded and unloaded CD1d tetramers were generously donated by Wilfried Ellmeier (Medical University, Vienna). For flow cytometry, single-cell suspensions were prepared from various tissues; splenocytes were depleted of red blood cells. Purified rat-anti-mouse CD16/CD32

(2.4G2) (BD Pharmingen) was added to avoid nonspecific binding of monoclonal antibodies to FcγR. For intracellular staining, cells were fixed and permeabilized with paraformaldehyd. Briefly, cells were fixed with 2% paraformaldehyde at room temperature (RT) for 10 minutes and incubated with ice-cold methanol for 20 minutes at -20°C. Fc-receptors were blocked, cells were incubated with STAT5 (C17; Santa Cruz) at 4°C over night under agitation and counterstained with PE-conjugated goat-anti-rabbit IgG (Santa Cruz). For *in vivo* BrdU incorporation assays, mice were intraperitoneally (i.p.) injected with 1 mg 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 BD Bioscience FACSCantoII and FACSDiva software.

NK cell purification, expansion and function

NK cells were purified and expanded as previously described.³² Briefly, single-cell suspensions were prepared from at least 4 spleens per genotype. For NK cell purification, cell suspensions were incubated with anti-DX5-coated MACS beads (Miltenyi Biotec) and purified by LS columns on a MACS separator (Miltenyi Biotec). NK cells were expanded for 6-10 days in media (RPMI 1640 containing 10% FCS, β-2ME, L-glutamine, penicillin/streptomycin) supplemented with rhIL-2 (henceforth IL-2) (5000 U/ml). The purity of NK cells was assessed by flow cytometry and was routinely >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 x 10⁵ cells/well) under IL-2 (5000 U/ml). Proliferation was measured by ³[H]-thymidine incorporation. NK cell cytotoxicity was analyzed by flow cytometry, as described previously.³³ Briefly, 1 x 10⁴ target cells/well were incubated with 5 μM CFSE (Invitrogen) for 6 minutes at 4°C in dark. Expanded NK cells at day 10 were co-incubated with CFSE labeled YAC-1, RMA and RMA-Rae1γ targets at indicated E:T ratios for 4 hours at 37°C and placed on ice. Propidium iodide (Sigma) was added to each well immediately before flow cytometry. Percent specific lysis was determined as described.³³ To check the effects of *Stat5* deletion in primary NK cells, *Stat5*^{fl/fl} NK cells were purified, expanded for 3 days under IL-2 and infected with adenovirus either expressing GFP (Ad/GFP) or Cre-GFP (Ad/Cre-GFP) (both were kindly provided by Wolfgang Mikulits, Medical University of Vienna) and 7 μg/ml polybrene. GFP expression of CD3⁻DX5⁺ cells was tracked via flow cytometry. The day of the highest GFP expression was defined as day0.

T cell stimulation

MACS sorted (Miltenyi Biotec) T cells (5×10^5 cells/well) were stimulated with plate-bound anti-CD3 ϵ (1 μ g/ml) (145-2C11; BD Biosciences) and anti-CD28 (2 μ g/ml) (37.51; BD Biosciences) on 48-well plates in the presence of IL-2 (100 U/ml) 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 DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin-streptomycin, 2 mmol/l L-glutamine and 5 μ mol/l β -mercaptoethanol. YAC-1, RMA and RMA-Rae1 γ cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin-streptomycin, 2 mmol/l L-glutamine and 5 μ mol/l β -mercaptoethanol. Mice were injected intravenously (i.v.) with 1×10^6 B16F10 and subcutaneously (s.c.) 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 μ g 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 was purified from PK136-cell supernatant and 53-6.72-cell supernatant, respectively. The effective deletion of the respective cell population was confirmed by flow cytometry of splenocytes.

Real time PCR

Total RNA was isolated using Tri Reagent (Sigma) according to the manufacturer's instructions. 1 μ g of total RNA was used for cDNA synthesis using the GeneAmp RNA PCR Kit (Roche) and used for the RT-PCR reaction performed on an Eppendorf RealPlex cycler using Taq DNA Polymerase (5Prime) and SYBR Green. All experiments were performed in triplicates. The following primers were used: *Stat5a* Forward: CAGATCAAGCAAGTGGTCCC3', *Stat5a* Reverse: TCGAGACTGTCCATGGGCC, *Stat5b* Forward: GGCAGGGTCAGTAACGGAAG, *Stat5b* Reverse: GGCTCTGCAAAGGCGTTGTC. Samples were normalized to GAPDH expression; *Gapdh* Forward: TCTCCTCTGACTTCAACAGCG, *Gapdh* Reverse: ACCACCCTGTTGCTGTAGCC

Statistics

Statistical analysis was performed by using Student's *t* test. The *P*-values were defined as following: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; n.s., not significant. Data are expressed as mean $\pm$ SEM and analyzed by Graph Pad[®] software (San Diego, CA).

Figure 1

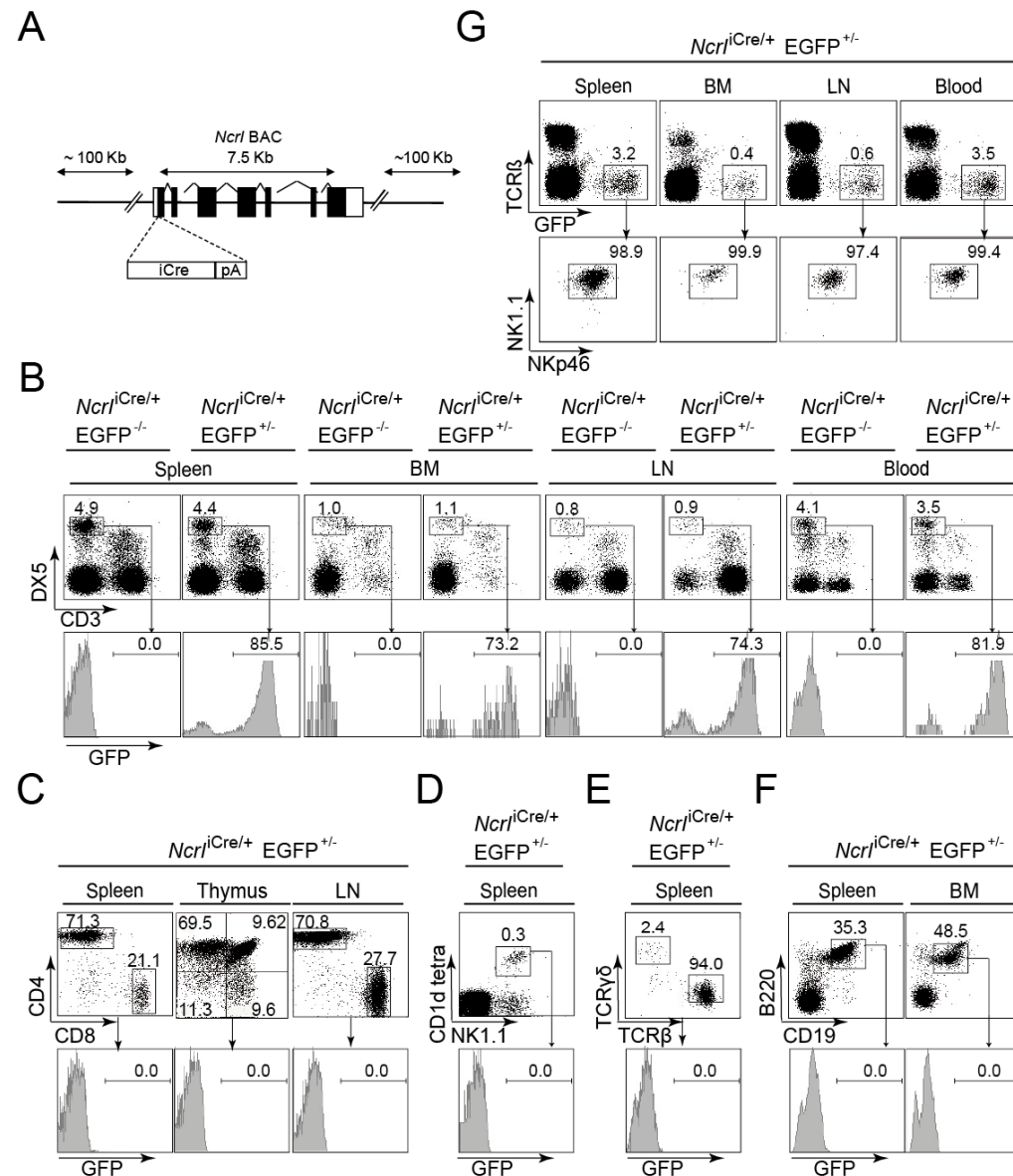


Figure 2. Generation and characterization of *Ncr1*^{iCre} mice.

(A) Schematic illustration of the modified *Ncr1* bacterial artificial chromosome (BAC). A codon improved Cre recombinase (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*^{iCre} EGFP^{LSL} 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 percentage of NK cells (CD3⁻DX5⁺) of various lymphoid organs. Histograms show the percentage of EGFP expression of gated NK cells. (C-F) Flow cytometry of *Ncr1*^{iCre} EGFP^{LSL} double transgenic mice and their littermate controls showing the absence of EGFP expression in hematopoietic cell lineages other than NK cells. Dot plots indicate percentages of (C) gated CD3⁺CD4⁺CD8⁻ cells (D) gated CD3⁻NK1.1⁺CD1d^{tetra}⁺ cells (E) gated CD3⁺TCRγδ⁺TCRβ⁺ cells (F) and B220⁺CD19⁺ cells. Histograms show the percentage of EGFP expression. (G) Almost all NK cells express EGFP. ~ 99% of gated TCRβ⁺EGFP⁺ cells are NK cells (NK1.1⁺NKp46⁺). (n≥4 per genotype). Data are representative of at least three independent experiments. BM: bone marrow, LN: lymph nodes

Figure 2

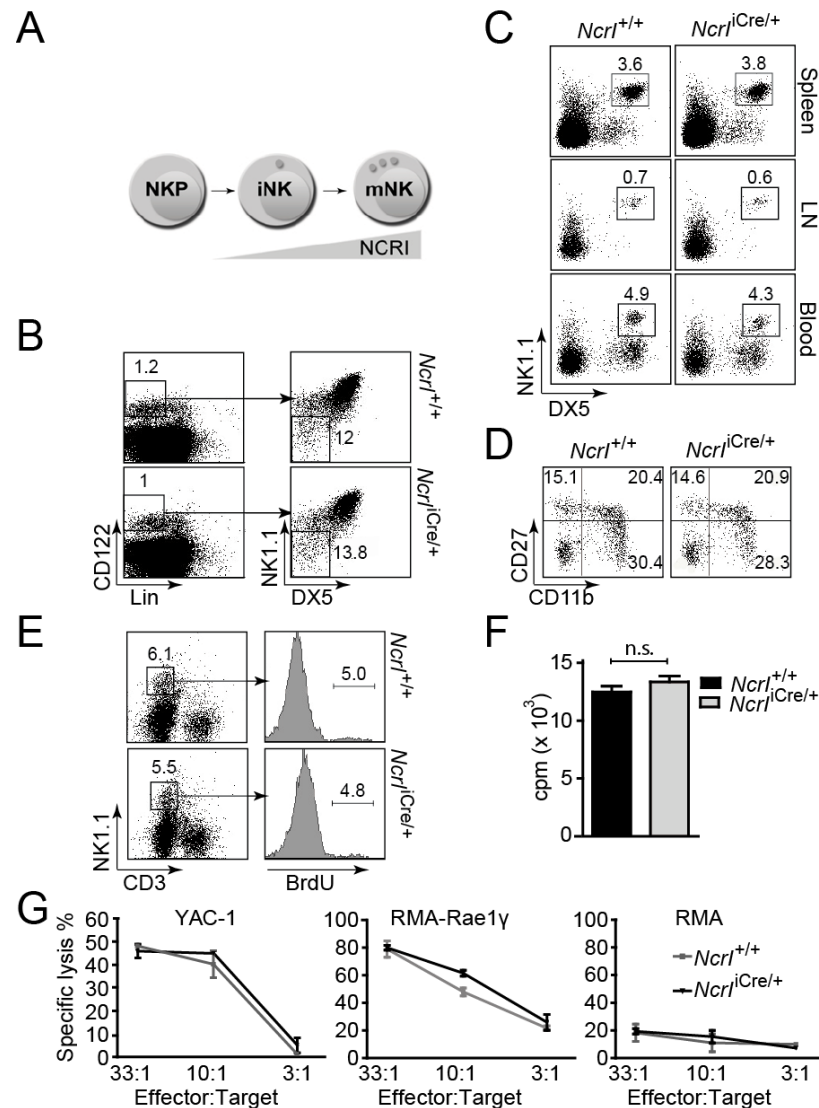


Figure 2. Development and function is not altered in Cre expressing NK cells.

Neither NK cell development nor their proliferation or cytotoxicity was influenced by Cre recombinase. (A) Simplified scheme of NCR1 expression in NK cell development. (B-E) Flow cytometry of *Ncr1^{Cre}* mice and littermate controls. Dot plots indicate percentage of (B) gated Lin⁻CD122⁺DX5⁻NK1.1⁻ NKPs in the BM (C) gated CD3⁻ NK cells (NK1.1⁺DX5⁺) in the periphery (D) and 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 three independent experiments. (E) *In vivo* proliferation of splenic NK cells. Mice were injected i.p. with BrdU. After 12 hours, the incorporation of BrdU in splenic NK cells was analyzed. Numbers adjacent to outlined areas in the dot plot indicate percent CD3⁺NK1.1⁺ cells. Histograms show the percentage of BrdU positive cells (n≥5 per genotype). Data are representative of two independent experiments. (F) *In vitro* proliferation of IL-2 expanded NK cells purified from the spleen of indicated genotypes. At day 6, NK cells were seeded in triplicates 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 two independent experiments. (G) Cytotoxicity of IL-2 expanded splenic NK cells purified from indicated genotypes. At day 10, NK cells were co-incubated in triplicates with CFSE labeled YAC-1, RMA-Rae1γ and RMA targets at indicated E:T ratios. Five mice per genotype were pooled. Data are representative of two independent experiments.

Figure 3

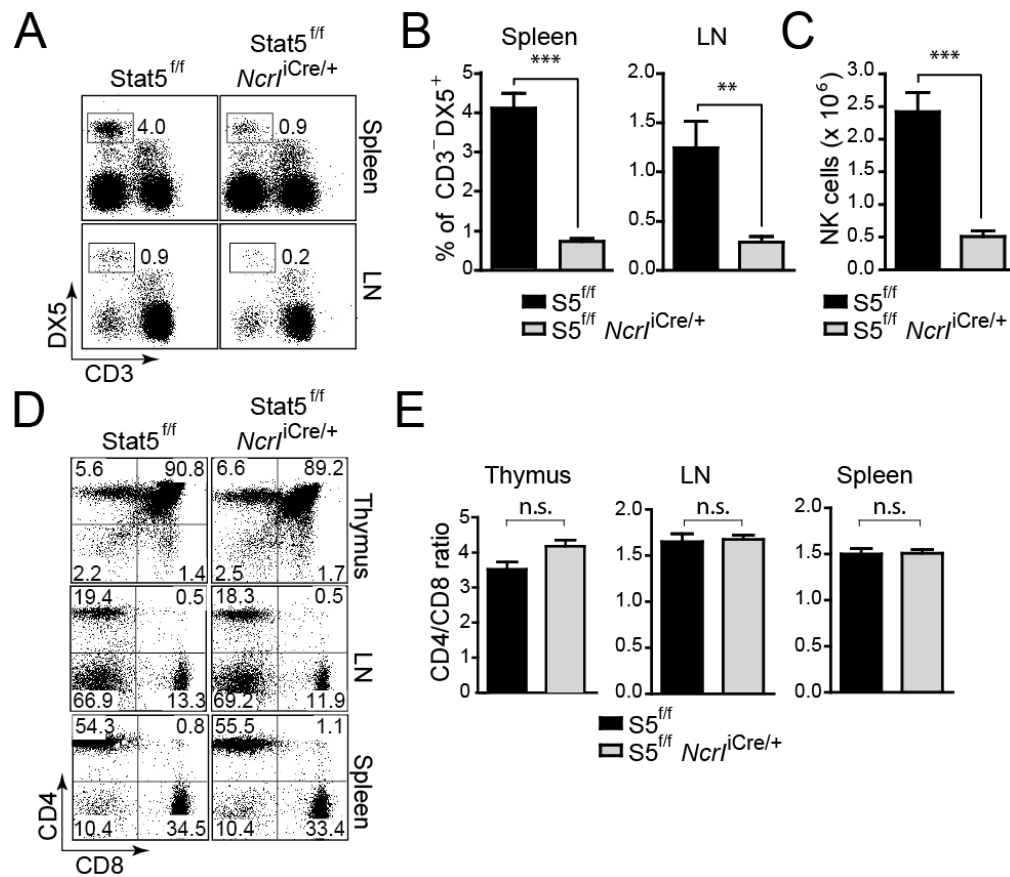


Figure 3. NK cells are severely reduced in *Stat5^{f/f} Ncr1^{iCre}* mice.

Deletion of *Stat5* results in severely diminished NK cell numbers. (A, D) Flow cytometry of *Stat5^{f/f} Ncr1^{iCre}* mice and littermate controls. Dot plots show percentage of (A) peripheral NK cells (D) and CD4 and CD8 expression on cells from various lymphoid organs. Cells were gated on total thymocytes (upper panel), CD3⁺ lymph node cells (middle panel) and CD3⁺ splenocytes (lower panel). (B) Bar graphs show percentage of peripheral NK cells (CD3⁺DX5⁺). (C) Bar graph indicates total splenic NK cell numbers. (E) Bar graphs show 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 three independent experiments. (n≥4 per genotype). (B, C, E) (n≥5 per genotype).

Figure 4

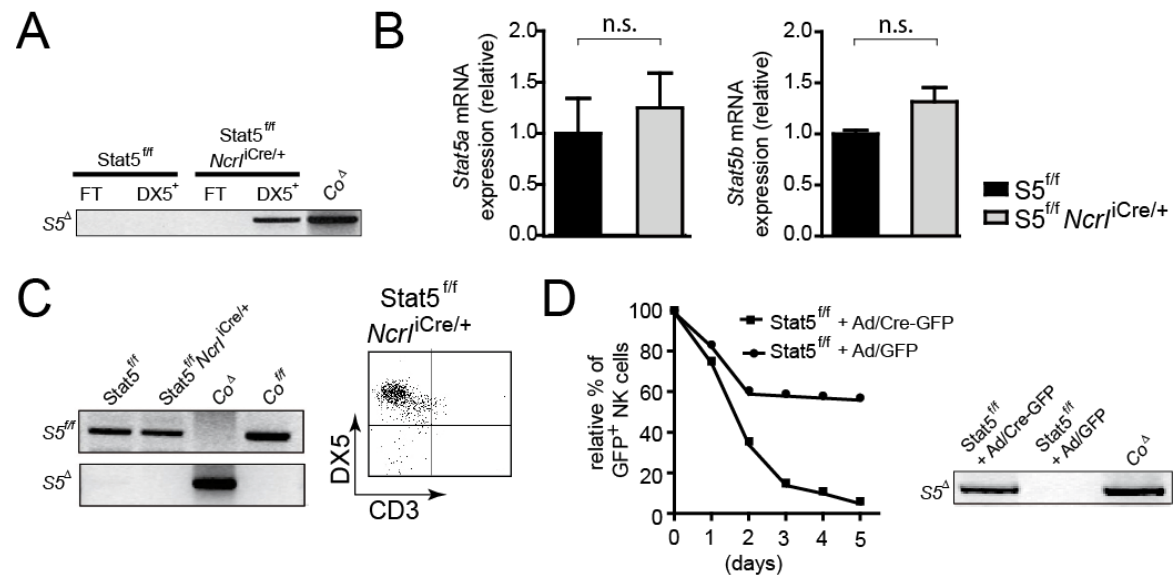


Figure 4. Loss of STAT5 is incompatible with NK cell viability.

(A) PCR genotyping of deleted *Stat5* alleles in MACS purified splenic NK cells of *Stat5*^{fl/fl} and *Stat5*^{fl/fl} *Ncr1*^{iCre} mice. (Four mice per genotype were pooled). (B) Real time PCR analysis of *Stat5a* and *Stat5b* mRNA levels of sorted splenic CD3⁺DX5⁺ NK cells. (Ten mice per genotype were pooled). (C) MACS purified splenic NK cells were cultured under IL-2. After 6 days of culture only those cells which express *Stat5* expanded as indicated by the lack of a *Stat5* deletion band via PCR analysis (left panel). Flow cytometry confirmed the NK cell nature of those cells (right panel). Dot plot indicates CD3⁺DX5⁺ cells. (Four mice per genotype were pooled). (D) MACS purified splenic NK cells from *Stat5*^{fl/fl} mice were cultured under IL-2 (four mice per genotype were pooled) and infected with Ad/Cre-GFP and mock infected. 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 two independent experiments. FT: flow-through; Co: Control.

Figure 5

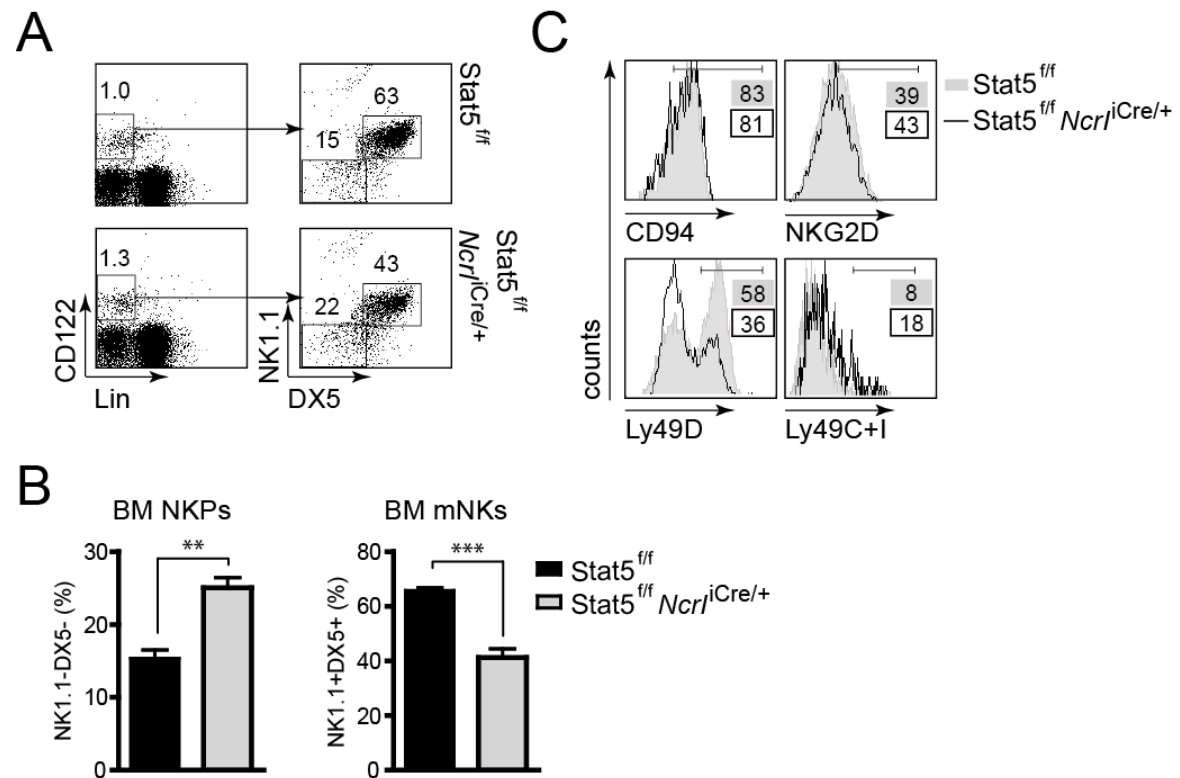


Figure 5. STAT5 is required for early NK cell development in the bone marrow.

(A) Flow cytometry of *Stat5*^{f/f} *Ncr1*^{iCre} mice and littermate controls. Dot plots show percentage of gated Lin⁺ CD122⁺ NKPs (DX5⁺ NK1.1⁺) and gated Lin⁺ CD122⁺ mNKs (DX5⁺ NK1.1⁺). (B) Bar graphs show percentage of NKPs and mNKs in the bone marrow (C) Histograms showing the expression of indicated differentiation markers in percent. (n≥4 per genotype). Data are representative of (A) four (C) and three independent experiments.

Figure 6

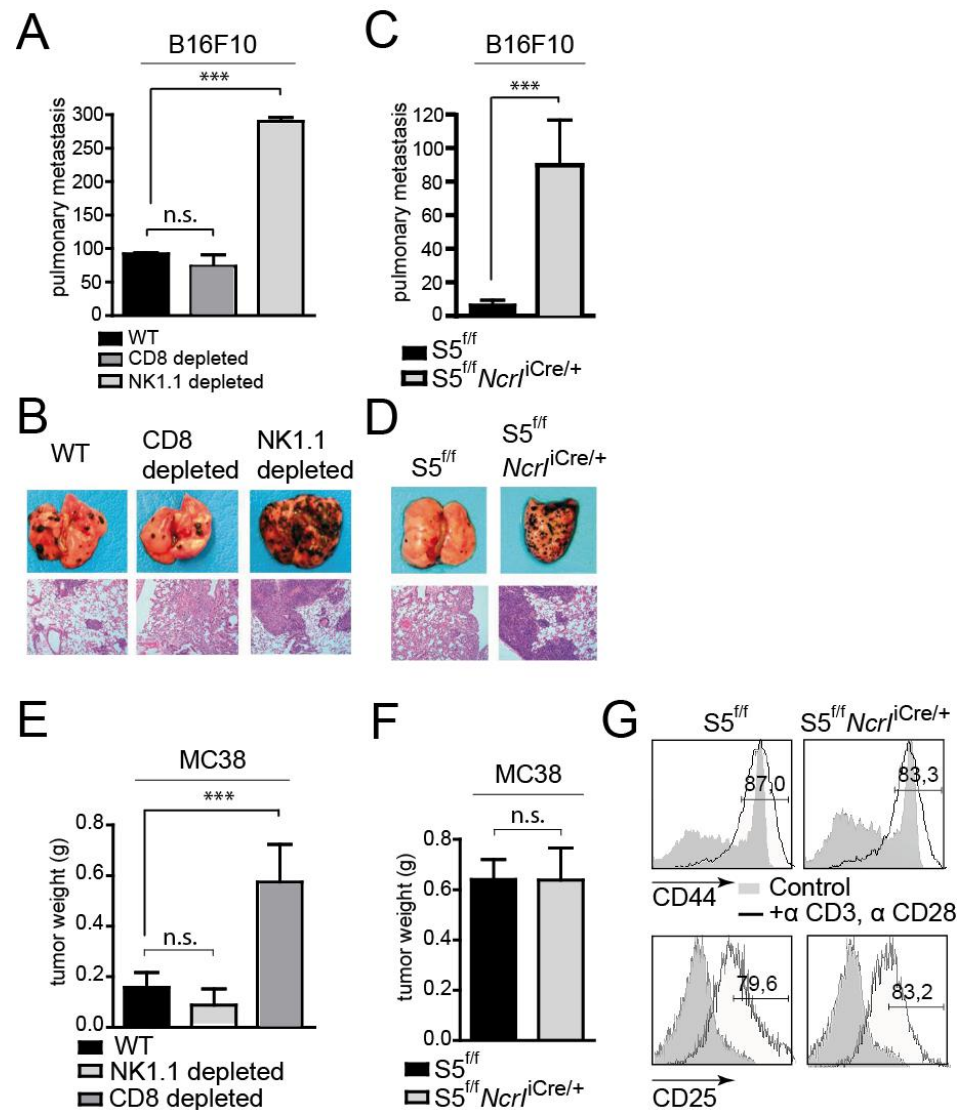


Figure 6. Tumor surveillance of NK cell controlled tumors is missing in $Stat5^{f/f} Ncr1^{iCre}$ mice.

(A-D) B16F10 cells were injected i.v. into (A) WT, WT depleted of CD8⁺ cells, and WT depleted of NK1.1⁺ cells (C) and $Stat5^{f/f}$ and $Stat5^{f/f} Ncr1^{iCre}$ mice. Numbers of metastatic infiltrates per lung were counted under the binocular microscope after (A) 21 days (C) and 12 days. (B, D) One representative example of an infiltrated lung of the indicated genotype is shown. Upper panel: photographs, digital camera, Canon EOS 300D. Lower panel: H&E stained histological lung sections; magnification, x100 Zeiss Axiolmager 21 (Jena, Germany) 10x objective, NA 0.25, air; camera: Pixelink Color, 1600 x 1200; Software: PixelNK Capture 3.0. (E-F) MC38 cells were injected s.c. into (E) WT, WT depleted of CD8⁺ cells, and WT depleted of NK1.1⁺ cells (F) and $Stat5^{f/f}$ and $Ncr1^{iCre} Stat5^{f/f}$. After 17 days of injection, tumor weights were analyzed. (G) Histograms showing CD44 (upper panel) and CD25 (lower panel) expression on in vitro activated T cells from indicated genotypes. CD8⁺ T cells were cultured under IL-2 and stimulated with plate-bound anti-CD3 plus anti-CD28 antibodies. Cells were gated on CD3⁺CD8⁺ populations. Open histograms indicate 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 three independent experiments.

Supplementary Figure 1

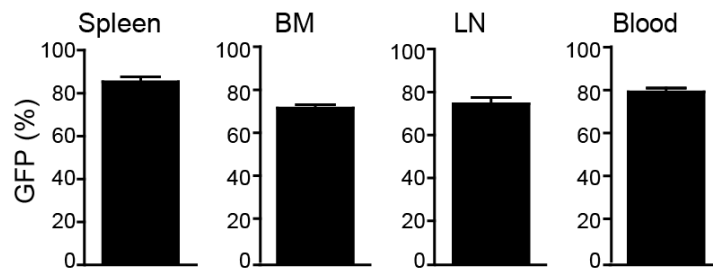


Figure S1. Percentage of GFP⁺ NK cells in various tissues of *Ncr1^{iCre/+} EGFP^{+/-}* mice.

Bar graphs showing the percentage of NK cells of various lymphoid tissues from *Ncr1^{iCre} EGFP^{LSL}* double transgenic mice. (n≥4 per genotype).

Supplementary Figure 2

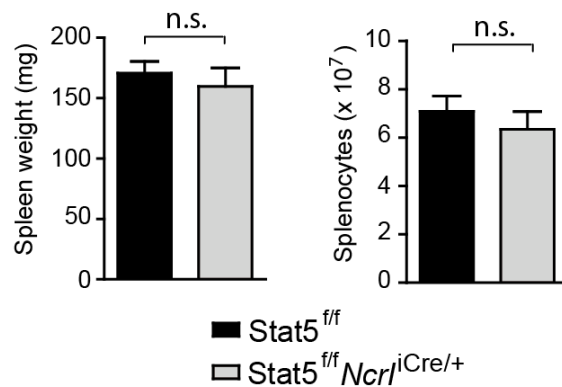


Figure S2. No differences in spleen weight or numbers of total splenocytes in *Stat5^{f/f} Ncr1^{iCre}* mice compared to *Stat5^{f/f}* mice.

Bar graphs show spleen weight (left panel) and total splenocytes (right panel) of indicated genotypes. (n≥6 per genotype)

Supplementary Figure 3

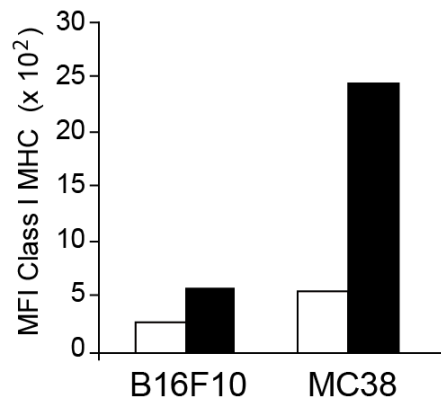


Figure S3. MHC class I levels of indicated tumor cell lines.

Bar graphs show mean fluorescence intensity (MFI) of class I MHC on B16F10 and MC38 cell clones determined by flow cytometry. White bar graphs indicate negative control; black bar graphs display the indicated cell clone.

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3.2 Natural immunity enhances the activity of a DR5 agonistic antibody and carboplatin in the treatment of ovarian cancer.

Manuscript published in Mol Cancer Ther. 2010 Apr;9(4):1007-18. Epub 2010 Apr 6.

I contributed to this project by producing and purifying NK1.1 antibodies for the *in vivo* NK cell depletion experiments, by performing flow cytometric analysis, and by helping with *in vivo* experiments.

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Natural Immunity Enhances the Activity of a DR5 Agonistic Antibody and Carboplatin in the Treatment of Ovarian Cancer

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Abstract

The tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) induces apoptosis specifically in cancer cells with little effect on normal cells. We have previously shown that TRAIL signaling is altered in most ovarian cancer patients and that resistance to TRAIL contributes to ovarian cancer progression. In this study, we investigated whether resistance to TRAIL may be overcome by a monoclonal TRAILR2 (DR5) agonistic antibody (AD5-10). We found that the joint presence of AD5-10 with TRAIL and natural killer (NK) cells expressing TRAIL resensitizes ovarian cancer cells to apoptosis *in vitro* and *in vivo*, respectively. The combination of AD5-10 with carboplatin exerts a more than additive effect *in vitro*, which may at least partially be explained by the fact that carboplatin triggers DR5 expression on ovarian cancer cells. Moreover, AD5-10 restores the sensitivity of platin-resistant ovarian cancer to carboplatin *in vivo*. In addition, we found that TRAIL expression and NK cells are abundant in the tumor microenvironment and that depletion of NK cells abolishes the antitumor activity of AD5-10. This indicates that NK-mediated immunosurveillance against ovarian cancer might be mediated by TRAIL and that apoptosis induced by AD5-10 requires the presence of NK cells. In conclusion, this study indicates a key role and strong antitumorigenic effect of DR5 and highlights a novel link between NK-mediated immunosurveillance and activation of DR5-mediated apoptosis in ovarian cancer. *Mol Cancer Ther*; 9(4); 1007–18. ©2010 AACR.

Introduction

Ovarian cancer is the most lethal gynecologic cancer and the fifth leading cause of cancer-related deaths among women in western industrialized countries (1, 2). Ovarian cancer originating from the ovarian surface epithelium is the most common form and displays a range of histologic subtypes (3). Whereas most ovarian cancers are sensitive to platin-based chemotherapy at the time of diagnosis, recurrence of the disease is frequent, and ultimately, platin-resistant disease develops in all patients.

Apoptosis is important for maintaining cellular homeostasis in normal tissues by eliminating disordered cells, and defects in the apoptosis pathway may lead to cancer (4). The apoptotic cascade can be stimulated by

death receptors, resulting in activation of caspases (5). Trimerization of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) functional receptors TRAILR1 (DR4) or TRAILR2 (DR5) by their ligands leads to the assembly of death-inducing signaling complex, which initiates apoptotic cascade (6). DR4 and DR5 are characterized by an extracellular cysteine-rich domain and an intracellular death domain, giving them the ability to trigger the assembly of the death-inducing signaling complex. We have previously found that TRAIL is highly expressed in the human ovarian cancer microenvironment, but that tumor tissues display a reduced number of TRAIL functional receptors (7).

One major physiologic role of TRAIL is the mediation of natural immunity and the elimination of developing tumors (8, 9). Previous studies have shown that soluble TRAIL or agonistic monoclonal antibodies specific for functional TRAIL receptors exhibit tumoricidal activities, a phenomenon that has been tested in clinical trials (10). The agonistic human DR5-specific monoclonal antibody AD5-10 used in this study was reported to mediate antitumor effects in various tumor cells and, due to its unique binding site, does not compete with TRAIL for binding to DR5 in contrast to other agonistic DR5 antibodies (11).

In the current study, we identified the functional role of DR5 in ovarian cancer progression and shed light on a novel strategy to eliminate ovarian cancer in a preclinical mouse model. Moreover, we show for the first time that the function of natural killer (NK) cells is necessary for

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Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

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doi: 10.1158/1535-7163.MCT-09-0933

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the activation of DR5-mediated apoptosis and that the presence of NK cells is highly correlated with a longer life span in xenograft mice.

Materials and Methods

Drugs

Carboplatin, paclitaxel (both EBEWE), bevacizumab (Roche), lapatinib (GlaxoSmithKline), agonistic DR5 monoclonal antibody (AD5-10) prepared as described previously (11), and recombinant human and mouse soluble TRAIL (Alexis) were used for stimulation of ovarian cancer cell lines at various concentrations.

Cell culture

The human ovarian cancer cell line MDAH-2774 (ovarian endometrioid adenocarcinoma-derived cell line, originating from the ascitic fluid of a patient; ref. 12) and a platin-resistant subline of the ovarian cancer cell line A2780 (2780^{CP8}, human epithelial ovarian cancer cell line established from tumor tissue; ref. 13) were cultured in RPMI 1640 (Invitrogen). The ovarian cancer cell line ES-2 (human ovarian clear cell carcinoma cell line taken from a 47-y-old woman; ref. 14) was cultured in McCoy's medium (Invitrogen). Medium was supplemented with 10% FCS (PAA Laboratories GmbH), 1 mmol/L glutamine, and 1% penicillin/streptomycin (PAA Laboratories GmbH). MDAH-2774 and ES-2 cell lines were obtained from American Type Culture Collection, and the cell line 2780^{CP8} was obtained from the European Collection of Animal Cell Cultures.

Determination of apoptosis

MDAH-2774, 2780^{CP8}, and ES-2 ovarian cancer cell lines were plated at a density of 5×10^4 cells per well in 24-well plates and incubated for 24 h before stimulation. Then cells were treated with either AD5-10 (1 μ g/mL), carboplatin (100 μ g/mL), paclitaxel (0.05 μ mol/L), bevacizumab (100 ng/mL), lapatinib (4 μ mol/L), or an antibody-drug combination for 24 h. For inhibition of caspase activation, the caspase-8 inhibitor *N*-benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone (zIETD.fmk; Bachem) was used. Cells were harvested by trypsinization using 0.05% trypsin and 0.02% EDTA without Ca^{2+} and Mg^{2+} (PAA Laboratories GmbH). Apoptosis was determined by an Annexin V-FITC apoptosis detection kit (Alexis) according to the manufacturer's instructions. Flow cytometry of Annexin V-FITC and propidium iodide staining was done using FACScan (Becton Dickinson) with CELLQuest Pro software. The mean background values for the three selected adherent ovarian cancer cell lines MDAH-2774, 2780^{CP8}, and ES-2 were 17%, 8%, and 13.6%, respectively.

Immunoblotting analysis

Western blot analysis was done according to the standard protocol. Briefly, proteins were extracted from cells lysed for 30 min at 4°C in radioimmunoprecipitation as-

say⁺ buffer supplemented with complete protease inhibitor (Roche) and 1 mmol/L orthovanadat, followed by high-speed centrifugation. Protein concentration was determined according to the method of Bradford (Sigma-Aldrich). Equal amounts of protein (~50 μ g protein per lane) were separated by 12% SDS-PAGE gel and electroblotted onto polyvinylidene difluoride membranes (GE Healthcare). The membrane proteins were incubated with the following primary antibodies: mouse anti-caspase-8 monoclonal antibody, 12F5 (recognizes human procaspase-8 and active human caspase-8, Alexis), mouse anti-caspase-3 monoclonal antibody, 31A1067 (recognizes human procaspase-3 and active human caspase-3, Imgenex), mouse anti-p53 monoclonal antibody (Cell Signaling), mouse monoclonal anti-human cellular Fas-associated death domain-like interleukin-1 β -converting enzyme-like inhibitory protein (c-FLIP; NF6, Alexis), and goat anti-actin polyclonal antibody (Santa Cruz). Detection was done with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz). The membranes were developed using enhanced chemiluminescence (Amersham).

Detection of TRAIL ligand and its functional receptor expression by flow cytometry

MDAH-2774, 2780^{CP8}, and ES-2 ovarian cancer cell lines were collected and washed in PBS. The cells were then incubated with mouse monoclonal antibodies against human TRAILR1, HS101 (Alexis), human TRAILR2, HS201 (Alexis), or mouse IgG1 isotype control (Ancell) for 1 h at 4°C. Afterwards, cells were washed in PBS, incubated with goat anti-mouse IgG-FITC (Santa Cruz) for 1 h at 4°C in the dark, washed with PBS, and analyzed by flow cytometry. TRAIL intracellular staining was done as described previously (15). Briefly, cells were washed with PBS and resuspended in 2% formaldehyde and incubated for 10 min at 4°C. Afterwards, cells were washed with PBS and resuspended in blocking solution (0.1% saponin and 20% serum) and incubated for 20 min at room temperature. Cells were then stained with mouse monoclonal antibody against human TRAIL, III6F (Alexis), or mouse IgG2b isotype control (Ancell) for 30 min at room temperature in staining buffer (0.1% saponin, 2% FCS serum). After that, cells were washed thrice with staining buffer, resuspended in secondary antibody goat anti-mouse IgG-FITC (Santa Cruz), and incubated for 1 h at 4°C in staining buffer. Then, cells were washed thrice with staining buffer and analyzed by flow cytometry. Flow cytometric analysis was done using FACScan (Becton Dickinson), and the resulting data were analyzed with CELLQuest Pro software.

Immunohistochemistry

Immunohistochemical analysis was done using paraffin-embedded sections as described previously (7). Briefly, tissue sections were deparaffinized and rehydrated. For epitope retrieval, specimens were incubated in 96°C prewarmed 10 mmol/L citrate buffer (pH 6.0) for 20 min.

Slides were then incubated with 0.3% H₂O₂/PBS for 10 min at room temperature to block endogenous peroxidase. After blocking the background staining with serum of the secondary antibody (diluted 1:10 in PBS), tissues were incubated for 1 h at room temperature with primary antibody diluted in serum/PBS. The following primary antibodies were used: rabbit anti-human cleaved caspase-3 monoclonal antibody Asp175 (dilution 1:50, Cell Signaling); mouse anti-human Ki-67 monoclonal antibody (dilution 1:200, Dako); goat anti-mouse TRAIL polyclonal antibody AF1121 (dilution 1:100, R&D Systems); rabbit anti-human TRAILR1 polyclonal antibody H130 (dilution 1:200, Santa Cruz); and rat anti-mouse Ly-49G2 monoclonal antibody 4D11 (ref. 16; dilution 1:400, eBioscience). The appropriate secondary biotinylated antibodies (Vector Laboratories) were diluted with the serum/PBS buffer (dilution 1:200) and incubated for 30 min at room temperature. Tissue sections were incubated with StreptABComplex/HRP (Dako) for 45 min at room temperature, then visualized with 3,3'-diaminobenzidine (Dako), and counterstained with Mayer's hematoxylin. Tissue sections were analyzed on an Olympus BX50 upright light microscope (Olympus Europe) equipped with the soft imaging system CC12.

Apoptosis was measured by using an *in situ* cell death detection kit (Roche) according to the manufacturer's instructions. Afterwards, the cells were analyzed on a fluorescence microscope (Nikon Eclipse 800) equipped with a Nikon DS-R1 camera, using the NIS-Elements software.

Negative controls were done by excluding incubation with primary antibody and yielded negative results. The percentage of positive cells was determined by a blinded operator.

Xenograft mouse model

Four- to six-week-old female athymic nude-Foxn1 nu/nu mice were obtained from Harlan (Italy) and maintained under specific pathogen-free conditions at the animal resource service of the Medical University of Vienna. Mice were s.c. inoculated with 2780^{CP8} ovarian cancer cells on both sides. Treatment started on day 2 by i.p. injection of PBS, AD5-10, carboplatin, or the latter two in combination. Tumor size was measured every second day by calipers. The tumor volume was calculated according to the formula $V = 4/3 \times \pi \times (L / 2 \times W / 2 \times W / 2)$; L, length; W, width (17). At the end of the experiment, tumors were recovered, weighed, and then prepared for histologic and pathologic analysis. Animal experiments were done according to protocols approved by the Austrian Federal Ministry for Education, Science, and Art. For NK depletion experiments, mice were treated with 20 μ l anti-asialo GM1 antibody (Wako) and 100 μ g NK1.1 antibody (prepared as described previously; ref. 18) 3 d before inoculation and then every 4 d.

Generation of splenocytes and analysis of NK cells

The spleen was removed from sacrificed mice and placed into 60-mm tissue culture dishes. A single-cell sus-

pension was made by passing the tissue through a 70- μ m nylon cell strainer (BD Bioscience) in PBS containing 2% FCS. Cells were centrifuged, and hypotonic lysis of RBC was done using ACK lysis buffer [0.15 mol/L NH₄Cl, 1 mmol/L KHCO₃, and 0.1 mmol/L Na₂EDTA (pH 7.2)], followed by resuspension in an appropriate volume of PBS containing 2% FCS. Cells were incubated at 4°C with Fc-block (BD Bioscience) for 5 min and then stained with PerCP hamster anti-mouse CD3e, APC mouse anti-mouse NK-1.1, FITC rat anti-mouse CD49b (BD Bioscience), and PE rat anti-mouse TRAIL (Santa Cruz) antibodies for 30 min at 4°C in PBS. After that, cells were washed thrice with PBS and analyzed by flow cytometry. Flow cytometric analysis was done on FACSCalibur (BD Biosciences). Data were analyzed using the CellQuest Pro software.

Statistical analysis

Two-sided Student's *t* tests were used to detect statistically significant differences between study groups and controls, using R and StatSoft's Statistica software. Where appropriate, a one-way ANOVA was used. Data were visualized with box plots or bar plots. *P* values below 0.05 were considered statistically significant, and *P* values below 0.005 were considered highly significant. The correlation between variables was estimated using the Pearson correlation coefficient.

Results

In vitro effect of combining AD5-10 with cytotoxic drugs

We have previously shown that the TRAIL signaling pathway plays a fundamental role in ovarian cancer progression (7, 19). In this study, we addressed the functional role of human DR5 using three distinct ovarian cancer cell lines (MDAH-2774, 2780^{CP8}, and ES-2). The selected ovarian cancer cell lines have accumulated different mutational and epigenetic changes that alter normal cell growth and survival pathway, e.g., mutated p53 in MDAH-2774 and ES-2, epigenetic silencing of DR4 in 2780^{CP8}, as well as upregulation c-FLIP in most of them (19–22). Moreover, they express different levels of DR5 (Fig. 1A), and all of them were resistant to TRAIL-induced apoptosis. We found that AD5-10 triggered tumor cell apoptosis to a detectable extent and had an additive effect when combined with different pharmacologic and cellular anticancer agents (paclitaxel, bevacizumab, lapatinib; Supplementary Fig. S1). In combination with carboplatin, AD5-10 showed a more than additive effect (Fig. 1B), illustrated by comparing the sum effect of AD5-10 and carboplatin to a combination of both. This particular effect was observed in three different carboplatin-resistant ovarian cancer cell lines and was confirmed by detection of active caspase cleavage fragments (Fig. 1B and C, top). To prove whether DR5-enhanced caspase activation leads to apoptosis, we used caspase-8 inhibitor zIETD.fmk. Apoptosis in

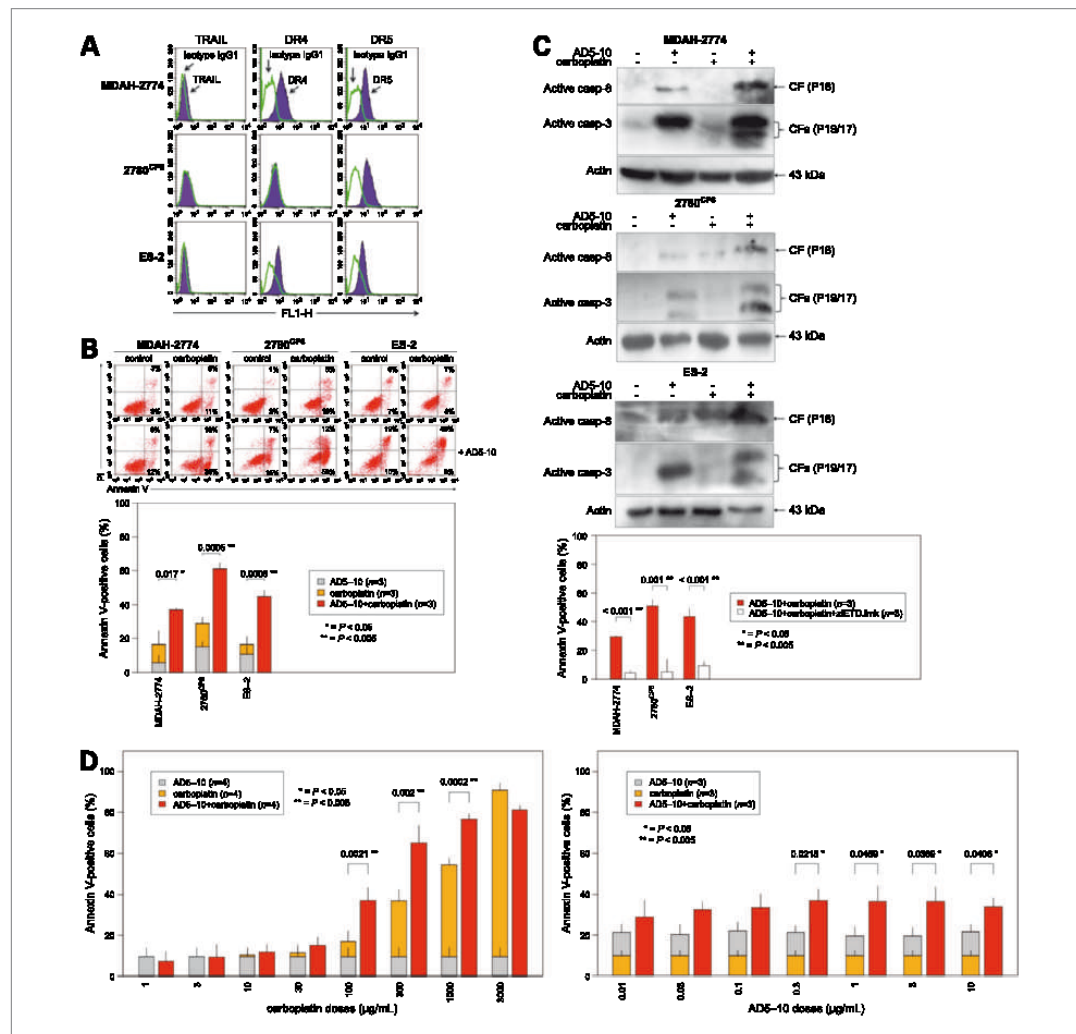


Figure 1. Effect of combining AD5-10 with carboplatin *in vitro*. **A**, histogram showing expression of the TRAIL ligand and as well as its functional receptors DR4 and DR5 on the surface of selected ovarian cancer cell lines as determined by flow cytometry. Selected ovarian cancer cell lines were stained with either TRAIL ligand (IIISF, Alexis), anti-DR4 (HS101, Alexis), anti-DR5 (HS201, Alexis) monoclonal antibody (colored histogram), or isotype control IgG1 monoclonal antibody (unfilled histogram). Data represent three independent experiments. **B**, top, MDAH-2774, 2780^{CP8}, and ES-2 ovarian cancer cell lines were either left untreated or treated with either AD5-10 (1 µg/mL), carboplatin (100 µg/mL), or both for 24 h. Apoptosis was determined by Annexin V and propidium iodide (PI) staining. Numbers in dot plot quadrants represent the percentage of stained apoptotic cells. Bottom, quantitative evaluation of top panel as well as the apoptosis rate of two additional experiments. Columns, mean of three independent experiments; bars, SEM. Statistically significant (*, $P < 0.05$) or highly significant (**, $P < 0.005$) differences were obtained by comparing the sum effect of AD5-10 and carboplatin to a combination of both. **C**, top, expression levels of activated caspase-8 and caspase-3 were determined by immunoblotting in untreated or treated ovarian cancer cells using AD5-10 (1 µg/mL), carboplatin (100 µg/mL), or both. Data represent two independent experiments. CF, cleavage form. Bottom, selected ovarian cancer cell lines were either untreated or treated with AD5-10 (1 µg/mL) and carboplatin (100 µg/mL) in absence or presence of 20 µmol/L zIETD.fmk (40 µmol/L for MDAH-2774) for 24 h, and apoptosis was determined. Columns, mean of three independent experiments; bars, SEM. Statistically highly significant (**, $P < 0.005$) differences obtained by comparing the apoptotic effect of AD5-10 and carboplatin in absence or presence of zIETD.fmk. **D**, left, dose-response curve of carboplatin is presented using MDAH-2774 cells. MDAH-2774 ovarian cancer cell line was incubated with the specified concentration of carboplatin, AD5-10 (1 µg/mL), or both for 24 h, and apoptosis was determined. Columns, mean of four independent experiments; bars, SEM. Statistically highly significant (**, $P < 0.005$) differences obtained by comparing the sum effect of AD5-10 and carboplatin to a combination of both. EC₅₀ values of carboplatin shifted from 105.5 to 172.6 µg/mL (95% confidence interval) in the presence of 1 µg/mL AD5-10. Right, dose-response curve of AD5-10 is presented using MDAH-2774 cells. MDAH-2774 ovarian cancer cell line was incubated with the specified concentration of AD5-10, carboplatin (100 µg/mL), or both for 24 h, and apoptosis was determined. Data are represented as indicated above in D (left).

response to combination of AD5-10 and carboplatin was almost completely blocked in the presence of zIETD.fmk in all three selected ovarian cancer cell lines (Fig. 1C, bottom). The dose-response curves for carboplatin in the presence of AD5-10 and the dose-response curves for AD5-10 in the presence of carboplatin confirmed the observed effects (Fig. 1D). Taken together, these findings suggest that AD5-10-mediated stimulation of DR5 in the presence of carboplatin efficiently sensitizes ovarian cancer cell lines to apoptosis.

Carboplatin cooperates with AD5-10 to trigger apoptosis in ovarian cancer cells by upregulation of DR5

We then focused on the mechanism by which carboplatin cooperates with AD5-10 signaling to mediate enhanced tumor cell death. DR5 is a transcriptional target of p53 (23), and carboplatin has been shown to induce the p53 tumor suppressor pathway (24). To identify whether carboplatin cooperates with AD5-10 via upregulation of DR5 expression, we treated MDAH-2774 cells (over-expressing a mutated form of p53, Arg273His; ref. 22), 2780^{CP8} cells (expressing wt p53; ref. 20), and ES-2 cells (expressing one mutant allele of p53, S241F, and the other wild type; ref. 21; Supplementary Fig. S2A) with carboplatin. Consequently, we analyzed the expression of

p53 and DR5 by immunoblotting and flow cytometry, respectively (Fig. 2A and B). The p53 status was confirmed by sequencing p53 (data not shown). DR5 expression levels were increased in all cell lines after carboplatin treatment, irrespective of the p53 status (Fig. 2A and B). Notably, DR5 expression (after treatment with carboplatin) and the apoptosis rate (after treatment with a combination of AD5-10 with carboplatin) in wild-type p53 cells were higher than in cells with mutant p53 (Figs. 1B and 2B, respectively). These observations indicate that carboplatin forces expression of DR5 on ovarian cancer cells, thereby enhancing their susceptibility to AD5-10-mediated apoptosis. In addition, MDAH-2774 harboring a mutant p53 allele also reacted with apoptosis in a clear dose-dependent manner (Fig. 1B and D). This illustrates that carboplatin cooperates with AD5-10 regardless of the p53 status in ovarian cancer cells. Because carboplatin treatment induced an increase in DR5 expression irrespective of the p53 status, p53 mutations do not limit the therapeutic usefulness of combining AD5-10 with carboplatin. Interestingly, we found no change in DR5 expression in MDAH-2774 and ES-2 ovarian cancer cell lines upon treatment with paclitaxel (Supplementary Fig. S2B). On the other hand, an increase in DR5 expression on 2780^{CP8} was detected (Supplementary Fig. S2B). These data are in line with our observation that

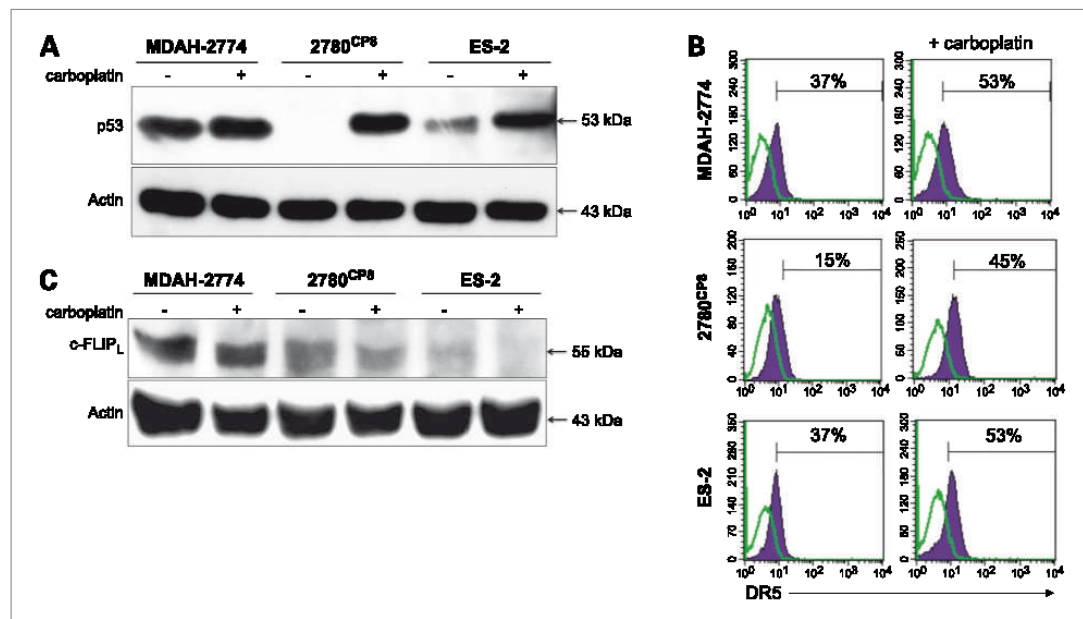


Figure 2. Carboplatin cooperates with AD5-10 to trigger apoptosis in ovarian cancer cells by upregulation of DR5. **A**, selected ovarian cancer cell lines were either untreated or treated with carboplatin (100 μ g/mL) for 24 h. Expression of p53 was determined by immunoblotting; data represent two independent experiments. **B**, flow cytometry analysis of DR5 expression on selected ovarian cancer cell lines either untreated or treated with carboplatin (100 μ g/mL) for 24 h; data represent three independent experiments. Mean fluorescent intensity value was increased from 10.35 to 12.54 in MDAH-2774, from 16.54 to 18.40 in 2780^{CP8}, and from 10.86 to 13.11 in ES2 after treatment with carboplatin. **C**, protein expression of c-FLIP_L was assessed by immunoblotting after 24 h incubation without or with carboplatin (100 μ g/mL). Data represent two independent experiments.

the combination of paclitaxel with AD5-10 in MDAH-2774 and ES-2 has no effect (Supplementary Fig. S1). In contrast, there is a more than additive effect in 2780^{CP8} ovarian cancer cell line.

c-FLIP can prevent the recruitment and activation of caspase-8 (25, 26). We previously showed that the long isoform of c-FLIP (c-FLIP_L) is highly expressed in more than two third of ovarian cancer patients (7). To determine whether c-FLIP_L expression, as DR5 modulators, is affected in our experimental system, we analyzed its expression before and after treatment with carboplatin by immunoblotting. Notably, ES-2 has a very low basal expression of c-FLIP_L, indicating that c-FLIP_L is of no importance in this cell line (Fig. 2C). Significant c-FLIP_L expression was detected in MDAH-2774 and 2780^{CP8} cells. Carboplatin treatment does not alter c-FLIP_L expression in MDAH-2774 but decreases c-FLIP_L expression in 2780^{CP8} cells (Fig. 2C), which is inline with the fact that active p53 inhibits c-FLIP expression (27). Altogether, our data indicate that combination of carboplatin-induced DR5 expression with AD5-10 potentiated activation of caspase-dependent cell death in a c-FLIP_L-independent manner.

Combination of AD5-10 and carboplatin eradicates tumors in a xenograft ovarian cancer mouse model

To assess the antitumor efficacy of AD5-10 alone and/or in combination with carboplatin, we applied a tumor model of ovarian cancer in xenograft-bearing mice. In this model, mice bearing s.c. established tumors were randomly grouped for treatment with vehicle, AD5-10, carboplatin, or a combination of AD5-10 and carboplatin. Treatment started on day 2, and tumor size was measured every second day after tumor inoculation. After 2 weeks, all tumor-bearing mice were sacrificed and tumor-free mice were maintained for another 2 weeks (Table 1).

Mice treated with AD5-10 showed a clear reduction in tumor progression compared with untreated mice (Fig. 3). As expected, the treatment with carboplatin alone had no significant effect on tumor growth. In contrast, the combination of AD5-10 with carboplatin significantly suppressed tumor growth. Six of seven treated mice (86%) remained tumor-free at the time of sacrificing (day 14). After 28 days, >50% of mice ($n = 4$) remained tumor-free following combined AD5-10 and carboplatin treatment (Table 1). Overall, our data suggest that AD5-10 in combination with carboplatin induces a high apoptosis rate in ovarian cancer cells *in vitro* and ovarian cancer rejection in a significant number of mice in a xenograft model, whereas carboplatin alone has no significant effect (Figs. 1B and 3; Table 1).

Significant differences in tumor volume between control and AD5-10-treated mice were also observed. The largest reduction in tumor volume compared with the control group of untreated mice was observed in the group treated with AD5-10 in combination with carboplatin (Fig. 3A and C; Table 1). Similar results were obtained for tumor weight (Fig. 3B). In contrast, mice receiving carboplatin alone did not display any statistically significant differences in tumor volume or tumor weight. Clearly, the tumor suppressive effect of AD5-10 alone and the elimination effect of AD5-10 in combination with carboplatin suggest that DR5 plays a strong antitumor role in carboplatin-resistant ovarian cancer.

AD5-10 enhances antitumor activity against ovarian cancer *in vivo*

We then evaluated the cytotoxic effect of given substances on tumor tissues by immunohistochemical analysis. We observed a significant increase in caspase-3 activation and consequently in the apoptosis rate [measured by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay] for AD5-10, but

Table 1. Antitumorigenic effect of AD5-10 alone and/or in combination with carboplatin in a xenograft nude ovarian cancer mouse model

Group	No. mice sacrificed upon tumor development				
	14th day	23rd day	25th day	28th day	Tumor-free mice after observation period
Control ($n = 7$)	7*	—	—	—	0 (0%)
AD5-10 ($n = 5$)	3	1	—	—	1 (20%)
Carboplatin ($n = 6$)	4	1†	—	—	1 (16.7%)
AD5-10 + carboplatin ($n = 7$)	1	1	1	—	4‡ (57.2%)

NOTE: (a) Two mice in the AD5-10 and one mouse in the carboplatin-treated group were excluded from the statistical analysis due to inoculation resulting in i.p. tumor. (b) On day 14, all tumor-bearing mice were sacrificed; the remaining mice were sacrificed upon reaching the final tumor volume of ~4,000 mm³.

*One tumor-bearing mouse died before sacrificing.

†Sacrificed due to subcutaneous tumor development and ascites.

‡One mouse without tumor was sacrificed for the photograph in Fig. 3C on day 14.

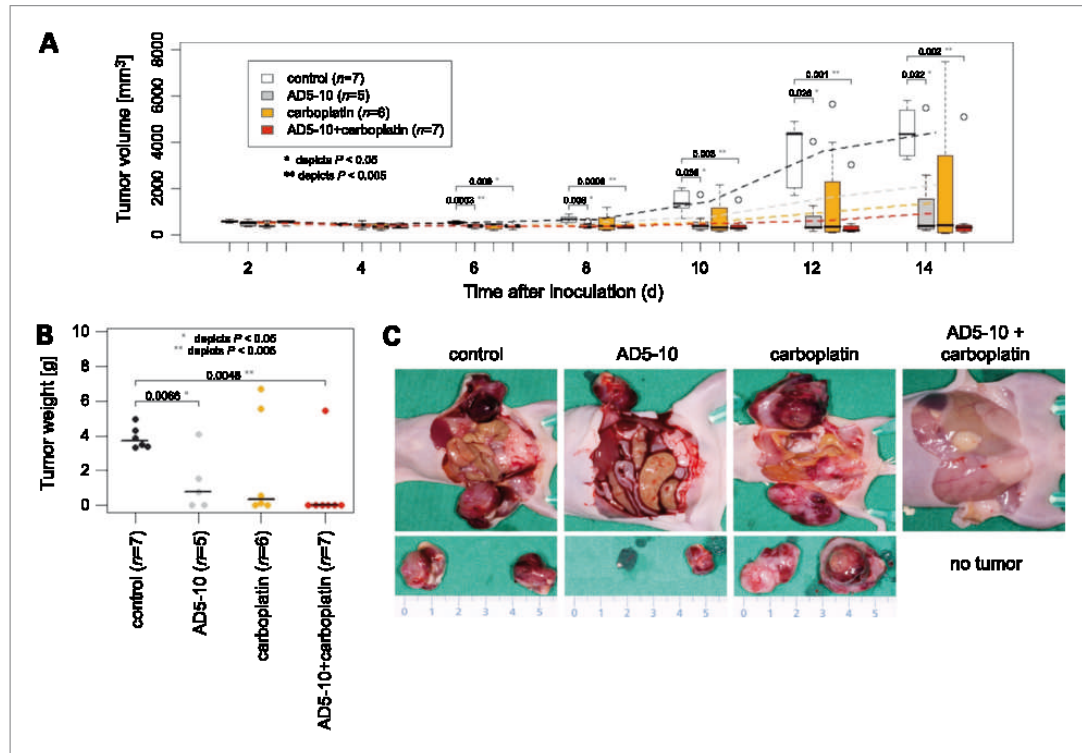


Figure 3. Combination of AD5-10 with carboplatin eradicates large, established tumors in a xenograft ovarian cancer mouse model. **A**, 28 nude/nude mice were inoculated s.c. with 1.5×10^7 2780^{CP8} ovarian cancer cell line into the left and right hind ventral flank (day 0). Random groups of seven mice each were then treated i.p. with either saline (control tumor) or AD5-10 (10 mg/kg) on days 2, 6, 9, 13, 16, and 20; carboplatin (100 mg/kg) on day 2; or a combination of AD5-10 and carboplatin at the respective doses and time as indicated above. Tumor volumes were measured every second day, and on day 14, all tumor-bearing mice were sacrificed. Each measurement in each group is the sum of both left and right tumor volumes per animal and is included in the box plots. Box plots depict median values and the interquartile range, whereas dotted lines depict mean values in each group. Statistically significant differences (*, $P < 0.05$; **, $P < 0.005$) were determined by comparing tumor volume in the control group with treated groups. A significant difference was also observed by ANOVA at the time points 6, 10, 12, and 14 d ($P = 0.024$, 0.019, 0.011, 0.024). **B**, tumor weight (after 14 d) in the four different groups; the median is indicated by a horizontal line. Each measurement in each group is the sum of both left and right tumor weight per animal. A significant difference was also observed by ANOVA ($P = 0.0305$). **C**, representative mice and tumors of the indicated groups were assessed on day 14.

not for carboplatin, when compared with a control group (Fig. 4A and B). No significant differences in proliferation were observed for AD5-10 or carboplatin treatment (Supplementary Fig. S3). These data indicate that AD5-10 treatment induces apoptosis, consequently inhibiting the growth of ovarian tumor tissue.

DR4 is epigenetically silenced in the 2780^{CP8} cells *in vitro* (19). To determine whether the observed effects are due to DR5 and not to DR4, e.g., by demethylation of the DR4 promoter and reconstitution of DR4 expression *in vivo*, we analyzed the expression of DR4 in tumor tissues. Immunohistochemical and immunoblotting analysis showed no reconstitution of DR4 expression *in vivo* (Fig. 4C), indicating that TRAIL-induced apoptosis is only mediated by DR5. Furthermore, we analyzed the immunohistochemical expression of murine TRAIL using anti-mouse TRAIL antibody. Interestingly, we found

murine TRAIL to be highly expressed in tumor tissues (Fig. 4C). Remarkably, selected human ovarian cancer cells do not express human TRAIL (Fig. 1A), and murine TRAIL was shown to have some cross-reactivity with human tumor cells (Fig. 5A; ref. 28). This finding might support our previous observation in humans that TRAIL is highly expressed in the tumor microenvironment of ovarian cancer patients (7). TRAIL is a key mediator of cytotoxic activity of activated NK cells and CD8⁺ lymphocytes (29) and is at least in part responsible for antitumorigenic activities *in vivo* (30, 31). Immunohistochemical analysis revealed a dense infiltration of the tumor tissues with NK cells (Fig. 4C). Moreover, flow cytometric analysis for mouse TRAIL surface positivity of NK cells within the tumor showed that ~60% of NK cells express mouse cell-bound TRAIL (data not shown). Together, our data suggest that NK cells mediate cytotoxicity in ovarian cancer by TRAIL-induced cell death.

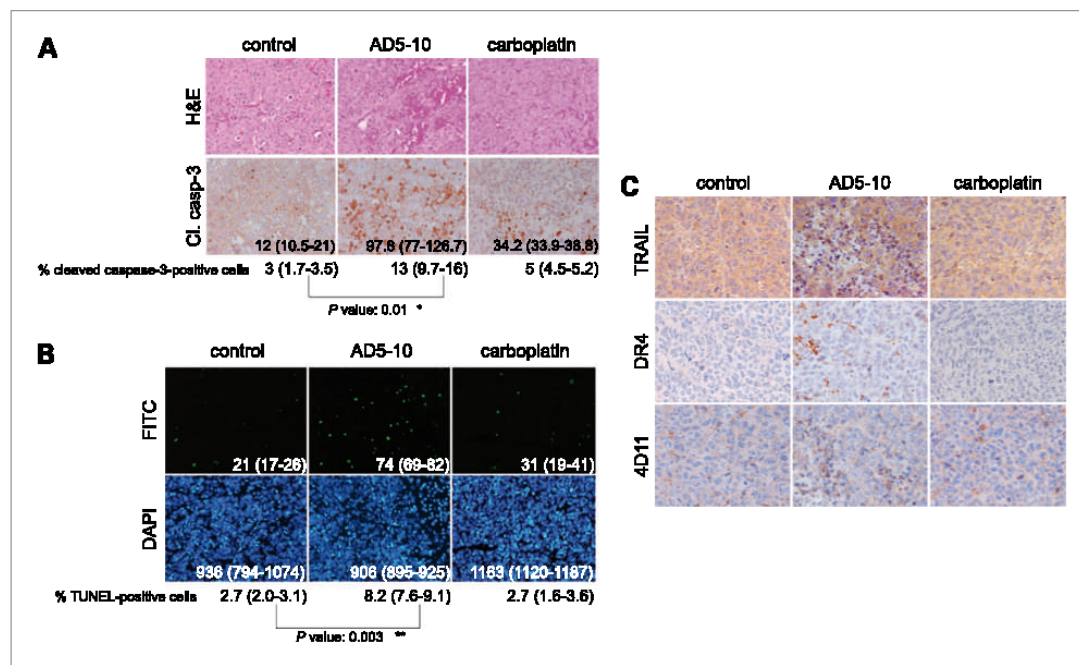


Figure 4. AD5-10 enhances sensitivity of ovarian tumors to apoptosis. Immunohistochemical analysis of tumors obtained from mice inoculated with 2780^{CP8} ovarian cancer cell line and sacrificed on day 14 (Fig. 3B) for H&E and caspase-3 staining (A); TUNEL assay (B); TRAIL ligand, DR4, and NK cell staining (C). Magnification, 20x. A, H&E staining showed vital solid intact tumor tissues in control and carboplatin-treated mice. In contrast, tumor cells from AD5-10-treated mice seemed damaged, leading to structural alteration and secondary bleeding. A and B, proportional cell numbers in each micrograph (in caspase-3 staining, numbers of cleaved caspase-3-positive cells) are given as median values, and the interquartile range of 12 subsections of four different tumor sections per group is indicated within brackets. The median and interquartile range of the percentage of cleaved caspase-3- and TUNEL-positive cells are given below micrographs. Significant differences between the control group and the various treated groups are indicated: *, $P < 0.05$; **, $P < 0.005$. C, TRAIL staining (done using goat anti-mouse TRAIL polyclonal antibody AF1121; R&D Systems): vital tumor cells with weak diffused background staining of murine TRAIL, probably representing the soluble form of the molecule in control and carboplatin-treated tumor tissues. However, damaged tumor cells were observed in TRAIL-positive AD5-10-treated tumor tissue. DR4 staining: tumor cells were consistently negative for DR4 in all tumor sections. Tissues from animals treated with AD5-10 showed granular intercellular staining, which probably represents unspecific staining of degenerated apoptotic tumor cells. 4D11 staining: monoclonal antibody 4D11 (anti-Ly-49G2) revealed specific reactivity for mouse NK cells (16). Specific staining for NK cells was observed in all tumor sections. Damaged tumor cells were observed close to positive NK cells staining in AD5-10-treated but not in control or carboplatin-treated tumors. Consecutive slides of the same tissue block for mouse TRAIL and 4D11 immunohistochemistry were used.

AD5-10 cooperates with NK cells to suppress tumor growth of established ovarian cancer in a xenograft mouse model

To gain more insight on the role of NK-mediated immunosurveillance mediated by TRAIL, we further explored the antitumor effect of AD5-10 in combination with mouse TRAIL. Combination of human or mouse TRAIL (at different concentrations) with AD5-10 enhances 2780^{CP8} cellular sensitivity to TRAIL-induced apoptosis *in vitro* (Supplementary Figs. S4A and S5A). Notably, AD5-10 antibody has been shown to bind to a different binding site than TRAIL (11). Therefore, AD5-10 does not compete with the TRAIL-binding site, which may explain a significantly increased rate of apoptosis observed after treatment with the combination of AD5-10 and mouse soluble TRAIL (1,000 ng/mL; Fig. 5A).

Finally, we determined whether NK cell-mediated cytotoxicity is involved in AD5-10-induced effects. To do so, we made use of NK cell-depleting antibodies. Four groups of nude mice were s.c. inoculated with 2780^{CP8} cells and were either left untreated or treated with AD5-10 and/or NK1.1- and anti-asialo-depleting antibodies (Supplementary Fig. S4B). Tumor development was observed for a period of 4 weeks. In NK cell-depleted mice, AD5-10 had only a minimal effect on tumor growth (Fig. 5B), whereas AD5-10 significantly reduced tumor volume in the presence of NK cells (Figs. 3A and 5B). This suggests that TRAIL expressed by active NK cells is required for AD5-10-induced apoptosis. This finding is in line with the fact that DR5 is more efficiently activated by secondary cross-linked trimers of TRAIL (32, 33). NK cell depletion alone had only a small effect on tumor growth, which might reflect the

fact that 2780^{CP8} cells are resistant to TRAIL-induced apoptosis (expressed via NK cells). Moreover, we found that NK cell numbers correlate with the survival of the animal, with a high NK cell number being protective (Fig. 5C). In summary, these data suggest that the antitumor activity of AD5-10 depends on NK in ovarian cancer.

Discussion

The TRAIL pathway has been extensively studied *in vitro* and *in vivo*, and molecules targeting this pathway have become attractive candidates for anticancer treatment (10). Preclinical studies in mice provided the first evidence that the soluble form of recombinant TRAIL suppresses the growth of human tumor xenografts with

no apparent systemic toxicity (34, 35). More recently, recombinant TRAIL has also entered clinical trials for the treatment of various malignancies (36). Although published phase 1 and phase 2 studies have indicated tolerated toxicity, the therapeutic efficiency is variable. In addition to the soluble ligand, several agonistic antibodies to the TRAIL functional receptors (DR4 or DR5) have been developed and entered into clinical trials in parallel (10). These agonistic antibodies may be more effective than the ligand at eradicating tumors for several reasons, one of them being the prolonged half-life time *in vivo* when compared with the recombinant proteins. Furthermore, the decoy receptors, which have been implicated in modulating the response to TRAIL, are not targeted by these ligands. Cross-linking therapeutic

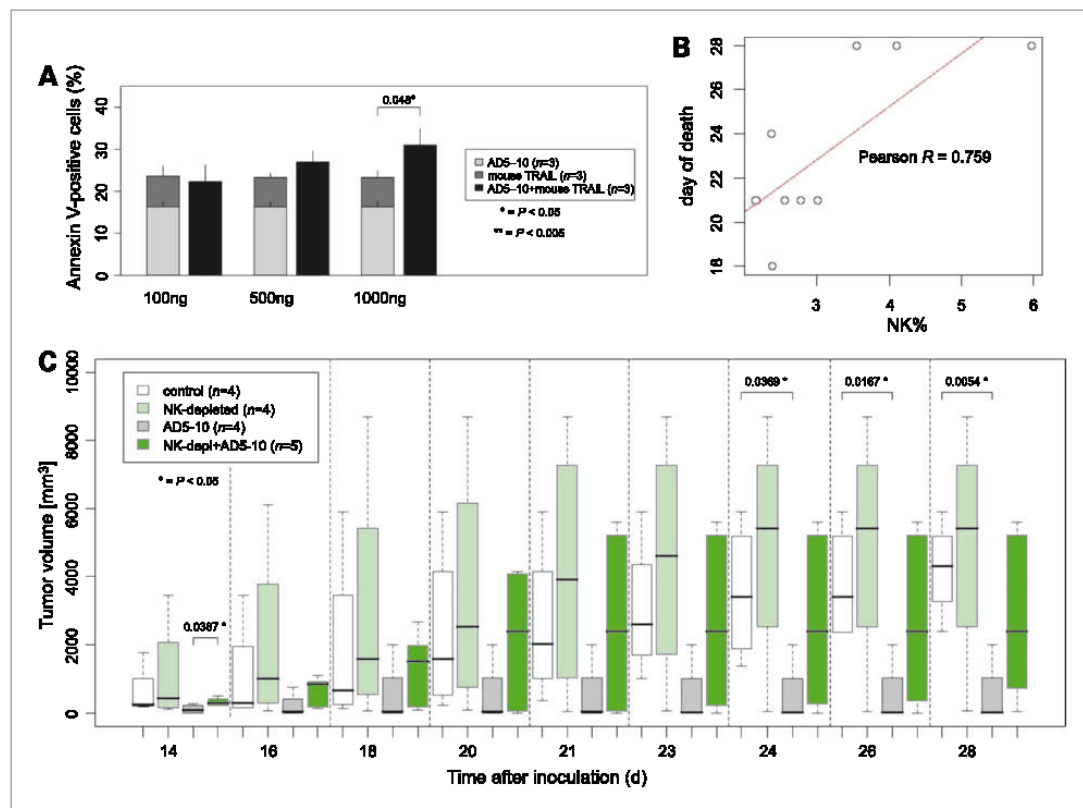


Figure 5. Depletion of NK cells suppresses the tumoricidal effect of AD5-10 on tumor outgrowth. **A**, 2780^{CP8} ovarian cancer cell line was either untreated or treated with the specified concentration of mouse soluble TRAIL, AD5-10 (1 µg/mL), or both for 24 h, and the apoptosis rate was measured. Columns, mean of three independent experiments; bars, SEM. Statistically significant (*, $P < 0.05$) difference obtained by comparing the sum effect of AD5-10 and mouse soluble TRAIL to a combination of both. **B**, two groups of mice were depleted of NK cells by administration of polyclonal rabbit anti-asialo GM1 antibody at a dose of 20 µl i.p. every 4 d starting on day 3 and NK1.1 antibody at a dose of 100 µg i.p. every third day starting at day 3. A total of 1×10^7 2780^{CP8} cells were inoculated s.c. into the left and right dorsal sides of the mice. Mice were treated i.p. with AD5-10 or saline as indicated in Fig. 3A. Tumor volumes were measured every second day. Mice were sacrificed when tumor size (at least in one flank) reached the required volume ($\sim 4,000 \text{ mm}^3$). Each measurement in each group is the sum of both left and right tumor volumes per animal. Data are represented as indicated in Fig. 3A. **C**, correlation between NK cells (%) in depleted mice and life span.

antibodies might also overcome the resistance mechanisms to TRAIL-induced apoptosis observed in ovarian cancer (5, 13), according to the data in our study, which provides a novel link between NK-mediated immunosurveillance and activation of DR5-mediated apoptosis.

DR5 was identified in 1997 (23, 37), and several DR5 agonistic antibodies exhibit a potent antitumor effect against TRAIL-sensitive tumor cells but not against TRAIL-resistant tumor cells. These are currently in phase II evaluations in patients with advanced malignancies (38, 39). In the present study, we used a DR5 agonistic antibody AD5-10 against TRAIL-resistant ovarian cancer cells, and our results shed light on the postulated critical role of DR5 and NK-mediated immunosurveillance in ovarian cancer progression. As expected, we observed limited cytotoxic effects on ovarian cancer cells resistant to TRAIL-induced apoptosis *in vitro* upon stimulation with AD5-10 alone (Fig. 1B and C). We hypothesized that combined therapy with anti-DR5 and different pharmacologic anticancer agents might induce more than additive antitumor activity. Our results showed that out of a number of agents used in the standard treatment of ovarian cancer only the combination of AD5-10 with carboplatin displayed more than additive effect *in vitro* (Supplementary Fig. S1A and B). This phenotype stimulated our interest in the nature of this mechanism. Consequently, we determined the cytotoxic effect of AD5-10 alone or in combination with carboplatin in a platin-resistant ovarian cancer mouse model. Whereas there was, as expected, no effect of carboplatin alone, an effect of AD5-10 was observed. Importantly, the combination of AD5-10 with carboplatin eventually eliminated ovarian cancer (Fig. 3; Table 1) in a significant number of animals. Chemotherapeutic agents have been shown to cooperate with the TRAIL ligand to enhance apoptosis (40). However, the molecular basis behind such cooperation has not yet been elucidated. One mechanism is based on our observation that carboplatin cooperates with AD5-10 by upregulating DR5 expression regardless of the p53 status (Fig. 2A and B). As a consequence, this DR5 upregulation in the presence of AD5-10 leads to increased induction of caspase-dependent cell death (Fig. 1C, top), because the resulting apoptosis is completely blocked in the presence of a caspase-8 inhibitor (Fig. 1C, bottom). The observed apoptotic effect upon combination of AD5-10 and carboplatin seems to be independent from the DR5 modulator c-FLIP_L (Figs. 1B and 2C). It has been recently shown that a modified TRAIL ligand that specifically binds to DR5 has a more pronounced apoptotic effect dependent on cisplatin-induced DR5 expression than TRAIL *in vitro* as well as in an orthotopic bioluminescent ovarian cancer mouse model (41). Collectively, the described data support the importance of drug-induced DR5 upregulation in the enhanced sensitivity of ovarian cancer cells to TRAIL-induced apoptosis.

As postulated previously, the TRAIL pathway plays a key role in tumor surveillance (7, 29–31). Under-

standing the activity of a potential agent targeting this pathway requires putting a spotlight on the activity of the hosts' immune system as reflected in the tumor microenvironment. Our previously published data support a key role of TRAIL and the functional TRAIL receptors in ovarian cancer, showing increased survival in patients expressing a high level of TRAIL in tissue adjacent to the tumor (7, 19). However, the origin of this expression has not been fully evaluated to date. In our current study we addressed the role of NK cell-mediated effects of DR5-induced apoptosis. We recorded that AD5-10 alone did not have a significant effect on apoptosis. However, it had a more than additive effect when combined with human or mouse soluble TRAIL *in vitro* to enhance TRAIL-mediated cytotoxicity (Supplementary Figs. S4A and S5A). Membrane-bound TRAIL is more active than soluble TRAIL in inducing apoptosis in tumor cells, which might explain the strong inhibitory effects we observed in mice treated with AD5-10 (Fig. 3; Table 1). Moreover, we observed that TRAIL expression and NK cells are abundant in the tumor microenvironment, and ~60% of tumor infiltrating NK cells in tumors expressed cell-bound TRAIL (Fig. 4C; data not shown). Furthermore, we determined that depletion of NK cells from mice bearing tumors led to increased tumor growth and also abolished the cytotoxic effect of AD5-10 (Fig. 5B and C). Taken together, these findings indicate that TRAIL expressed on active NK cells might cross-link AD5-10, suggesting that DR5 is more efficiently activated by secondary cross-linked trimers of soluble TRAIL than by non-cross-linked molecules (Fig. 5A; refs. 32, 33). Also, our above-mentioned observations might support a role for TRAIL in mediating NK immunosurveillance against ovarian-transformed cells *in vivo*. Nevertheless, further experiments are still needed to fully prove that TRAIL alone or in combination with other molecules like perforin and granzymes mediates spontaneous and activated NK-induced cytotoxicity against ovarian cancer (42, 43). Notably, AD5-10 antibody binds to a different binding site than TRAIL (11), and murine TRAIL was shown to have some cross-reactivity with human tumor cells (Fig. 5A; ref. 28). However, other mechanisms like upregulation of DR5 on ovarian cancer cells by carboplatin and targeting by AD5-10 efficiently activated cell killing without the need for secondary cross-linking (Fig. 1B and C). In fact, according to our data, forced expression of DR5 in the presence of TRAIL-expressing NK cells provides an ideal environment for highly efficient activation of DR5 by AD5-10. This fact is supported by eradication of implanted tumors in an NK-competent xenograft ovarian cancer mouse model (Fig. 3; Table 1) and by the abolished cytotoxic activity of AD5-10 in NK-depleted mice (Fig. 5B). This finding clearly confirms the importance of NK-mediated immunosurveillance in ovarian cancer in a preclinical mouse model. Agonistic DR5 antibodies probably act together with naturally occurring TRAIL

ligand to overcome the resistance mechanisms that develop during tumorigenesis.

Our study highlights the lack of knowledge regarding the interplay between therapeutic agents, host immunity, and the evasion mechanisms developed by individual tumors. Only an improved understanding of these basic mechanisms will ultimately lead to tailored patient selection and the choice of combination partners for drugs targeting the TRAIL pathway. Carboplatin and AD5-10 seem to be a promising regimen for future clinical trials in platin-resistant ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acknowledgments

We thank Prof. U. Losert and his colleagues in the animal facility for taking good care of our mice, Bettina Pichlhoefer (Department of Pathology, Medical University of Vienna) for sequencing p53 in our ovarian cancer cell lines, and Martin Hilgarth (Department of Medicine I, Medical University of Vienna) for technical assistance.

Grant Support

Austrian Science Fund (FWF) grant P20715.

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Received 10/08/2009; revised 01/21/2010; accepted 02/09/2010; published OnlineFirst 04/06/2010.

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3.3 STAT5 is an important mediator of imatinib resistance in Abelson-induced leukemia

Manuscript in revision (Blood).

I contributed to this project by performing PCR experiments, Western Blots, and by establishing cell lines for the revision process.

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ABSTRACT

In *BCR-ABL1*⁺ leukemia, drug resistance is often associated with upregulation of BCR-ABL1 or multidrug transporters as well as *BCR-ABL1* mutations. Here we show that the expression level of the transcription factor STAT5 is another parameter that determines the sensitivity of *BCR-ABL1*⁺ cells against BCR-ABL1 tyrosine kinase inhibitors (TKI) such as imatinib, nilotinib or dasatinib. Abelson-transformed cells expressing high levels of STAT5 were found to be significantly less sensitive to TKI-induced apoptosis *in vitro* and *in vivo*. This protection requires tyrosine-phosphorylation of STAT5 and transcriptional activity. In support of this concept, under imatinib treatment and with disease progression, STAT5 mRNA and protein levels increased in patients with Ph⁺ chronic myeloid leukemia. Based on our data, we propose a model in which disease progression in *BCR-ABL1*⁺ leukemia leads to an upregulated expression of STAT5, which subsequently confers protection against TKI-induced apoptosis. This process provokes the selection of cells with high STAT5-expression during treatment with TKIs and suggests that STAT5 may serve as a novel, attractive target to overcome imatinib resistance in *BCR-ABL1*⁺ leukemia.

INTRODUCTION

More than 99% of all patients with chronic myeloid leukemia (CML) and about 30% of acute lymphoid leukemia (ALL) are characterized by the t(9;22)(q34;q11) translocation and the so called Philadelphia chromosome. Two different chimeric oncogenic tyrosine kinase products, p185^{BCR-ABL1} or p210^{BCR-ABL1}, may be generated.^{1,2} Whereas p210^{BCR-ABL1} is associated with CML, p185^{BCR-ABL1} is almost exclusively found in ALL.³ The BCR-ABL1 oncoprotein promotes leukemia development by activating multiple signal transduction pathways that regulate cell proliferation, transformation and survival of hematopoietic cells. BCR-ABL1-induced ALL is characterized by an excess of lymphoblasts and usually shows rapid disease progression, whereas *BCR-ABL1*⁺ CML is a stem cell-derived disease with three distinct phases: (i) a chronic phase (CP) that may last for several years, (ii) an accelerated phase (AP), and (iii) a blast crisis (BC).⁴

Therapy of BCR-ABL1-induced diseases was significantly improved by the development and application of small molecular weight inhibitors blocking the activity of the ABL1 kinase. Imatinib was the first substance, which became available by 2001, and was soon followed by other tyrosine kinase inhibitors (TKIs) such as dasatinib and nilotinib.⁵⁻⁷ All these TKIs target the enzymatic activity of the ABL1 tyrosine kinases, including c-ABL, v-ABL, and BCR-ABL1.^{6,8,9} Imatinib has now become the standard first line therapy for all patients with CML. However, not all patients with CML respond equally well to imatinib.¹⁰ Moreover, about 15-25% of the patients that initially responded well acquire resistance against imatinib during therapy. The percentage of non-responders is even higher in an advanced phase of CML.¹⁰⁻¹² These patients are treated with an increased imatinib dosage (600-800 mg per day), second generation BCR-ABL1 inhibitors, or with stem cell transplantation.¹³⁻¹⁸ The most frequently reported causes for resistance to TKIs in CML are mutations in the kinase domain of BCR-ABL1 with a reported frequency of 40 to 90%.^{13,15,16} Other mechanisms include the upregulation of BCR-ABL1 or increased expression of the drug transporter ABCB1.^{14,18} Apart from these mechanisms, deregulation in signaling cascades and activation of specific signaling molecules like LYN have also been discussed in contributing to drug resistance in CML.^{19,20}

Signaling pathways activated by BCR-ABL1 include the PI3K-mTOR pathway, RAS/RAF/MEK/ERK pathway, and the JAK-STAT pathway.²¹⁻²⁴ We have recently shown that STAT5 is absolutely required for the transformation of hematopoietic cells by *BCR-ABL1* oncogenes.^{22,25} In this study, we investigated the effect of STAT5 on imatinib response in *BCR-ABL1*⁺ cells. We show that

imatinib resistance increases upon elevated STAT5 protein expression. Interestingly, this correlates with an upregulation of STAT5 in imatinib-resistant patients and patients with advanced stages of disease. Thereby our study defines STAT5 as a clinically relevant modulator of imatinib responsiveness. This may have implications for the understanding of drug resistance development and the evolution of CML from CP to advanced phases, as well as in the design of new drugs and new treatment approaches.

RESULTS

Increased imatinib resistance in Abelson transformed cells upon maintenance in tissue culture

Transformation of hematopoietic cells with p185^{BCR-ABL1} and v-ABL results in the outgrowth of growth-factor independent CD19⁺CD43⁺B220⁺ cells. We noted that different v-ABL transformed lymphoid pro B-cell clones (v-*AbI*⁺ cells) varied significantly in their response to imatinib. We asked whether these differences might be related to the time the cells had been in culture. V-*AbI*⁺ cells that had been propagated for less than three months after the initial transformation event were defined as short term cultures (STCs). They were opposed by long term cultures (LTCs) that had been cultured for more than eight months. First, we compared cell numbers of LTCs and STCs upon imatinib treatment. Since LTCs proliferated slightly faster than STCs (Figure S1A), we set the individual controls to 100% to allow their comparison. As depicted in Figure 1A, STCs reacted with a pronounced decrease in cell numbers to 100 nM imatinib, whereas LTCs were affected to a significantly minor degree. This difference in imatinib sensitivity also became obvious when the cells were plated in growth-factor free methylcellulose (Figure 1B, summarized in Figure S1B). Whereas the ability of LTCs to form colonies in the presence of 100 nM or 200 nM imatinib remained largely unchanged, a significant decrease was observed for STCs. Dose response curves confirmed the decreased imatinib-sensitivity of LTCs (IC₅₀STC = 0.35 μM, IC₅₀ LTC = 5.05 μM; Figure 1C). Immunoblots revealed that several molecules were expressed at elevated levels in LTCs compared to STCs (Figure 1D). Among the factors that were up-regulated were the STAT transcription factors. Quantitative analysis by ImageJ revealed a highly significant up-regulation of STAT5A/B. STAT1, STAT3 and c-ABL showed a less consistent pattern (Figure 1D, quantitative analysis Figure S1C). We asked whether the elevated expression of STAT transcription factors contributes to the reduced imatinib sensitivity in LTCs. Several factors have been shown to confer TKI resistance, but nothing was known about STAT factors. To test whether the up-regulation of individual STAT proteins are on their own of functional relevance, *Stat1*^{-/-} and *Stat3*^{Δ/Δ} leukemic cell lines were treated with increasing concentrations of imatinib for 24 hours. No differences on imatinib-sensitivity were detectable (Figure S1D, S1E and S1F).

Low STAT5 protein levels are related to increased imatinib sensitivity in p210^{BCR-ABL1} and v-ABL dependent cells

Stat5^{null/null} cells cannot be transformed by BCR-ABL1 and v-ABL.²² To evaluate the impact of STAT5 on imatinib sensitivity, we prepared single cell suspensions of bone marrow (BM) from

Stat5^{+/+} and *Stat5*^{null/+} mice. Differences in the STAT5 protein levels of the BMs were verified via immunoblot (Figure S2A). These cells were infected with *pMSCV-p210*^{BCR-ABL1}-IRES-GFP in StemPro medium. The initial infection rate was comparable between *Stat5*^{+/+} and *Stat5*^{null/+} cells and varied between 10-20% in individual experiments. After three days the cells were kept under cytokine free conditions which increased the percentage of GFP⁺ cells to 30-50% after five days. The majority of the GFP⁺ and hence BCR-ABL1⁺ cells expressed the myeloid surface-marker MAC-1 (Figure S2B). The cells were then treated with increasing concentrations of imatinib for 48 hours. Imatinib reduced the viability and cell numbers of the BCR-ABL1⁺/GFP⁺ cells without affecting the BCR-ABL1 negative population. As additional control naive BM was treated with Imatinib, which did not exert any effect irrespective of the genotype. In contrast and as depicted in Figure 2A and 2B *Stat5*^{null/+} cells showed a significantly stronger reduction of viable BCR-ABL1⁺/GFP⁺ cells when compared to BCR-ABL1⁺/GFP⁺ *Stat5*^{+/+} cells. Similar results were obtained with two individual p210^{BCR-ABL1} transformed *Stat5*^{fl/+} Rosa-Cre-ER^{T2} cell lines that express the progenitor markers c-KIT and SCA-1 (Figure S2C and S2D). Deletion of one *Stat5a/b* allele by Tamoxifen treatment enhanced cell death to imatinib exposure.

Our observations were confirmed in v-ABL transformed lymphoid cells. Cell lines derived from *Stat5*^{null/+} fetal livers display a ~ 50% decreased STAT5 protein expression when quantified by ImageJ software when compared to v-Abi⁺ *Stat5*^{+/+} cells (Figure 2C). In contrast, no differences in v-ABL expression were detected. Determination of IC₅₀ values upon imatinib treatment in the *Stat5*^{null/+} STC revealed a more than 10-fold difference (IC₅₀ *Stat5*^{null/+} = 31 nM, IC₅₀ wild type = 353 nM; Figure 2D). The increased imatinib sensitivity of *Stat5*^{null/+} cells was also prominent when cells were seeded in growth-factor free methylcellulose in the presence of 10 or 100 nM imatinib (Figure 2E). AnnexinV stainings suggest that the underlying mechanism relies on increased apoptosis in *Stat5*^{null/+} cells. Significantly more cells underwent apoptosis in *Stat5*^{null/+} STCs compared to wild type controls upon treatment with 100 nM imatinib for 24 hours (Figure 2F and 2G). These experiments implicate STAT5 as a regulator of imatinib responsiveness.

Enhanced expression of STAT5A reduces TKI -induced cytotoxicity

Stat5^{null/+} cells may harbor additional developmental alterations that account for the differences in imatinib sensitivity. To exclude this possibility, we undertook the following experiment: We infected *p185*^{BCR-ABL1+} or v-Abi⁺ STCs with a *pMSCV-Stat5A-eGFP* based retrovirus to generate transformed B-cells that differ from vector controls solely by an elevated STAT5A expression.

One representative experiment is depicted in Figure 3A. We obtained an infection rate of about 20% irrespective whether we used the empty vector or the vector encoding STAT5A (resulting in “S5a^{high} cells”). Subsequently, we challenged the resulting mixed cell population of non-transduced and transduced cells to increasing concentrations of imatinib. Of the viable cells, the percentage of *GFP*⁺ cells was analyzed using FACS 24 hours thereafter. The treatment of cells with imatinib did not change the percentage of *GFP*⁺ cells infected with empty vector (Figure 3A, lower panel). In contrast, cells expressing elevated levels of STAT5A were selected in the presence of imatinib. This was indicated by the increase in percentage of *GFP*⁺ - S5a^{high} cells (Figure 3A, upper panel). The effect was most evident at a concentration of 1000 nM imatinib, where the percentage of S5a^{high} cells increased from 20% to 56% within 24 hours. The experiment was repeated with comparable results using individually derived *v-Abl*⁺ cell lines (n = 10) or *p185^{BCR-ABL1}* cell lines (n = 5) (summarized in Figure 3B and 3C). The effects were reinforced when imatinib incubation time of *v-Abl*⁺ cells, at a concentration of 100 nM, was extended to 48 and 72 hours (Figure S3A). Similarly, long term incubations using low concentrations of imatinib (10 nM) were capable to select for S5a^{high} cells over a period of two weeks (Figure S3B).

To exclude that interaction of S5a^{high} cells with non-infected cells in the mixed populations interferes with the imatinib response, *v-Abl*⁺ cells were sorted for GFP-expressing (S5a^{high}) and non-expressing (S5a^{low}) cells. FACS analysis for intracellular STAT5A/B and immunoblots confirmed higher STAT5 protein level in S5a^{high} cells compared to S5a^{low} cells (Figure 3D, inserted histograms and Figure S3C). The differences in imatinib sensitivity were even more pronounced than in the mixed cell populations. Starting at concentrations of 300 nM imatinib, S5a^{high} cell lines had a significantly higher viability. All S5a^{low} cells underwent apoptosis when exposed to 1000 nM imatinib, whereas still ~60% of the S5a^{high} cells remained viable after 48 hours (Figure 3D). Cytospins confirmed these results (Figure 3E). These experiments clearly link enforced STAT5A expression to decreased imatinib sensitivity.

We next tested whether the protective effect of elevated STAT5A protein levels is specific for imatinib or extends to other BCR-ABL1 kinase inhibitors. As depicted in Figure 3F, the protective effect of STAT5A was also observed in experiments employing dasatinib and nilotinib. Most importantly, the protective effect neither restricted to one BCR-ABL1 kinase inhibitor nor to our cellular system. We took advantage of p210^{BCR-ABL1} transformed Ba/F3 cells that additionally express a doxycycline-inducible dominant negative variant of STAT5A (S5a^{Δ749}).²⁷ The expression

of S5a^{Δ749} was induced for 48 hours by doxycycline treatment. A dosage of doxycycline was defined in preliminary experiments that induced S5a^{Δ749} expression but had only a minor impact on the viability of the cells (data not shown). To delineate the combined effects of BCR-ABL1 kinase inhibition and STAT5 inhibition, concentrations of the TKIs only slightly affecting the cells were used. The inhibitors imatinib, nilotinib or dasatinib were added 24 hours after doxycycline. As depicted in Figure 3G, the combined inhibition of STAT5 and p210^{BCR-ABL1} resulted in a significantly decreased viability compared to the inhibition of p210^{BCR-ABL1} alone. These experiments suggested that transcriptionally competent STAT5 regulates the sensitivity of v-ABL and BCR-ABL1 transformed cells towards BCR-ABL1 kinase inhibition.

Transcriptional activity of STAT5 is required to confer protection from imatinib-induced apoptosis

For transcriptional regulation, STAT5 is tyrosine phosphorylated, dimerizes and translocates to the nucleus, where it binds to DNA. To investigate whether enforced expression of STAT5 in Abelson transformed cells is accompanied by increased tyrosine phosphorylation, DNA binding, and transcriptional activity of STAT5, we undertook the following experiments: First, we quantified tyrosine phosphorylated STAT5 using intracellular FACS. As depicted in Figure 4A (left panel), higher levels of phosphorylated STAT5 were present in sorted S5a^{high} cells compared to S5a^{low} cells. Accordingly, S5a^{high} cells showed increased amounts of DNA-bound-STAT5 verified by EMSA (Figure 4A, right panel). We next tested mRNA expression levels of critical STAT5 target genes implicated in survival or proliferation promotion of hematopoietic cells. Real-time (RT)-PCRs for the STAT5 target genes *Osm*, *Pim-1*, *Cis*, *c-Myc*, *Cyclin D2*, *Bcl-2* and *Socs2* were performed. The mRNA levels of S5a^{low} cells served as baseline. A clear upregulation of all analyzed mRNAs was detected in S5a^{high} cell lines (Figure 4B). A recent publication describes the downregulation of STAT5 target genes as one of the earliest events after dasatinib treatment.³⁴ In line with this report, treatment with high concentrations of dasatinib for 3 hours strongly reduced STAT5 target gene mRNA levels, irrespective of the STAT5A expression level (Figure 4B). To investigate whether the transcriptional function of STAT5A accounts for the STAT5A mediated resistance against imatinib, we took advantage of different STAT5A mutants (depicted in Figure 4C). cS5^F is a constitutively active STAT5A mutant due to a S711F point mutation. S5a^{ΔN} lacks the N-terminal domain required for oligomerization, but is still capable of functioning as a transcription factor for some STAT5 target genes.^{29,32} S5a^{Y/F} lacks the critical tyrosine phosphorylation (pY) site on position aa693 and does not efficiently translocate to the nucleus,

S5a^{EE/AA} has DNA-binding domain mutations (EE437/438AA) blocking efficient DNA binding to STAT5 response elements.³⁰ Retroviral infection was used to express these mutants in wild type STCs. Cells were treated for 24 hours with imatinib and viable cells were analyzed for GFP-expression by FACS. Wild type STAT5A, cS5^F and S5a^{ΔN} mediated growth advantage compared to untransfected cells. In contrast, infection with the empty vector or STAT5A mutants incapable of DNA binding had no effect (Figure 4D). This experiment provided evidence that tyrosine phosphorylation and DNA binding of STAT5 are prerequisites for the protective effect against imatinib. In line with this finding, sorted S5a^{high} cells showed only an insignificant reduction of pSTAT5 levels when treated with imatinib up to concentrations of 300 nM for 24 hours (Figure 4E, upper panel). The pSTAT5 levels in S5a^{high} cells were reduced to the levels observed in S5a^{low} cells, only when we used concentrations of imatinib as high as 1000 nM. This was accompanied by apoptosis after 72 hours of treatment, underscoring a close correlation of pSTAT5 level and imatinib sensitivity (Figure 4E, lower panel). In contrast, phosphorylation of STAT5 in S5a^{low} cells was already blocked at an imatinib concentration of 300 nM, accompanied by apoptosis. We conclude that only tyrosine phosphorylated and transcriptional active STAT5 confers protection against imatinib and that a certain threshold of pSTAT5 is required to enable Abelson transformed cells to survive.

STAT5 expression in leukemic cells of CML patients increases during disease progression

We next analyzed STAT5 levels during disease progression in order to determine the significance of our data for patients suffering from CML. To address this, peripheral blood (PB) or BM derived leukemic cells of untreated patients at the time point of diagnosis were collected. Thirteen patients were in CP and seven in AP at the time point of collection. *STAT5A* mRNA levels were determined by RT-PCR and BM from three healthy patients served as control. A significant increase of *STAT5A* mRNA was found in samples obtained from patients in AP compared to CP (Figure 5A). These data correlate with the recommended dosage of imatinib for CP, which is 400-600 mg/day and increases to 600-800 mg/day when the leukemia progresses to AP. In addition, we tested eight samples from imatinib treated patients, six in CP, one in AP and one in BC that had developed imatinib resistance and relapsed. 40% - 90% of cases of imatinib resistance are a result of mutations within the kinase domain of BCR-ABL1.³⁵ Although all of our analyzed imatinib-resistant CML patients were tested positive for BCR-ABL1 mutations, a significant up-regulation of *STAT5A* mRNA compared to patients with imatinib sensitive CP was registered. The international scale (IS) value for *BCR-ABL1* mRNA level and detected *BCR-ABL1* mutations are

depicted (analyzed *BCR-ABL1* mutations listed in Figure S4A). Similar results were obtained for *STAT5B* mRNA when samples from three healthy and nineteen CML diseased patients (eight in CP, six in AP and five imatinib-resistant patients, three of them in CP, one in AP and one in BC) were analyzed (Figure S4B). The increase in *STAT5* mRNA is reflected by changes in protein expression levels: patients suffering from CML in AP or imatinib-resistant CP display higher *STAT5* protein levels (Figure 5B). In one sample (patient #32) we failed to detect *STAT5* protein even after prolonged exposure for unknown reasons. To control for the quality of the patient samples additional to β -Actin we analyzed the protein expression of CDK2, CDK4 and α -Tubulin which were comparable between the disease phases (Figure S4C). Expression of activated *STAT5* (p*STAT5*) in primary CML cells was further confirmed by immunocytochemistry. In these experiments, the anti-p*STAT5* antibody AX1 was found to react with primary CML cells. The percentage of p*STAT5*⁺ cells varied among patients. In most patients with CP, only a few cells stained positive for p*STAT5*, whereas higher counts were recorded in AP and BC (Figure 5C and Table 1) consistent with our previous findings. Furthermore, the p*STAT5*-intensity, which reflects the amount of *STAT5* proteins in untreated cells, was elevated in AP and BC. Interestingly, even in BC, not all clonal cells (blasts) were found to react with the anti-p*STAT5* antibody.

The upregulation of *STAT5* was restricted to *BCR-ABL1*⁺ disease, since two samples of CML patients in *BCR-ABL1*-negative BC (48% and 50% PB blasts) and two patients with secondary AML (26% and 85% PB blasts) did not show significant changes in *STAT5* mRNA levels (Figure 5D). The upregulation of *STAT5* can therefore not be explained by a shift in cellular populations such as the presence of increased numbers of immature cells and blasts in the *BCR-ABL1*⁺ samples investigated. Since in these *BCR-ABL1*-negative cells the *BCR-ABL1*-driven constitutive activation of *STAT5* is missing, we speculated that activated *STAT5* promotes its own transcription, leading to its upregulation. First evidence supports this concept; the enforced expression of wild type *STAT5A* or constitutive active *STAT5A* (cS5a^F) in v-ABL of p210^{BCR-ABL1} dependent cells induces *STAT5B* expression and vice versa (Figure S4D). It is attractive to speculate that this feed-forward loop, combined with an imatinib-induced selection pressure, accounts for the upregulation of *STAT5* as observed in CML-patients.

Ectopic expression of STAT5A renders v-ABL transformed cells resistant to *in vivo* treatment with imatinib

The data obtained so far encouraged us to challenge our concept in an *in vivo* experimental setting. To mimic the situation of leukemic patients undergoing imatinib treatment, we injected NOD/SCID mice with *v-Abl*⁺ cells. We prepared wild type STCs that contained 99% of uninfected cells (S5a^{low}) enriched by 1% of cells infected with *pMSCV-Stat5A-IRES-GFP* (S5a^{high}). Thus, S5a^{high} cells are indicated by GFP expression (Figure 6A, upper middle panel). 2x10⁶ cells/mouse were injected intravenously and the animals showed first signs of disease such as reduced mobility and scruffy fur 13 days thereafter. Blood samples were taken, which revealed the presence of significant amounts of leukemic cells. About 80% of the cells in the PB expressed CD19 and were therefore classified as leukemic cells. No change in the composition of S5a^{high} versus S5a^{low} cells was detectable. Still, 1% of the leukemic cells were *GFP*⁺, indicating high STAT5A expression levels (Figure 6A, top right panel). Hence, under conditions where the leukemic cells spread and proliferate in the animal, the enforced expression of STAT5A did not confer any selective advantage. Treatment with imatinib was initiated with 100 mg/kg once per day by gavage. During therapy, PB samples were checked daily by FACS for the presence of leukemic cells. Importantly, the “regular” S5a^{low} leukemic cells steadily declined under imatinib therapy. In contrast, the S5a^{high} cells remained unaffected and steadily increased (Figure 6B). Nine days after treatment initiation, the animals had to be sacrificed because they displayed signs of sickness with pronounced weight loss and hind leg ataxia. Pathological analysis revealed that the mice suffered from a leukemia densely infiltrating spleen, BM and lymph nodes. FACS analysis showed that the leukemic cells consisted nearly exclusively of *GFP*⁺ S5a^{high} cells (Figure 6B, right panel). This was observed in all mice analyzed (n = 6; Figure 6C). In contrast, no selection of S5a^{high} cells was detected in untreated animals (Figure S5A). Interestingly, we did not only see a selection of S5a^{high} cells over S5a^{low} cells, but also a selection process within the S5a^{high} population with an enrichment of the brightest *GFP*⁺ S5a^{high} cells indicating very high levels of STAT5A protein. The GFP fluorescence intensity correlates with the amount of ectopically expressed STAT5A (Figure S5B). We observed a 46 fold higher GFP-mean-fluorescence intensity (GFP-MFI) in the S5a^{high} population compared to the GFP-negative cells which remained constant during the progression of the leukemia before imatinib treatment (Figure 6D, top and middle panel). However, 9 days after imatinib-treatment the GFP-MFI value directly indicating STAT5A expression had significantly increased from 46-fold to 97-fold (Figure 6D, bottom panel, summarized in Figure S5C). Next, we questioned if CML cells derived from the murine transplant do have high pSTAT5

levels despite the continuous presence of imatinib. As depicted in Figure 6E, S5a^{high} leukemic cells that evolved in the presence of imatinib displayed pSTAT5 levels that were only slightly reduced compared to untreated cells *in vitro*.

METHODS

Mice

Stat5a/b^{null/+}, *Stat5^{fl/+}*, *Stat3^{fl/fl}*, *Mx1Cre* (all mixed *C57BL/6J* x *Sv129*), *Stat1^{-/-}* (*C57BL/6J*), *Cre^{ER^{t2}}* (FVB/NJ) and *NOD.CB17-Prkdc^{scid}/NCrHsd* (NOD/SCID, Harlan Laboratories) mice were maintained at the Biomedical Research Institute (Medical University of Vienna) and genotyped as described previously.²⁶ NOD/SCID mice were used for leukemia engraftment and imatinib *in vivo* studies. All animal experiments were carried out in accordance with protocols approved by Austrian law.

Tissue culture conditions, infections, and *in vitro* imatinib sensibility assays

Tissue culture conditions, virus preparation, infection of fetal liver cells with viral supernatant from A010 cells or gp + E86 producer cell lines and establishment of cell lines was described previously.²²

Immunoblotting and EMSA

Immunoblots and EMSA on the β -casein promoter were carried out as described previously.^{22,27} Membranes were probed with antibodies directed against Stat1 (sc-592X), Stat3 (sc-482X), STAT5A/B (sc-835X), CDK2 (sc-163), CDK4 (sc-260), c-ABL (sc-23, all purchased from Santa Cruz), β -Actin (A5441) and α -Tubulin (T9026, both Sigma). Antisera against STAT5A and STAT5B have been described previously.²⁸

Plasmids

p210^{BCR-ABL1}, the construct of wild type *Stat5a*, the *Stat5a* mutants *cS5^F*, *S5a^{ΔN}*²⁹, *S5a^{Δ749}*²⁷, *S5a^{EE/AA}*, *S5a^{Y/F}*³⁰, and the *Stat5b* mutant *S5b1*6*³¹ were expressed in the retroviral vector *pMSCV-IRES-eGFP*. *V-Abl* and *p185^{BCR-ABL1}* were cloned into a *pMSCV* backbone. Ecotropic, replication incompetent gp + E86 producers were generated and selected for high virus titer production by FACS as described previously.^{29,32}

Imatinib treatment of *p210^{BCR-ABL1}-IRES-GFP* infected bone marrow

For imatinib-sensitivity studies of *p210^{Bcr-Abl1+}* cells, two different approaches were used. (i) Bone marrow cells derived from *Stat5^{+/+}* or *Stat5^{null/+}* mice were co-cultivated on *p210^{Bcr-Abl1}* retroviral producer cells for 48 hours in the presence of StemPro-34 serum-free medium (Life

Technologies) supplemented with IL-3 [10 ng/ml], IL-6 [5 ng/ml], GM-CSF [1 ng/ml], FLT3-ligand [20 ng/ml], IGF-1 [40 ng/ml] SCF [100 ng/ml], Dexamethasone and polybrene [7 µg/ml]. Three days after infection, StemPro medium was replaced with cytokine-free RPMI supplemented with 10% FCS, 1% penicillin/streptomycin and 0.1% β-mercaptoethanol. Five days after StemPro medium deprivation, cells were treated with imatinib for 48 hours and analyzed via FACS for GFP and MAC-1 expression. (ii) Bone marrow cells derived from *Stat5^{fl/+} Rosa Cre-ER^{t2}* and *Stat5^{+/+} Rosa Cre-ER^{t2}* mice were co-cultivated on *p210^{Bcr-Abl1}* retroviral producer cells for 48 hours in the presence of IL-3 [25 ng/ml], IL-6 [50 ng/ml], SCF [50 ng/ml] and polybrene [7 µg/ml] and injected via the tail vein into lethally irradiated (10 Gy) wt recipients (1×10^6 cells/mouse). After disease onset, the mice were sacrificed and leukemic cells isolated from bone marrow and blood. After two weeks of culture time in RPMI complete, growth factor independent leukemic cells were analyzed for expression of SCA-1 and c-KIT via FACS. For expression of CRE and deletion of the floxed *Stat5* allele, cell lines were treated with 1µM tamoxifen-OH. To test for TKI-sensitivity, cells were treated with imatinib for 24 hours and percentage PI positive cells were analyzed.

Primary patient samples

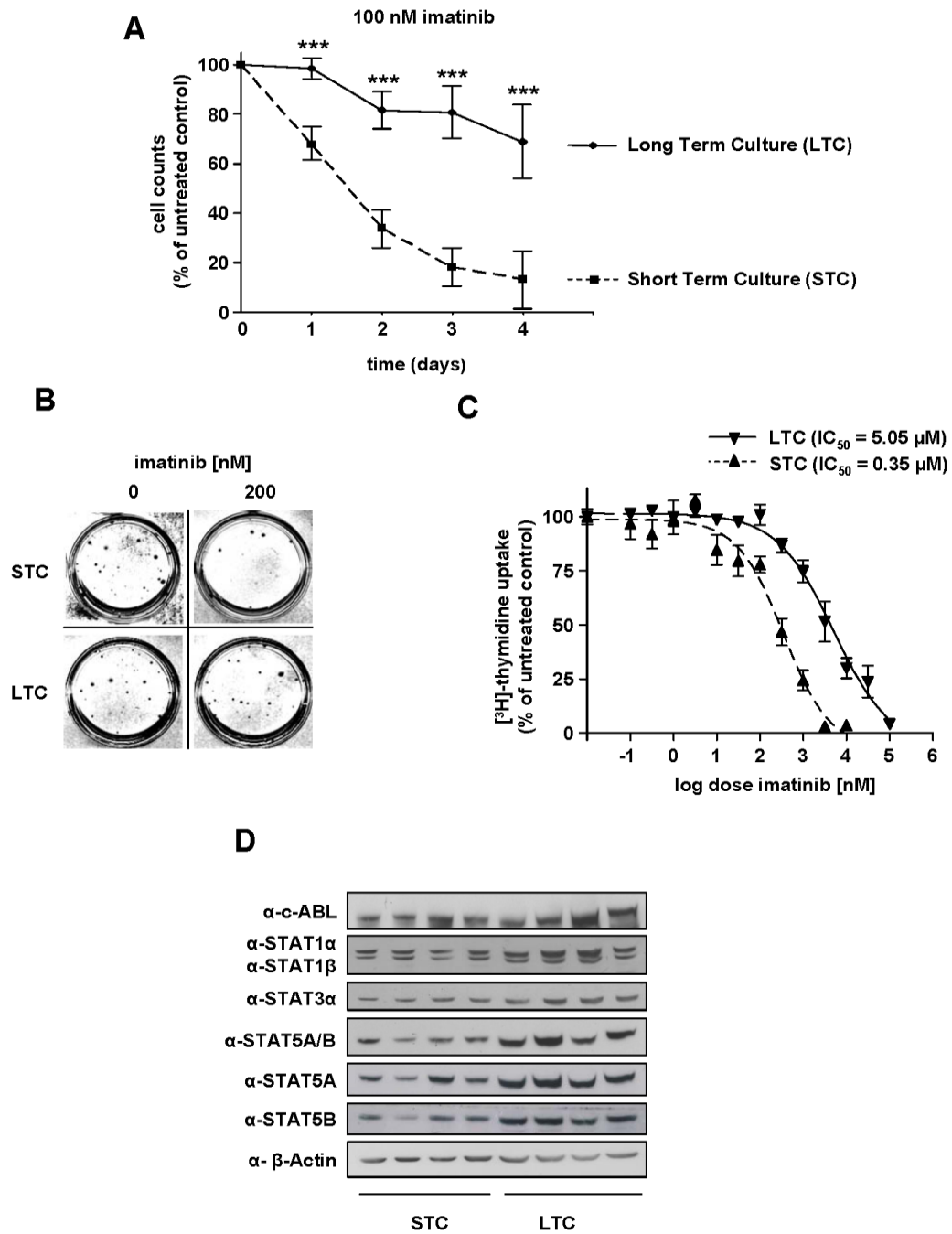
Primary cells were obtained from patients treated at the General Hospital, Vienna, Austria. Cells were obtained from patients with CML (n = 36) or secondary AML (n = 2) at routine blood and bone marrow examinations after informed consent was given. Peripheral blood and bone marrow mononuclear cells (MNC) were isolated using Ficoll. Samples were analyzed for *BCR-ABL1* mutations (complete list of analyzed mutations see Figure S4A) and *BCR-ABL1* mRNA level according to the international standard protocol. Use of human samples was approved by the Ethical Committee of the Medical University of Vienna and is in compliance with Austrian legislation.

Immunocytochemistry was performed as described in ³³

Statistics

Two-tailed Student's *t* tests were used for statistical analysis. Difference was considered statistically significant when *P* < 0.05. The data are represented as mean ± SD of the number of the determinations and were analyzed by Graph Pad® software (San Diego, CA).

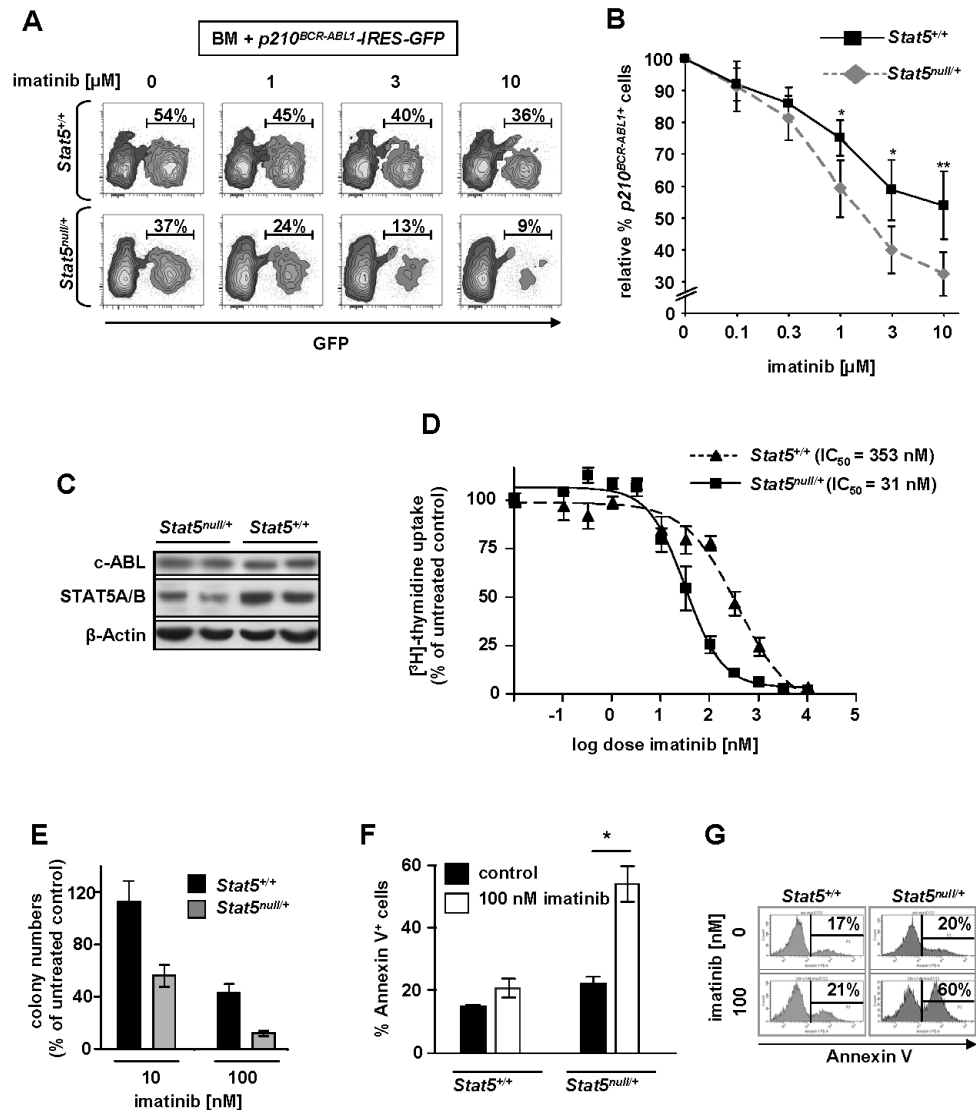
Figure 1



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Figure 1. Prolonged culture times of *v-Abl*⁺ lymphoid cells correlate with increased STAT5 protein level and decreased imatinib sensitivity. (A) Cell counts of LTCs (n = 4) and STCs (n = 6) treated with 100 nM imatinib. (B) Representative colony forming assays of LTCs and STCs in growth-factor free methylcellulose without and supplemented with 200 nM imatinib. Pictures of colonies were taken after 14 days (LTCs) and 21 days (STCs). (C) Dose response curves determined by [³H]-thymidine incorporation of LTCs (n = 6) and STCs (n = 4) treated with imatinib for 24 hours. Control values in the absence of imatinib were set to 100%. (D) Immunoblot analysis of STCs and LTCs for STAT transcription factors and v-ABL. (A, C: Error bars represent mean $\pm$ SD. *** P < 0.001).

Figure 2



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Figure 2. *Stat5*^{null/+} cells depending on p210^{BCR-ABL1} or v-ABL are highly susceptible to imatinib-induced apoptosis. (A) BM derived from *Stat5*^{null/+} (n = 4) and *Stat5*^{+/+} (n = 6) mice were infected with a retrovirus encoding for p210^{BCR-ABL1}-IRES-GFP and subsequently treated for 48 hours with increasing dosages of imatinib as indicated. Percentages of BCR-ABL1⁺/GFP⁺ cells were analyzed via FACS to evaluate differences in imatinib response. *Stat5*^{null/+} cells reacted significantly more sensitive on imatinib treatment compared to *Stat5*^{+/+} cells. Depicted is one representative FACS-profile. (B) Summary of all experiments described in (A). (C) Immunoblot analysis of STAT5A/B and v-ABL protein expression in *Stat5*^{null/+} and *Stat5*^{+/+} cells. (D) Dose response curves determined by [³H]-thymidine incorporation of v-ABL transformed *Stat5*^{null/+} and *Stat5*^{+/+} cells treated with imatinib for 24 hours (n = 4/genotype). (E) Summary of colony forming assays of v-ABL⁺ *Stat5*^{null/+} and *Stat5*^{+/+} cells grown for 21 days in growth-factor free methylcellulose enriched with 10 or 100 nM imatinib (n = 3/genotype). (F,G) Annexin V stained v-ABL transformed *Stat5*^{null/+} and *Stat5*^{+/+} cells challenged with 100 nM imatinib for 24 hours (right, n = 3/genotype). One representative FACS profile is depicted in panel G. (Data are means ± SD, * P < 0.05, ** P < 0.001).

Figure 3

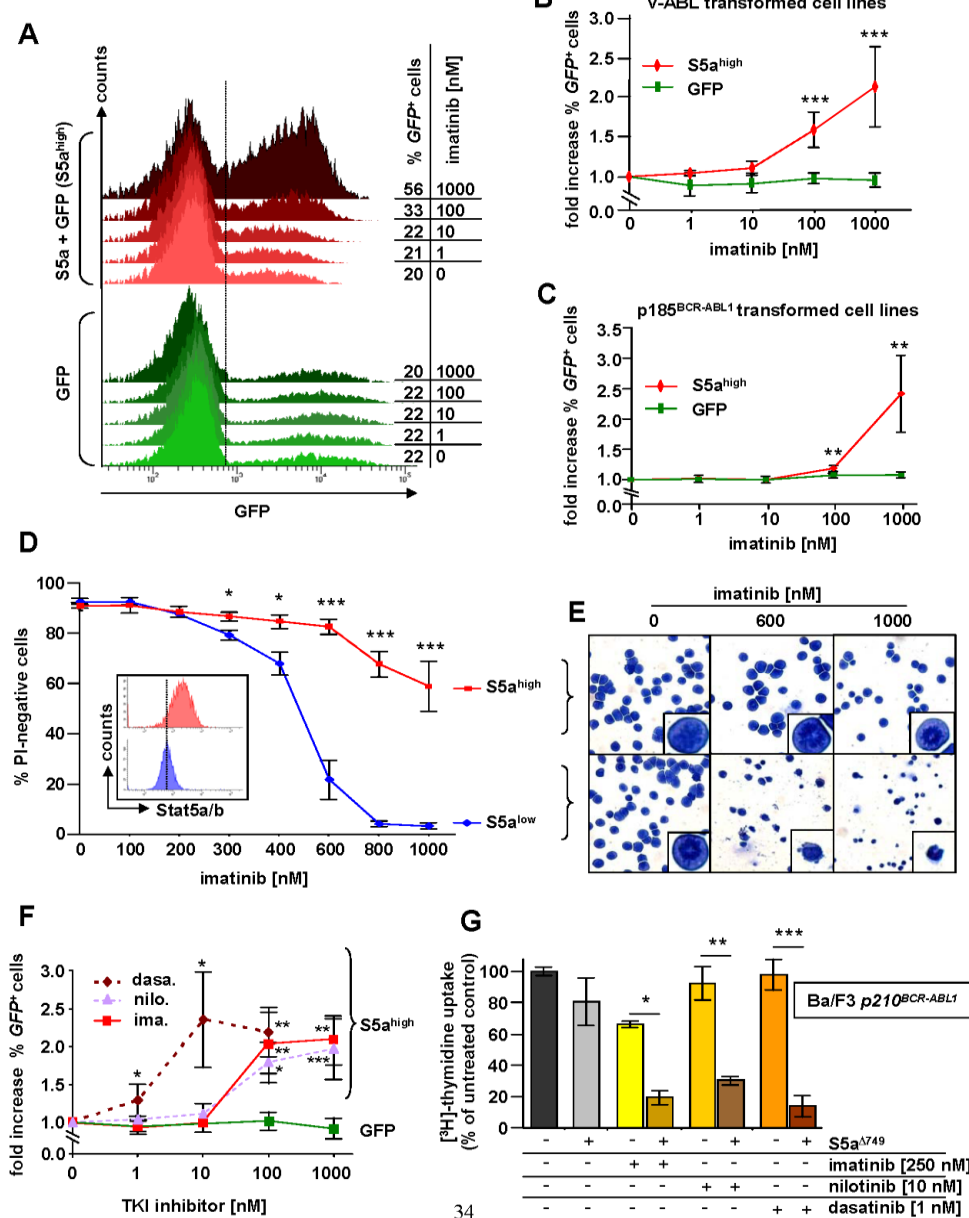


Figure 3. Enhanced STAT5A expression renders cells less susceptible to imatinib-induced apoptosis. (A) FACS profiles of *v-Abl*⁺ cells ectopically expressing STAT5A and GFP (top profile) and vector control cells expressing only GFP (bottom profile) treated 24 hours with imatinib. The percentage of GFP⁺ cells and the dosage of imatinib administered are indicated. (B, C) Summary of the experiment depicted in panel A repeated with 10 individual *v-Abl*⁺ (B) and 5 individual *p185^{BCR-ABL1}* (C) cell lines. (D) FACS analysis for PI-uptake of *v-Abl*⁺ cell lines sorted for *Stat5a-IRES-eGFP* expressing- (*S5a^{high}*) and control (*S5a^{low}*) cells treated imatinib for 48 hours (n = 3/group). STAT5A/B expression levels were determined by intracellular FACS staining and are depicted in the insert. (E) Representative cytopins of *S5a^{high}* and *S5a^{low}* cells 48 hours after imatinib treatment. (F) *v-ABL* transformed STCs were infected with *pMSCV-Stat5a-IRES-eGFP* and treated with imatinib, dasatinib and nilotinib. The experiment was performed as outlined in Figure 3A. The summary of three individually performed experiments is depicted. (G) [³H]-thymidine incorporation assay of Ba/F3 *p210^{BCR-ABL1}* cells harboring a doxycycline inducible dominant negative STAT5 (*S5a^{Δ749}*). [³H]-thymidine uptake was measured 48 hours after induction of *S5a^{Δ749}* expression and 24 hours after TKI treatment.

Figure 4

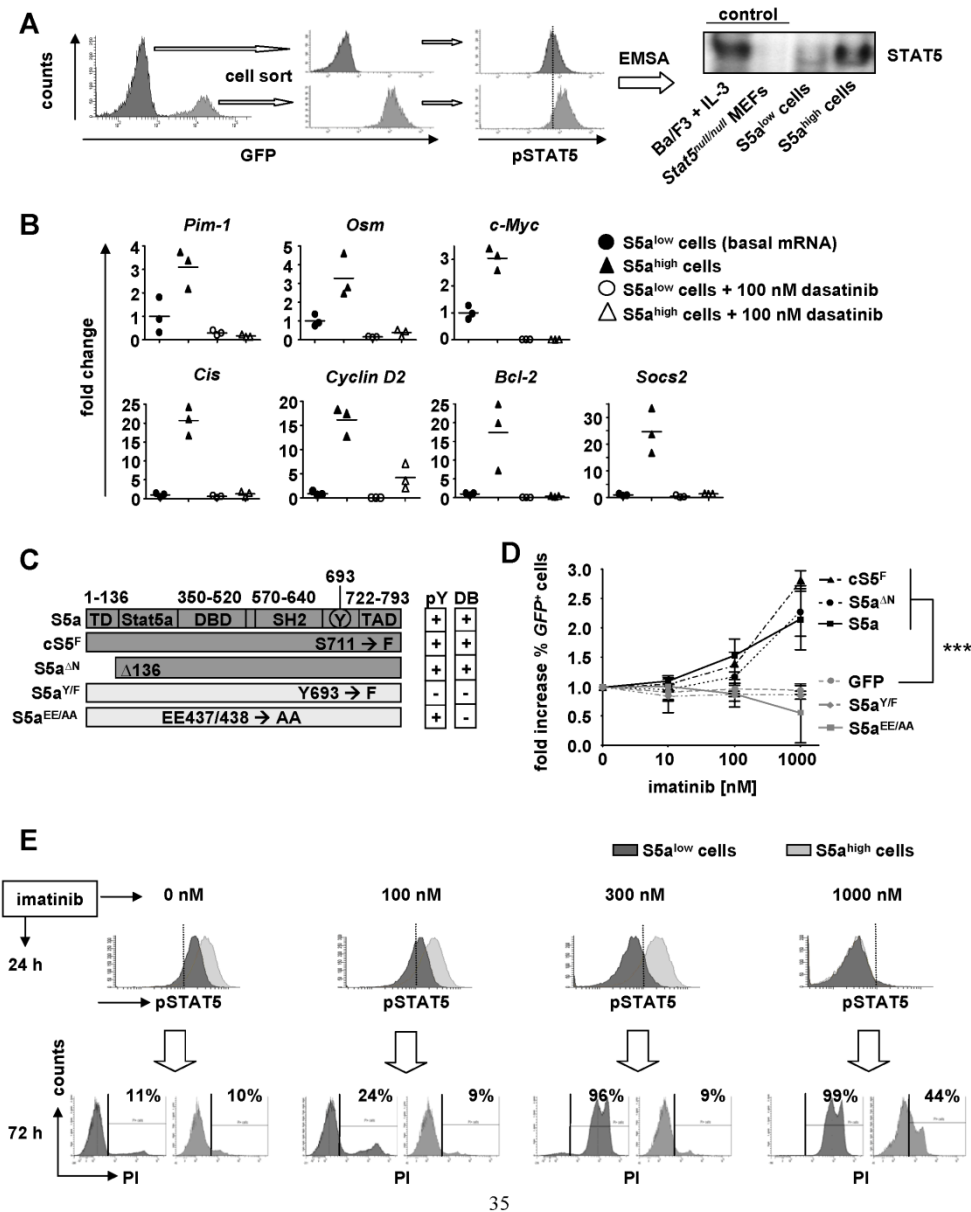


Figure 4. Survival of imatinib treated *v-Abi^f* cells depends on the phosphorylation-status of STAT5. (A) FACS profiles of cells infected with *pMSCV-Stat5a-IRES-eGFP* retrovirus before (left histogram) and after sort for GFP (middle histograms). Tyrosine phosphorylated STAT5 is depicted in the right histograms. The right panel depicts an EMSA of sorted *GFP⁺* (S5a^{high}) and *GFP⁻* (S5a^{low}) cells for STAT5. *Stat5^{null/null}* MEFs were used as negative control and Ba/F3 stimulated with IL-3 were used as positive control. (B) RT-PCR of S5a^{high} and S5a^{low} cells before and after treatment with 100 nM dasatinib for 3 hours. Results were normalized by *Gapdh* mRNA expression. The fold change compared to untreated S5a^{low} cell mRNA levels is shown (n = 3/group). (C) Scheme of murine wild type and mutant STAT5A variants. Mutants lacking the tyrosine phosphorylation (pY) site or having impaired DNA-binding (DB) are indicated by either (+) or (-). (D) *V-Abi^f* cell lines expressing STAT5A, STAT5A mutants or vector control along with GFP 24 hours after imatinib administration. The fold increase of GFP expressing cells is shown. Error bars represent means ± SD. *** P < 0.001 for a dosage of 1000 nM imatinib (n ≥ 4/group). (E) Upper Panel: intracellular pSTAT5 staining of S5a^{low} and S5a^{high} cells 24 hours after imatinib treatment as indicated. The dashed line displays the predicted threshold of pSTAT5 level essential for survival. Lower panel: FACS-analysis for PI-uptake 72 hours after imatinib treatment as indicated. Percentages of PI-positive cells are shown.

Figure 5

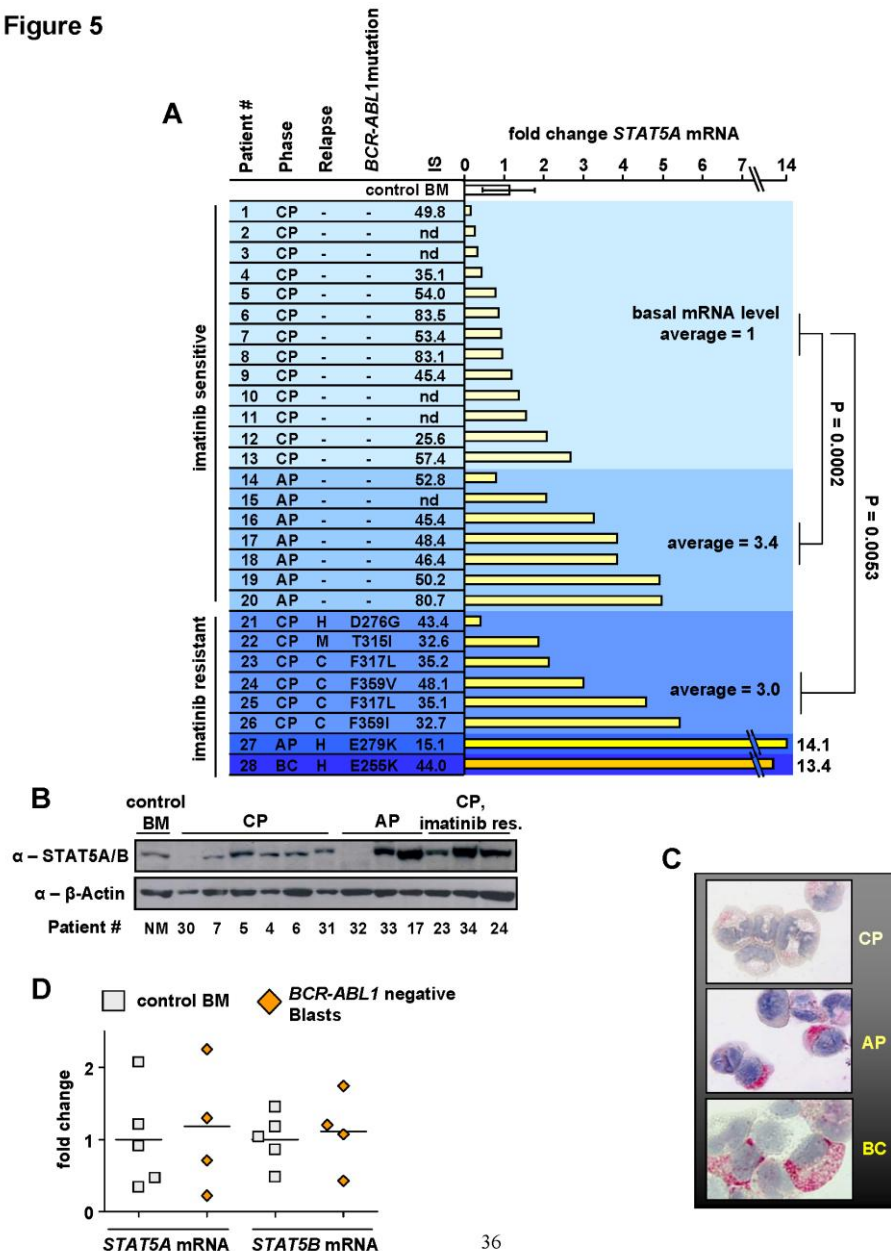


Figure 5. Increased STAT5 expression during disease progression and under imatinib treatment in human CML-patients. (A) RT-PCR of human *STAT5A* mRNA transcripts of BM derived from healthy control patients (n = 3), PB derived from untreated CML patients in CP (n = 13), AP (n = 7) and relapsed imatinib-resistant patients (n = 6 for CP, one in AP and one in BC). CML phase, kind of relaps (C, cytogenetic; H, hematologic; M, molecular), *BCR-ABL1* mutations, IS-value for *BCR-ABL1* mRNA level and fold change *STAT5A* mRNA levels compared to mean *STAT5A* mRNA level of imatinib sensitive CP samples are indicated. Each bar represents data derived from an individual patient. (B) *STAT5A/B* immunoblot of BM derived from a healthy patient (control BM) and PB derived from CML diseased patients in CP (n = 6), AP (n = 3) and imatinib-resistant CP (n = 3). Each lane represents an individual patient. (C) Expression of pSTAT5 in primary CML cells. Mononuclear BM cells from three CML patients, one in CP (upper image), one in AP (middle image), and one in BC (C, lower image) were spun on cytospin slides and stained with anti-pSTAT5 antibody AX1. Subsets of CML cells in AP and BC were found to stain positive for pSTAT5, whereas fewer cells expressed pSTAT5 in CP. Representative examples are depicted. (D) RT-PCR for *STAT5A* and *STAT5B* of control BM (n = 5) and PB derived from patients in *BCR-ABL1*-negative BC (n = 2) or secondary AML (n = 2).

Figure 6

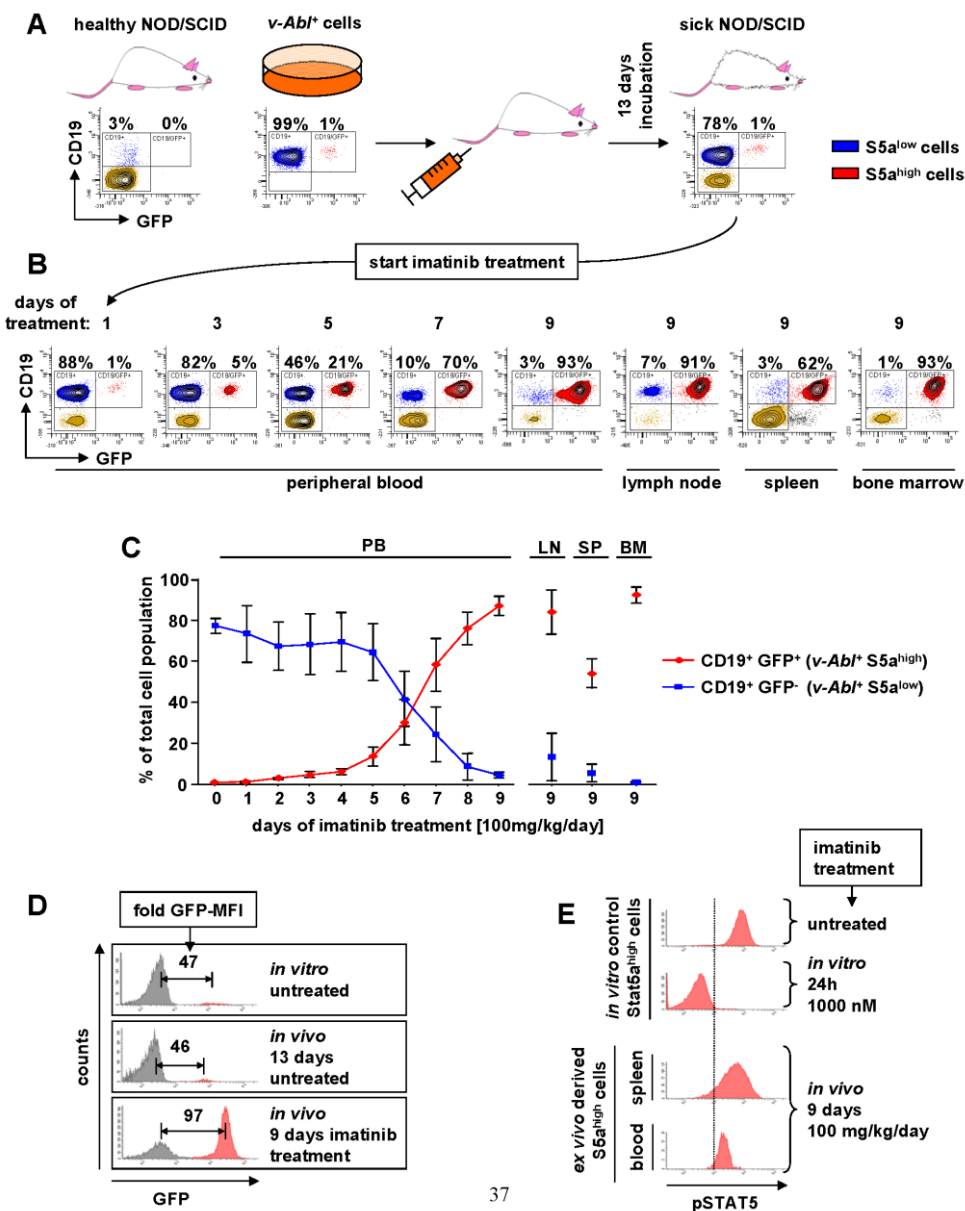


Figure 6. Treatment of leukemic mice with imatinib selects for STAT5A overexpressing cells. (A) Top panel, FACS analysis of CD19 and GFP from PB derived cells of a healthy NOD/SCID mouse (left panel), freshly established stable $v\text{-}Abl^+$ cells infected with $p\text{MSCV-eGFP}$ based retrovirus encoding for murine STAT5A showing 1% infection efficiency (middle panel) and blood derived cells from a NOD/SCID mouse 13 days after injection of the $v\text{-}Abl^+$ cells (right panel). (B) FACS analysis of PB, lymph nodes (LN), spleen (SP) and BM after imatinib treatment by gavage [100 mg/kg/day]. Percentage of CD19⁺/GFP⁺ (blue) and CD19⁺/GFP⁺ (red) cells is shown. (C) Summary of the *in vivo* experiment depicted in panel A and panel B for a total of 6 mice. Error bars represent means $\pm$ SD. (D) Representative FACS profile of $v\text{-}Abl^+$ cells before injection (top panel), 13 days after injection (middle panel) and 9 days after *in vivo* imatinib treatment (bottom panel). Fold difference of GFP-mean fluorescence intensity of GFP⁺ ($S5a^{\text{high}}$) to GFP⁺ ($S5a^{\text{low}}$) cells are indicated. (E) pSTAT5 level of *ex vivo* derived $S5a^{\text{high}}$ cells 9 days after *in vivo* imatinib [100 mg/kg/day] treatment (lower histograms) and *in vitro* control $S5a^{\text{high}}$ cells before and after 24 hours after imatinib [1000 nM] addition (upper histograms).

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3.4 C-JUN promotes BCR-ABL induced lymphoid leukemia by inhibiting methylation of the 5' region of *Cdk6*.

Manuscript under review (Blood).

I contributed to this project by isolating several times bone marrow cells, splenocytes and blood cells of the respective mice tissues, by performing flow cytometric experiments, and by helping with *in vivo* experiments.

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ABSTRACT

The transcription factor c-JUN and its upstream kinase JNK1 have been implicated in BCR-ABL induced leukemogenesis. JNK1 has been shown to regulate BCL₂ expression thereby altering leukemogenesis, but the impact of c-JUN remained unclear. In this study we show that JNK1 and c-JUN promote leukemogenesis via separate pathways, since lack of c-JUN impairs proliferation of *p185^{BCR-ABL}* transformed cells without affecting viability. The decreased proliferation of *c-Jun^{Δ/Δ}* cells is associated with the loss of cyclin dependent kinase 6 (CDK6) expression. In *c-Jun^{Δ/Δ}* cells CDK6 expression becomes down-regulated upon BCR-ABL induced transformation which correlates with CpG island methylation within the 5' region of *Cdk6*. We verified the impact of *Cdk6* deficiency by using *Cdk6^{-/-}* mice that developed BCR-ABL induced B-lymphoid leukemia with significantly increased latency and an attenuated disease phenotype. In addition we show that re-expression of CDK6 in BCR-ABL transformed *c-Jun^{Δ/Δ}* cells reconstitutes proliferation and tumor formation in *Nu/Nu* mice. In summary, our study reveals a novel function for the AP-1 transcription factor c-JUN in leukemogenesis by antagonizing promoter methylation. Moreover, we identify CDK6 as relevant and critical target of AP-1 regulated DNA methylation upon BCR-ABL induced transformation, thereby accelerating leukemogenesis.

INTRODUCTION

Activating protein 1 (AP-1) functions as a dimeric transcription factor in important cellular processes such as proliferation, differentiation and apoptosis.¹⁻³ This wide range of different roles is made possible by variable compositions of many homo- and heterodimers which are formed by the JUN, FOS, ATF and MAF proteins that comprise the AP-1 transcription factors.^{4,5}

C-JUN was originally identified as the normal cellular counterpart of the avian sarcoma JUN oncoprotein (v-JUN). Mouse model experiments revealed the essential role of c-JUN for development. *C-Jun*^{-/-} mice die at embryonic day 12.5 to 13.5 displaying multiple defects in liver, heart and neural crest.^{6,7} C-JUN was shown to transform cells in culture⁶⁻⁸ and is commonly expressed at high levels in human malignancies.^{9,10} High levels of c-JUN have been described in a large fraction of human melanoma samples¹¹, liposarcomas¹², lymphomas¹³ and are important players in skin and liver tumorigenesis.^{14,15} The tumor promoting roles of c-JUN have been attributed to growth accelerating effects via the regulation of CYCLIN expression¹⁶ or attributed to anti-apoptotic properties. In hepatocytes, c-JUN antagonizes the function of the tumor suppressor protein p53 and protects transformed hepatocytes from cell death.¹⁷ In T-cells, c-JUN is involved in the expression of FAS ligand (FASL) triggering apoptosis through the FAS receptor.¹⁸

In BCR-ABL driven leukemogenesis both, c-JUN and its upstream regulator JNK1 have been described as tumor promoters.¹⁹⁻²² For *JNK1*^{-/-} transformed cells, the underlying mechanism has been elucidated: *JNK1*^{-/-} cells express severely reduced levels of the anti-apoptotic protein BCL₂ which leads to a significantly delayed leukemogenesis in BCR-ABL transformed cells.²¹ However, it remained unclear whether this effect was mediated via c-JUN dependent phosphorylation.

In this report we describe a novel mechanism how AP-1 transcription factors modulate tumorigenesis. In B-lymphoid cells c-JUN counteracts the BCR-ABL induced DNA methylation within the 5' region of *Cdk6*. Thereby c-JUN prevents the BCR-ABL induced silencing of the CDK6 gene. A lack or down regulation of CDK6 is associated with a pronounced increase of disease latency as verified by the use of *Cdk6*^{-/-} mice. Our study thereby reveals primary insights in the role of AP-1 transcription factors for oncogene induced gene-silencing. Moreover, we provide the first conclusive evidence for a non-redundant tumor promoting role of CDK6 in lymphoid malignancies.

MATERIALS AND METHODS

Mice and infection of neonatal mice with Ab-MuLV

c-Jun^{fl/fl} ²⁷, *CD19*^{Cre/+} mice ²⁸, *Jnk1*^{-/-} ²⁹, *c-Jun*^{AA/AA} ³⁰, *p53*^{-/-} ³¹, *Cdk6*^{-/-} ³², *Nu/Nu* and *Rag2*^{-/-} mice ³³, were described previously. Animal experiments were performed in accordance with protocols approved by the Animal Welfare Committee (MUW). Newborn mice were injected intraperitoneally with 50µL of replication-incompetent ecotropic retrovirus encoding for Ab-MuLV.³⁴ Sick mice were sacrificed. Peripheral blood, lymphoid and hematopoietic organs were analysed for leukemic cells by FACS and by histopathology.

Cell culture, infection of fetal liver cells and expression vectors

Tissue culture conditions, virus preparation, infections, transformation-assays and establishment of cell-lines were performed as described previously.^{13,34,35} The expression vectors used for the experiments were *pMSCV-puro*, *pMSCV-Cdk6-puro* and *pMSCV-Bcl₂-puro*.

Transplantation of tumor cells into *RAG2*^{-/-} and *Nu/Nu* mice

For tail vein injections, a defined cell number from independently derived *pMSCV-p185^{BCR-ABL}-IRES-GFP*-transformed cell lines were injected into *Rag2*^{-/-} mice. For subcutaneous injections, 1 x 10⁶ cells were injected into *Nu/Nu* mice. Sick mice were killed and analyzed for spleen weights, white blood cell counts and the presence of leukemic cells in bone marrow, spleen, liver and blood by FACS analysis.

[³H]-thymidine incorporation

5 x 10⁴ cells were plated in triplets in 96-well plates and [³H]-thymidine (0.1µCi/well [MBq/well]) was added. After 12h incubation, analysis was performed with Ultima Gold MV scintillation fluid (Packard Instruments) by a scintillation counter.

Flow cytometric analysis

FACS analysis was done by a FACSCanto flow cytometer using FACSDiva software. *B-cell development staining*: We used different antibodies to determine the specific B-lineage maturation stages: B220 (CD45R; RA3-6B2), CD43 (1B11), CD19 (1D3), BP-1 (6C3), IgM (R6-60.2) and IgD^b (IgH-5b; 217-170) (all BD Pharmingen).

Cell cycle analysis: 5×10^6 cells were stained with PI (50 $\mu\text{g/ml}$) in a hypotonic lysis solution (0.1% sodium citrate, 0.1% triton X-100, 100 $\mu\text{g/ml}$ RNase) and incubated at 37°C for 30 minutes.

Protein analysis and Western blotting

Cells were lysed in a buffer containing protease and phosphatase inhibitors (50 mM Hepes, pH 7.5, 0.1% Tween-20, 150 mM NaCl, 1 mM EDTA, 20 mM β -glycero-phosphate, 0.1 mM sodium vanadate, 1 mM sodium fluoride, 10 $\mu\text{g/ml}$ aprotinin, leupeptin and 1 mM PMSF, respectively). Protein concentrations were determined using a BCA-kit (Pierce). Proteins (100 μg) were separated on 12% SDS-polyacrylamide gels and transferred onto Immobilon membranes. Membranes were probed with antibodies directed against CDK6 (C8343), β -ACTIN (A-4700) (Sigma Aldrich Inc.), c-JUN (sc-1694x), p53 (sc-6243), BCL₂ (sc-7382), CDK4 (sc-260), p21 (sc-471), p27 (sc-1641), p15 (sc-612), p16 (sc-1207) (Santa Cruz Biotechnologies Inc.). Immunoreactive bands were visualized by chemiluminescent detection (ECL detection kit; Amersham). *Treatment with Aza-dC (Sigma Aldrich Inc.):* Cells were seeded in 1 μM Aza-dC. After 12, 24, 36 and 48 hours incubation western blot analysis was performed.

RNA-isolation and Real time-PCR analysis

RNA was isolated using TriZol (Invitrogen). First-strand cDNA-synthesis and PCR-amplification were performed using a reverse transcriptase–polymerase chain reaction (RT-PCR) kit (GeneAmp RNAPCRkit; Applied Biosystems) according to the manufacturer. Real time PCR was performed on an Eppendorf RealPlex cyler using RealMasterMix (Eppendorf) and SYBR Green as described before ³⁶. The following primer pairs were used: *Gapdh*: 5'-AGAAGGTGGTGAAGCAGGCATC-3' and 5'-CGGCATCGAAGGTGGAAGAGTG-3' and *Cdk6*: 5'-GCTTCGTGGCTCTGAAGCGCG-3' and 5'-TGGTTTCTGTGGGTACGCCGG-3'. *Real time-PCR for Dnmt*: 1 μg of total RNA was used for cDNA preparation using the Omniscript RT Kit (Qiagen, Hilden, Germany). Real-Time RT-PCR of *Dnmt1*, *Dnmt3a* and *Dnmt3b* was performed using Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA) as recommended by the manufacturer. *Gapdh* was used as reference gene for normalization of RT-PCR data.

Nucleic acid isolation, methylation-specific PCR (MSP) and bisulfite genomic sequencing

Genomic DNA was isolated from murine *c-Jun^{fl/fl}* and *c-Jun ^{Δ/Δ}* cell lines by digestion with Proteinase K, followed by standard phenol-chloroform extraction and ethanol precipitation as

reported previously (Sambrook, Fritsch et al. 2. Edition). Afterwards, 1µg of genomic DNA was used for chemical modification by sodium bisulfite using the Epitect kit (Qiagen, Hilden, Germany). The methylation status of the region 5' to the coding sequence of *Cdk6* was analyzed by methylation-specific PCR (MSP). In brief, we performed a search for CpG islands in the 5' region of *Cdk6* (ENSMUSG00000040274, www.ensembl.org, release 52) using the CpGplot tool (<http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html>) and found a CpG island which is located at -187 bp to +279 bp relative to the transcription start site of *Cdk6* (Figure 5A). MSP primers were designed to amplify a region at -40 bp to +193 bp relative to the transcription start site of *Cdk6*. MSP primers sequences are as follows: *Cdk6m*-fwd, 5'-TAGTTCGGCGGTTCGCGAGTTCG-3', *Cdk6m*-rev, 5'-CGCACGCGCTTCAAAACCACG-3', *Cdk6u*-fwd, 5'-TAGTTTGGTGGTTGTGAGTTTG-3' and *Cdk6u*-rev, 5'-TCACACACACTTCAAAACCACA-3'. The PCR was performed using following conditions: initial denaturation for 12 minutes at 95°C followed by 38 cycles of denaturation for 30 seconds at 95°C, annealing for 40 seconds at 64°C and extension for 30 seconds at 72°C with a final extension for 7 minutes at 72°C. MSP products were separated in 2% agarose gels stained with GelRed™ (Biotium, Hayward, CA, USA) and visualized under UV spectrophotometry. DNA extracted from murine cell lines was treated with Sss1 CpG methylase (New England Biolabs, Beverly, MA, USA) and used as a positive control for methylated alleles. Water blanks were used as negative controls. For bisulfite genomic sequencing PCR primers were designed to anneal at both methylated and unmethylated bisulfite converted DNA. Primer sequences are available upon request. The PCR products were sequenced using the 3100 Genetic Analyzer (PE Applied Biosystems).

Statistical Analysis

Data are reported as mean values $\pm$ SEM, biochemical experiments were performed in triplicates and a minimum of three independent experiments were evaluated. Differences were assessed for statistical significance by an unpaired two-tailed *t* test or the log rank test (for Kaplan-Meier plots).

RESULTS

JNK1 and c-JUN are involved in BCR-ABL induced transformation

The AP-1 upstream kinase JNK1, as well as the AP-1 transcription factor c-JUN have been implicated in BCR-ABL driven leukemogenesis.^{19,21,22} We confirmed these previous observations by performing colony formation assays in growth factor free methylcellulose. Fetal liver cells were prepared from *Jnk1*^{-/-} and *c-Jun*^{fl/fl}*CD19*^{Cre/+} embryos and appropriate controls and infected with a retrovirus encoding *p185*^{BCR-ABL} (*pMSCV-p185*^{BCR-ABL}-*IRES-GFP*). As expected, a significant reduction of growth factor independent colony numbers was detected when *Jnk1*^{-/-} or *c-Jun*^{fl/fl}*CD19*^{Cre/+} hematopoietic cells were used (**Figure 1A and 1B**). Following transformation, *CD19*⁺/*CD43*⁺ leukemic cell lines were generated from all cultures. The lack of *Jnk1* has been reported to be associated with an increased disease latency.²¹ In order to study tumor cell intrinsic characteristics *in vivo*, we made use of *Rag2*^{-/-} mice. The lack of lymphoid cells in *Rag2*^{-/-} mice makes them particularly suitable to study lymphoid malignancies. Upon injection of *c-Jun*^{fl/fl}*CD19*^{Cre/+} (*c-Jun*^{ΔΔ}) and *c-Jun*^{fl/fl} stable cell lines via tail vein the mice developed leukemia with infiltrations of leukemic cells in bone marrow, spleen, liver and lymph nodes (**data not shown**). As summarized in **Figure 1C**, leukemia evolved with a significantly enhanced latency in mice that had received *c-Jun*^{ΔΔ} cells. The signs of disease were attenuated as evident from the reduced spleen weight of mice suffering from a *c-Jun* deficient leukemia (**Figure 1D**).

JNK1 and c-JUN act via different pathways in promoting BCR-ABL induced leukemia

The lack of *Jnk1* in BCR-ABL transformed cells was reported to decrease BCL₂ expression, which was verified as the major cause for the reduced malignancy of *Jnk1*^{-/-} cells upon BCR-ABL transformation. Transgenic expression of BCL₂ in these cells rescued apoptosis and restored tumors.²¹ Indeed, BCL₂ protein expression was also reduced in *c-Jun*^{ΔΔ} cells when compared to *c-Jun*^{fl/fl} control cells (**Figure 2A**). To explore whether the reduction in BCL₂ expression accounts for a delayed tumorigenesis in *c-Jun*^{ΔΔ} cells, we expressed BCL₂, encoded by a retrovirus, in BCR-ABL transformed *c-Jun*^{ΔΔ} and *c-Jun*^{fl/fl} cells (**Figure 2B, lower panel**). Leukemia formation was then investigated by injecting these cells into *Rag2*^{-/-} animals via the tail vein. Surprisingly, disease latency was unaffected irrespective whether we injected *c-Jun*^{ΔΔ} cells or *c-Jun*^{fl/fl} cells expressing low or high levels of BCL₂ (**Figure 2B, upper panel and S1A**). To further investigate whether JNK1 and c-JUN act in a signaling cascade independent of BCL₂ we made use of *c-Jun*^{AA/AA} mice harbouring point mutations at the critical serine sites subjected to phosphorylation by JNK.

Newborn *c-Jun*^{AA/AA} mice and appropriate controls were infected intraperitoneally with the replication deficient retrovirus Ab-MuLV encoding *v-Abl*. As additional control, we used *c-Jun*^{fl/fl}*CD19*^{Cre/+} and compared them to *c-Jun*^{fl/fl} mice. Ab-MuLV infects B-lymphoid precursors and induces a slowly evolving pro-B-cell leukemia in mice. As depicted in **Figure 2C**, *c-Jun*^{fl/fl}*CD19*^{Cre/+} developed leukemia with a significantly enhanced latency compared to *c-Jun*^{fl/fl} mice, which is clearly in line with the tumor promoting effect of c-JUN. However, no effect was observed when we compared *c-Jun*^{AA/AA} mice to control animals (**Figure 2D**). The slight difference in latency between the control mice in these two experiments are caused by minor background differences of the animals. In line, we failed to detect any alteration in the outgrowth of *c-Jun*^{AA/AA} and control *p185*^{BCR-ABL} transformed cell lines (**data not shown**). These data indicate that JNK1 and c-JUN promote BCR-ABL driven tumorigenesis independently by using different mechanisms.

c-Jun deficient cells show a proliferative defect

AP-1 members regulate survival, differentiation and proliferation.¹⁵ Since the enforced expression of BCL₂ did not accelerate leukemogenesis in *c-Jun*^{Δ/Δ} cells, we next investigated cell growth of BCR-ABL transformed *c-Jun*^{Δ/Δ} cells. Six individually derived stable cell lines lacking *c-Jun* and corresponding control cell lines were generated. [³H]-thymidine incorporation assays as well as growth curves showed a significant difference in the proliferation of *c-Jun*^{Δ/Δ} and *c-Jun*^{fl/fl} cells. *C-Jun*^{Δ/Δ} cells expanded significantly slower (**Figure 3A and 3B**). This was also evident from the DNA content of asynchronously proliferating cells: *c-Jun*^{Δ/Δ} cells showed a significantly increased proportion of cells in G1-phase and a decreased proportion in S-phase (**Figure 3C**). In order to monitor tumor growth *in vivo*, we made use of *Nu/Nu* mice. Tumor cells were injected subcutaneously to allow monitoring of tumor growth. These *in vivo* experiments recapitulated the *in vitro* observations. *C-Jun*^{Δ/Δ} tumors evolved significantly slower in *Nu/Nu* mice. The experiment was terminated after 10 days when the first tumor reached 1 cm diameter in size. At that time point, the weight of the *c-Jun*^{Δ/Δ} tumors compared to the *c-Jun*^{fl/fl} tumors was significantly smaller (**Figure 3D**). In hepatocytes, c-JUN accelerates tumor formation by antagonizing the pro-apoptotic activity of p53.¹⁷ In order to investigate whether this mechanism occurs in B-lymphoid leukemogenesis, we crossed *c-Jun*^{fl/fl} and *c-Jun*^{fl/fl}*CD19*^{Cre/+} mice with *p53*^{-/-} mice and subsequently assessed leukemia formation. Newborn *c-Jun*^{fl/fl}*p53*^{-/-} and *c-Jun*^{fl/fl}*CD19*^{Cre/+}*p53*^{-/-} mice were challenged with Ab-MuLV. As depicted in **Figure 2E**, *p53* deficiency was not capable to counteract or change the prolongation of disease latency induced

by the loss of *c-Jun*. These experiments point out the involvement of c-JUN in growth control in *p185^{BCR-ABL}* positive tumor cells.

Transformed *c-Jun*^{ΔΔ} cells down-regulate the expression of the cell cycle kinase CDK6

When we analyzed several cell cycle components (**Figure 4A**) we found that the loss of c-JUN was associated with the down-regulation of CDK6 expression in leukemic B-cells whereas other cell cycle components showed no consistent alterations. Loss of CDK6 was restricted to stable *c-Jun*^{ΔΔ} cell lines and not observed in *p185^{BCR-ABL}* transformed *c-Jun*^{AA/AA} cells, supporting the independence of JNK phosphorylation (**Figure 4B**). The protein expression of CDK6 steadily declined after 2, 4, 6 and 8 weeks of the initial transformation event by retroviral infection with *p185^{BCR-ABL}* in *c-Jun*^{ΔΔ} cells (**Figure 4C**). This constant protein reduction correlated with a decrease in the proliferative capacity of the cells (**Figure 4D**). It is important to mention that already after 2 weeks, the cell cultures consisted of more than 95% GFP⁺ and so BCR-ABL⁺ cells. A decreasing contamination with non transformed cells can therefore be ruled out. qPCR experiments revealed that the reduction of CDK6 protein was accompanied by a loss of *Cdk6* mRNA (**Figure 4E**).

The lack of c-JUN leads to the methylation within the 5' region of *Cdk6* in *p185^{BCR-ABL}* transformed cells

To determine whether epigenetic changes may be responsible for the down-regulation of CDK6 in *p185^{BCR-ABL}* transformed cells we developed assays for DNA methylation analysis of the 5' region of the *Cdk6* gene. Using a CpG island search tool we identified a 1000bp long CpG island comprising the transcription start site of *Cdk6* (**Figure 4A**). This region was used as target for methylation-specific PCR (MSP) analysis and bisulfite genomic sequencing. As analyzed by MSP, the time dependent reduction of *Cdk6* mRNA co-incided with DNA methylation within the 5' region of *Cdk6* (**Figure 5B, upper panel**). While *Cdk6* methylation was detected in all stable cell lines lacking c-JUN, the *c-Jun*^{fl/fl} cells were not methylated for this gene (**Figure 5B, lower level**). Results obtained by MSP were confirmed by bisulfite genomic sequencing of the *Cdk6* 5' region in *c-Jun*^{ΔΔ} and *c-Jun*^{fl/fl} cells (**Figure 5C**). Further confirmation was obtained when we treated the BCR-ABL transformed *c-Jun*^{ΔΔ} cells with the demethylating agent 5-aza-2'-deoxycytidine (Aza-dC) for 48 hours. As expected, Aza-dC induced demethylation of DNA resulted in re-expression of CDK6 already 12 hours after treatment (**Figure 5D**). True to form, most of the cells turn into G0/G1-phase (**Figure S2A**) and began to die 12 hours after Aza-dC addition (**Figure 5E**). Analyzing

the mRNA levels of DNA methyltransferases (*Dnmt1*, *Dnmt3a*, *Dnmt3b*) in *c-Jun*^{Δ/Δ} and *c-Jun*^{fl/fl} cells by real-time PCR showed a significant higher amount of *Dnmt3b* mRNA in *c-Jun*^{Δ/Δ} cells compared to *c-Jun*^{fl/fl} cells (**Figure 5F**).

Loss of CDK6 in BCR-ABL transformed cells recapitulates the phenotype of c-Jun deficiency

We next asked whether the loss of CDK6 accounts for the reduced proliferation of BCR-ABL transformed *c-Jun*^{Δ/Δ} cells and provokes the increased disease latency. To study the contribution of CDK6 to BCR-ABL induced tumor formation we employed *Cdk6*^{-/-} animals. Since no information on a potential role of CDK6 in B-lymphoid development was available, we first monitored the emergence of individual defined B-cell stages. As depicted in **Figure 6A**, *Cdk6*^{-/-} mice had significantly elevated levels of pre-pro B-cells. The total cell number of peripheral mature B-cells was unaltered, indicating that this partial block was compensated *in vivo*. Mature *Cdk6*^{-/-} B-lymphocytes responded equally well to mitogenic stimuli as compared to control cells (**Figure S3A**). We next transformed fetal liver derived *Cdk6*^{-/-} cells and wild type controls with a *pMSCV-p185^{BCR-ABL}-IRES-GFP* retrovirus. GFP⁺/CD43⁺/CD19⁺/B220⁺ colonies and cell lines were obtained with equal frequencies (**Figure S3B**). However a significant difference became obvious in a [³H]-thymidine incorporation assay; a decreased proliferation was also documented by growth curves (**Figure 6B and C**). These data point towards an essential non-redundant role for CDK6 in the transformed B-cells. This is in contrast to the observation in non-transformed mature B-cells, where the lack of CDK6 did not impair proliferation (**Figure S3A**).

To assess tumor formation *in vivo* we injected a replication-incompetent retrovirus encoding Ab-MuLV in newborn mice thereby inducing a slowly emerging lymphoid leukemia. The outcome of the experiment is summarized in **Figure 6D**: strikingly, *Cdk6*^{-/-} animals developed disease significantly later and survived the oncogenic challenge up to five months, whereas all *Cdk6*^{+/+} and *Cdk6*^{+/-} mice succumbed to leukemia within ~ 2 months. A slightly decreased infiltration of spleens with leukemic cells was found in *Cdk6*^{-/-} mice, nevertheless the difference did not meet the criteria of being statistically significant (**Figure 6E**). This experiment highlighted a non-redundant role of CDK6 as a tumor promoter for *p185^{BCR-ABL}*-induced B-cell leukemia. In summary, these data revealed a non-redundant tumor promoting role for CDK6 in *p185^{BCR-ABL}* driven leukemogenesis.

Re-expression of CDK6 reconstitutes proliferation in $c\text{-Jun}^{\Delta/\Delta}$ cells

To define whether the loss of CDK6 accounts for the decreased proliferation and tumor formation of BCR-ABL transformed $c\text{-Jun}^{\Delta/\Delta}$ cells we re-expressed CDK6, with a *pMSCV-Cdk6-puro* retrovirus in $c\text{-Jun}^{\Delta/\Delta}$ cells (**Figure 7A**). As depicted in **Figure 7B**, $c\text{-Jun}^{\Delta/\Delta}$ cells proliferated significantly faster after CDK6 expression compared to control cells that had been infected with the empty vector. Additionally, the proportion of these cells in G1- and S-phase showed no significant difference compared to $c\text{-Jun}^{fl/fl}$ (**Figure 7C**). In line with this, tumor formation in *Nu/Nu* mice was assessed with $c\text{-Jun}^{\Delta/\Delta}\text{-Cdk6-puro}$ cells compared to $c\text{-Jun}^{\Delta/\Delta}$ cells. In this *in vivo* experiment an increased tumor size of $c\text{-Jun}^{\Delta/\Delta}\text{-Cdk6-puro}$ cells was detected (**Figure 7D**). We concluded that re-expression of CDK6 in $c\text{-Jun}^{\Delta/\Delta}$ cells restores proliferation to levels observed in wild type control cells and accelerates tumor formation *in vivo*.

DISCUSSION

In this report we show that the AP-1 transcription factor c-JUN promotes BCR-ABL induced leukemogenesis by maintaining the expression of the cell cycle kinase (CDK6). C-JUN is required to protect the CpG island in the 5' region of *Cdk6* from getting methylated. Thereby we define a novel mechanism how AP-1 transcription factors that are commonly up-regulated in transformed cells contribute to tumorigenesis. Furthermore we define for the first time a tumor promoting role for CDK6 in B-lymphoid leukemogenesis.

Although c-JUN has unequivocally been enrolled in the regulation of apoptosis during tumorigenesis¹⁷, these effects have been excluded for BCR-ABL induced B-lymphoid leukemogenesis. Neither the enforced expression of BCL₂ nor the lack of *p53* counteracted or influenced the effects on disease latency that occurred in the absence of c-JUN during leukemia progression. This opposes the situation in *Jnk1*^{-/-} cells, where the enforced expression of BCL₂ reconstituted leukemogenesis.²¹ This also led us to conclude that JNK1 exerts its effects independently of c-JUN. Further proof was obtained by investigating BCR-ABL induced leukemogenesis in *c-Jun*^{AA/AA} mice that did not display any obvious alterations or phenotypes when challenged with the *p185^{BCR-ABL}* oncogene. How JNK1 regulates BCL₂ expression and promotes survival, independently of c-JUN, remains to be determined.

However, we cannot entirely rule out that c-Jun exerts a survival function during the initial transformation event. Similar to *Jnk1*^{-/-}, *c-Jun*^{ΔΔ} cells gave rise to significantly reduced colony numbers when plated in growth factor free methylcellulose. This effect cannot be attributed to CDK6 which does not display a reduction in colony numbers in the initial transformation event. Obviously transformation induces rewiring of signaling pathways and changes the signaling network required for survival and proliferation. Hence, factors that are required within the initial transformation process may become irrelevant for tumor cell maintenance. This has been recently demonstrated for the transcription factor STAT3.³⁷ In contrast, its close relative STAT5 comes into a privileged position and is essential for the survival of BCR-ABL⁺ cells.³⁸ One might speculate that initially c-JUN exerts a role in survival which is subsequently taken over by STAT5.

Interestingly, non transformed B-lymphoid cells lacking c-JUN express regular levels of CDK6. Only upon transformation with the BCR-ABL oncogene the 5' region of *Cdk6* becomes methylated, if not protected by c-JUN. Thus, our results suggest that methylation of the 5' region of *Cdk6* may be prevented by c-JUN. Hundreds of possibly epigenetically silenced genes may

exist in individual tumors. While a selection for stochastic events certainly exists and selects for dominant tumor clones in individual tumors it is unlikely that all of these events arise in a random fashion. The current model suggests that the existence of multiple epigenetically silenced genes rather reflects a general program of epigenetic control abnormalities. These early epigenetic silencing events could represent primary alterations induced by the initial transformation event. Our data support this model since the BCR-ABL induced transformation consistently provoked the silencing of the *Cdk6* gene by DNA methylation. This BCR-ABL induced methylation was only apparent in c-JUN deficient cells, since it is efficiently counteracted by c-JUN in a wild type background. We envision the *Cdk6* promoter being only one among many silenced genes due to a general reprogramming that is triggered by the initial BCR-ABL induced transformation. Alternatively one might suggest that c-JUN is exclusively required for CDK6 expression. In the absence of c-JUN *Cdk6* transcription comes to a stop which then induces DNA methylation. The fact that we found increased mRNA levels of the DNA methyltransferase *Dnmt1*, *Dnmt3a* and *Dnmt3b* in BCR-ABL transformed *c-Jun^{ΔΔ}* cells point at a general mechanism not specific for *Cdk6*. Of interest is the fact that the *de novo* methyltransferase *Dnmt3b* was significantly high in the absence of c-JUN. The enzymes DNMT3a and DNMT3b, account for the somatic methylation pattern during embryogenesis and favour semi- and un-methylated DNA substrates.^{39,40} In contrast DNMT1 specifies on the copying of already existing methylation patterns.^{39,41}

What singles out CDK6 silencing is the fact that CDK6 significantly contributes to the tumor promoting role of c-JUN. The reduced growth of BCR-ABL transformed *c-Jun^{ΔΔ}* cells can be largely overridden by the enforced expression of CDK6. This re-expression reconstitutes the proliferative ability of the cells and thereby enhances tumor formation in *Nu/Nu* mice. It is also noteworthy that the expression of CDK6 is not only subject to a regulation by c-JUN but also by the related protein JUNB. We have recently described that JUNB suppresses the transcription of *Cdk6* in BCR-ABL transformed cells.³⁵ As a consequence the loss or down regulation of JUNB – as observed in human myeloid and B-lymphoid malignancies – provokes an increased CDK6 expression. In summary a shift of AP-1 composition occurs in B-lymphoid malignancies: c-JUN becomes up-regulated whereas JUNB remains constant or gets down-regulated.¹³ Based on these alterations of AP-1 expression pattern one might therefore expect an overall increased expression of CDK6 in B-lymphoid malignancies. Indeed, an enhanced CDK6 protein expression has been documented in lymphoma and leukemia in people.⁴²⁻⁴⁵ In particular, several reports documented chromosomal translocations in patients suffering from B-lymphoid malignancies

involving CDK6. In these patients, the aberrant and increased expression of CDK6 had been proposed to represent a cause and/or driving force for the B-cell disease.⁴⁶⁻⁴⁸ Additionally, a recent study describes a down-regulation of the microRNA, hsa-miR-124a, during acute lymphoblastic leukemia resulting in an up-regulation of CDK6.⁴⁹ Nevertheless, it remained unclear up to now, whether the enforced expression of CDK6 represents a bystander alteration or is indeed a driving force in B-lymphoid leukemogenesis. We now provide first evidence for the tumor promoting role since the absence of CDK6 drastically prolonged tumorigenesis *in vivo*. CDK6 advanced the growth of transformed B-lymphoid cells: *p185^{BCR-ABL}*-induced leukemia was significantly delayed in *Cdk6*^{-/-} mice, the lifespan of the affected animals again nearly doubled. Hence, in B-lymphoid malignancies, CDK6 exerts a unique and non-redundant tumor promoting role. This is in contrast to non transformed B-cells that are independent from CDK6 for their development. CDK6 comes into this privileged position only upon BCR-ABL induced transformation where it takes over the dominant role during G1-phase of the cell cycle. It is attractive to speculate that this alteration might be the direct consequence of the changed AP-1 expression profiles and result from the combination of low or missing JUNB combined with elevated c-JUN expression.

In summary we provide first insights into a role for the AP-1 transcription factor as modulators of epigenetic reprogramming occurring in transformed cells. Our data reveal a linear axis for c-JUN and CDK6 in the regulation of proliferation in transformed B-lymphoid cells. In this scenario CDK6 gets into a privileged and unique position and exerts a non redundant tumor promoting role.

ACKNOWLEDGEMENTS

We thank Udo Losert and the staff of the Biomedical Research Institute (MUV) as well as Gabriele Schöppl and the mouse facility of the Institute of Pharmacology (MUV) for taking excellent care of the mice. We are grateful to Gerda Egger, Latifa Bakiri, Robert Eferl, Denise Barlow and Michael Freissmuth for valuable discussions. We thank Erwin F. Wagner for providing essential reagents.

AUTHORSHIP

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

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

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Figure 1

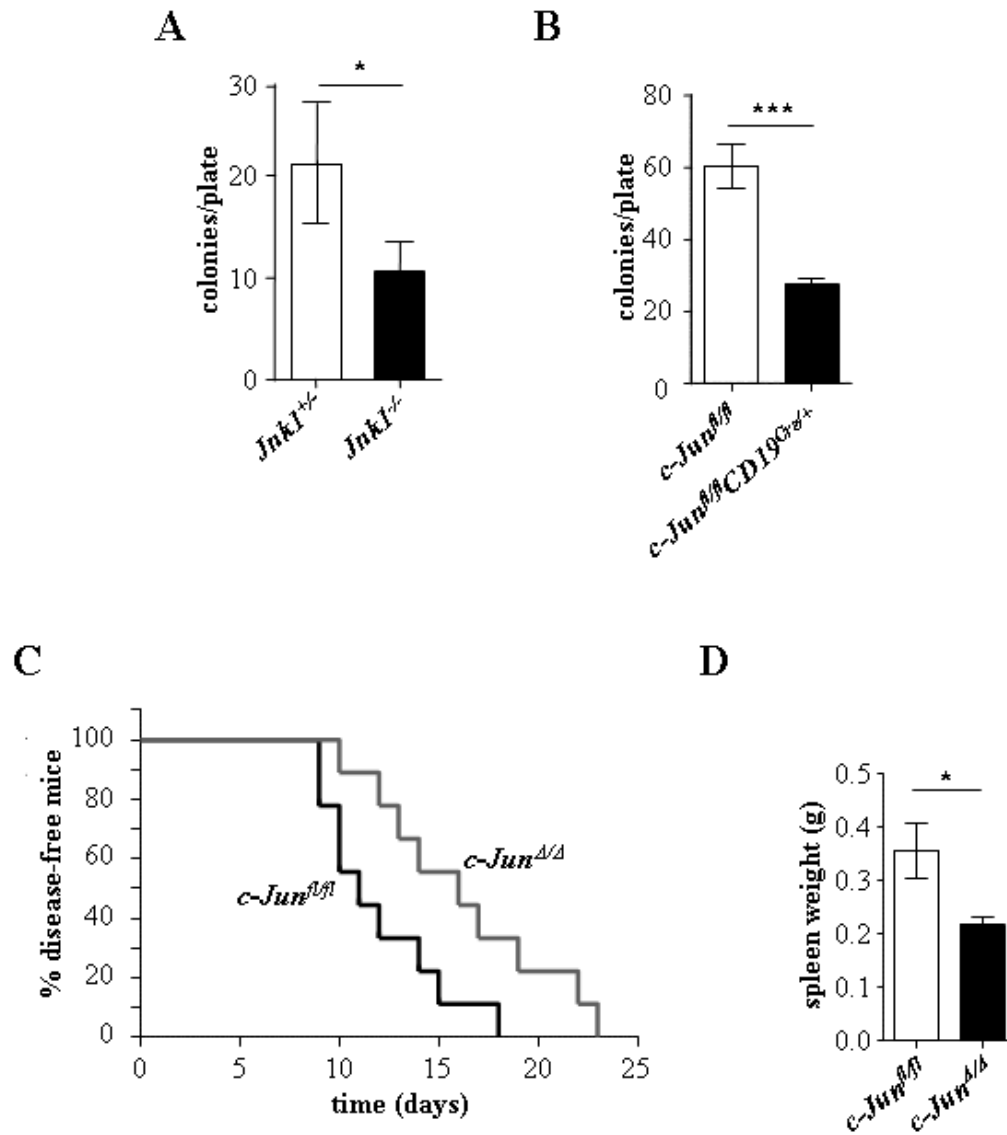


Figure 1: c-JUN is concerned in *p185^{BCR-ABL}* induced transformation and leukemogenesis. Colony formation assays (CFA) were performed using 1×10^6 **AJNK1^{+/+} and *JNK1*^{-/-} ($n = 8$; two-tailed; 49.2 ± 7.9 vs. 28.9 ± 4.1 colonies/ 10^7 fetal liver cells, $p = 0.0401$) and **Bc-Jun^{fl/fl} and *c-Jun*^{fl/fl}*CD19*^{Cre/+} ($n = 6$; two-tailed; 60.5 ± 6.1 vs. 27.3 ± 1.9 colonies/ 10^7 fetal liver cells, $p = 0.0004$) fetal liver cells after infection with a *pMSCV-p185^{BCR-ABL}-IRES-GFP* retrovirus in growth factor free methylcellulose. **C**) Transplantation of 1×10^5 *c-Jun*^{fl/fl} and *c-Jun*^{Δ/Δ} cells into *Rag2*^{-/-} mice. 2 independent cell lines per each cell type were injected in 9 mice (mean survival, 11 vs. 16 days in mice injected with *c-Jun*^{fl/fl} and *c-Jun*^{Δ/Δ} cells, respectively; $p = 0.0307$). **D**) Spleen weights of diseased recipient *Rag2*^{-/-} mice were analyzed (*c-Jun*^{fl/fl} $n = 9$ and *c-Jun*^{Δ/Δ} $n = 9$; two-tailed; $p = 0.0169$).****

Figure 2

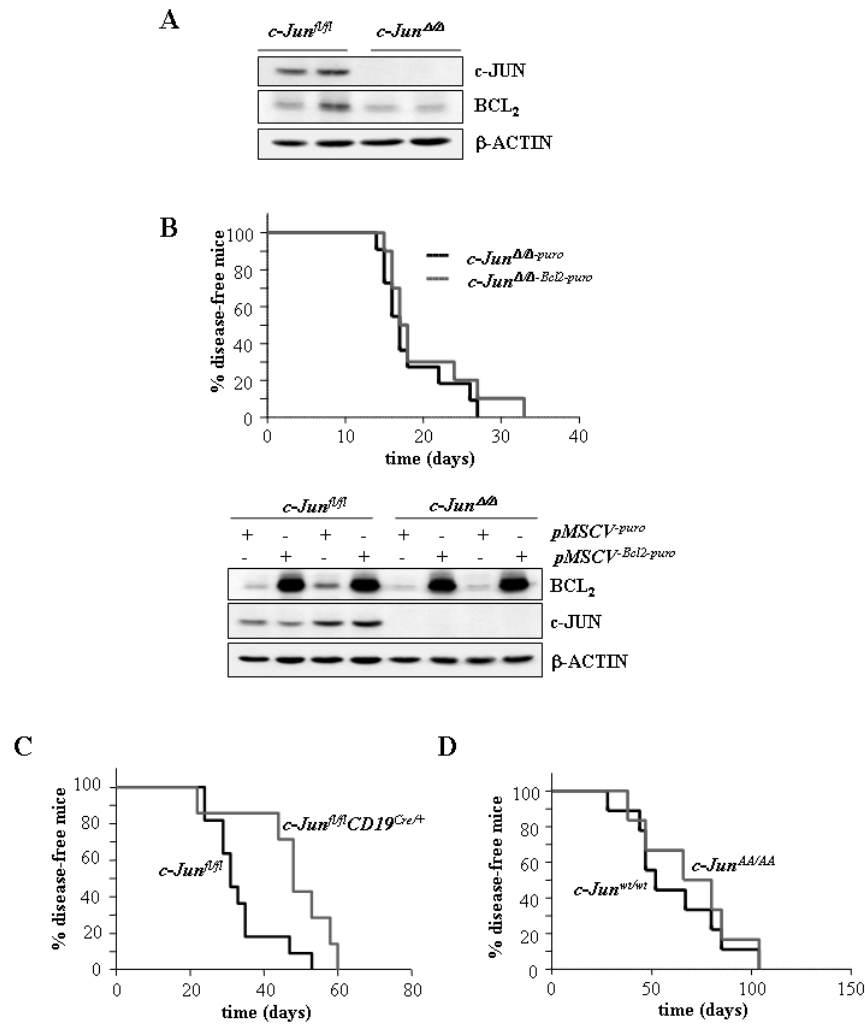


Figure 2: Enforced BCL₂ expression doesn't alter c-JUN deficient leukemogenesis. A) Protein levels of c-JUN and BCL₂ in *c-Jun^{fl/fl}* and *c-Jun^{ΔΔ} p185^{BCR-ABL}*-transformed cells. β-ACTIN served as loading control. **B)** 1 x 10⁵ leukemic *c-Jun^{ΔΔ}* B-cells, transduced either with *pMSCV-puro* or *pMSCV-Bcl₂-puro*, were injected intravenously into *Rag2^{-/-}* mice. (n = 11 and n = 13, respectively; mean survival, 17 vs. 17.5 days in mice injected with *c-Jun^{ΔΔ}-puro* and *c-Jun^{ΔΔ}-Bcl₂-puro* cells; p = 0.3552) (**upper panel**). Immunoblot analysis showing enforced expression of BCL₂ upon a *pMSCV-Bcl₂-puro* retrovirus infection in *c-Jun^{fl/fl}* and *c-Jun^{ΔΔ}* cells. β-ACTIN served as loading control (**lower panel**). **C)** Injection of *c-Jun^{fl/fl}* (n = 11) and *c-Jun^{fl/fl}CD19^{Cre/+}* (n = 7) newborn mice with a replication-deficient Ab-MuLV encoding retrovirus resulted in B-lymphoid leukemia/lymphoma (mean survival 31 vs. 48 days in *c-Jun^{fl/fl}* and *c-Jun^{fl/fl}CD19^{Cre/+}* mice, respectively; p = 0.0173). **D)** Injection of *c-Jun^{fl/fl}* (n = 9) and *c-Jun^{AA/AA}* (n = 6) newborn mice with a replication-deficient Ab-MuLV encoding retrovirus resulted in B-lymphoid leukemia/lymphoma (mean survival 52 vs. 73 days in *c-Jun^{fl/fl}* and *c-Jun^{AA/AA}* mice, respectively; p = 0.6168).

Figure 3

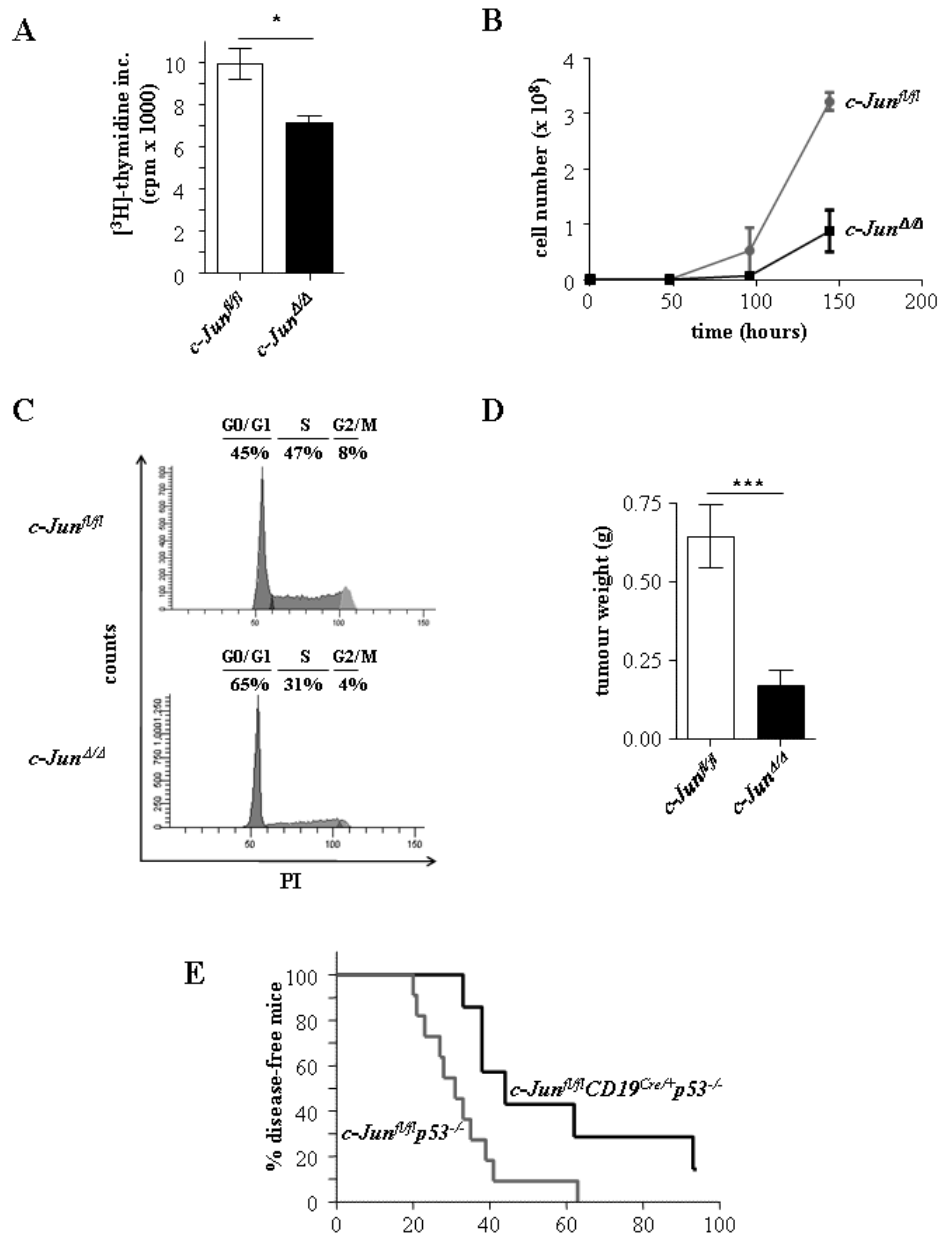


Figure 3: *c-Jun^{Δ/Δ} p185^{BCR-ABL}*-transformed cell lines demonstrate a proliferative defect. **A)** [³H]-thymidine incorporation of fetal liver-derived *c-Jun^{fl/fl}* (n = 3) and *c-Jun^{Δ/Δ}* (n = 4; two-tailed; p = 0.0142) *p185^{BCR-ABL}*-transformed cell lines. **B)** 1 x 10⁵ *p185^{BCR-ABL}*-transformed *c-Jun^{fl/fl}* (n = 3) and *c-Jun^{Δ/Δ}* cells (n = 3) were plated and total cell numbers were determined after 48, 96 and 144 hours. **C)** Cell cycle profiles of *c-Jun^{fl/fl}* (n = 5) and *c-Jun^{Δ/Δ}* (n = 4) cells (two-tailed; G1-phase: p = 0.0333 and S-phase: p = 0.0173), gated on living cells. One representative set of data is depicted. **D)** Tumor weights of *Nu/Nu* mice that were subcutaneously injected with 1 x 10⁶ *c-Jun^{fl/fl}* and *c-Jun^{Δ/Δ}* leukemic cells. 3 independent cell lines per each cell type were injected into 9 mice (two-tailed; p = 0.0007). **E)** Injection of *c-Jun^{fl/fl}p53^{-/-}* (n = 11) and *c-Jun^{fl/fl}CD19^{Cre/+}p53^{-/-}* (n = 7) newborn mice with a replication-deficient Ab-MuLV encoding retrovirus resulted in B-lymphoid leukemia/lymphoma (mean survival, 31 vs. 44 days in *c-Jun^{fl/fl}p53^{-/-}* and *c-Jun^{fl/fl}CD19^{Cre/+}p53^{-/-}* mice; p = 0.0213).

Figure 4

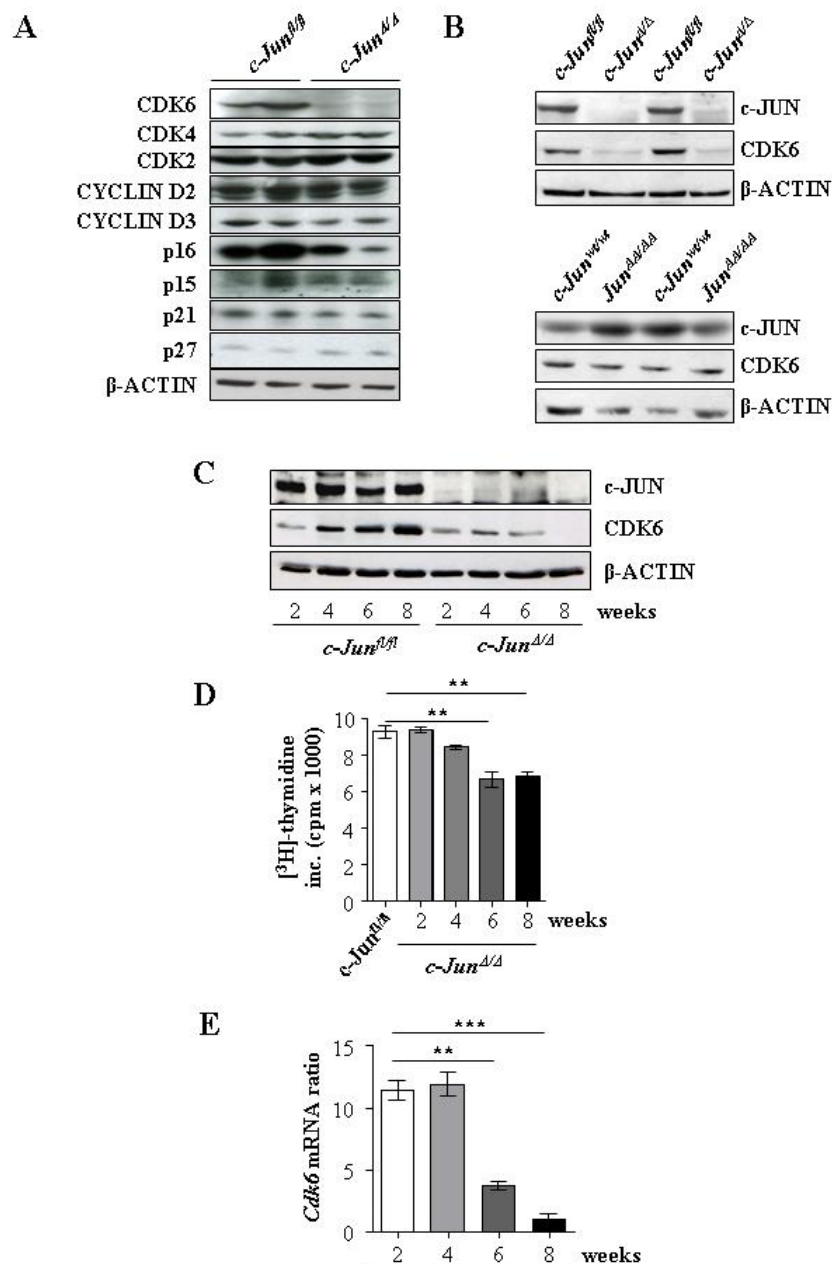


Figure 4: CDK6 protein levels are down-regulated in *c-Jun*^{Δ/Δ} *p185*^{BCR-ABL}-transformed cell lines. **A)** Immunoblot analysis for CDK6, CDK4, CDK2, Cyclin D2, Cyclin D3; p16^{INK4a}, p15^{INK4b}, p21 and p27 and protein expression in *c-Jun*^{fl/fl} and *c-Jun*^{Δ/Δ} *p185*^{BCR-ABL}-transformed cells. β-ACTIN served as loading control. **B)** Immunoblot analysis for c-JUN and CDK6 protein expression in *c-Jun*^{fl/fl} and *c-Jun*^{Δ/Δ} (**upper panel**) and *c-Jun*^{fl/fl} and *c-Jun*^{AA/AA} (**lower panel**) *p185*^{BCR-ABL}-transformed cells. β-ACTIN served as loading control. **C)** c-JUN and CDK6 protein levels of *c-Jun*^{Δ/Δ} and *c-Jun*^{fl/fl} cell lines after 2, 4, 6 and 8 weeks of transformation. β-ACTIN served as loading control. One representative set of data is depicted. **D)** [³H]-thymidine incorporation of the same cell lines was measured (n = 3; two tailed: *c-Jun*^{fl/fl} vs. *c-Jun*^{Δ/Δ} 6 weeks, p = 0.002; *c-Jun*^{fl/fl} vs. *c-Jun*^{Δ/Δ} 8 weeks, p = 0.0016). **E)** *Cdk6* mRNA levels of *c-Jun*^{Δ/Δ} cells 2, 4, 6 and 8 weeks after *p185*^{BCR-ABL} transformation were analyzed by q-PCR (n = 3; two tailed: *c-Jun*^{Δ/Δ} 2 weeks vs. *c-Jun*^{Δ/Δ} 6 weeks, p = 0.0011; *c-Jun*^{Δ/Δ} 2 weeks vs. *c-Jun*^{Δ/Δ} 8 weeks, p = 0.0004). The fold change compared to *c-Jun*^{Δ/Δ}

Figure 5

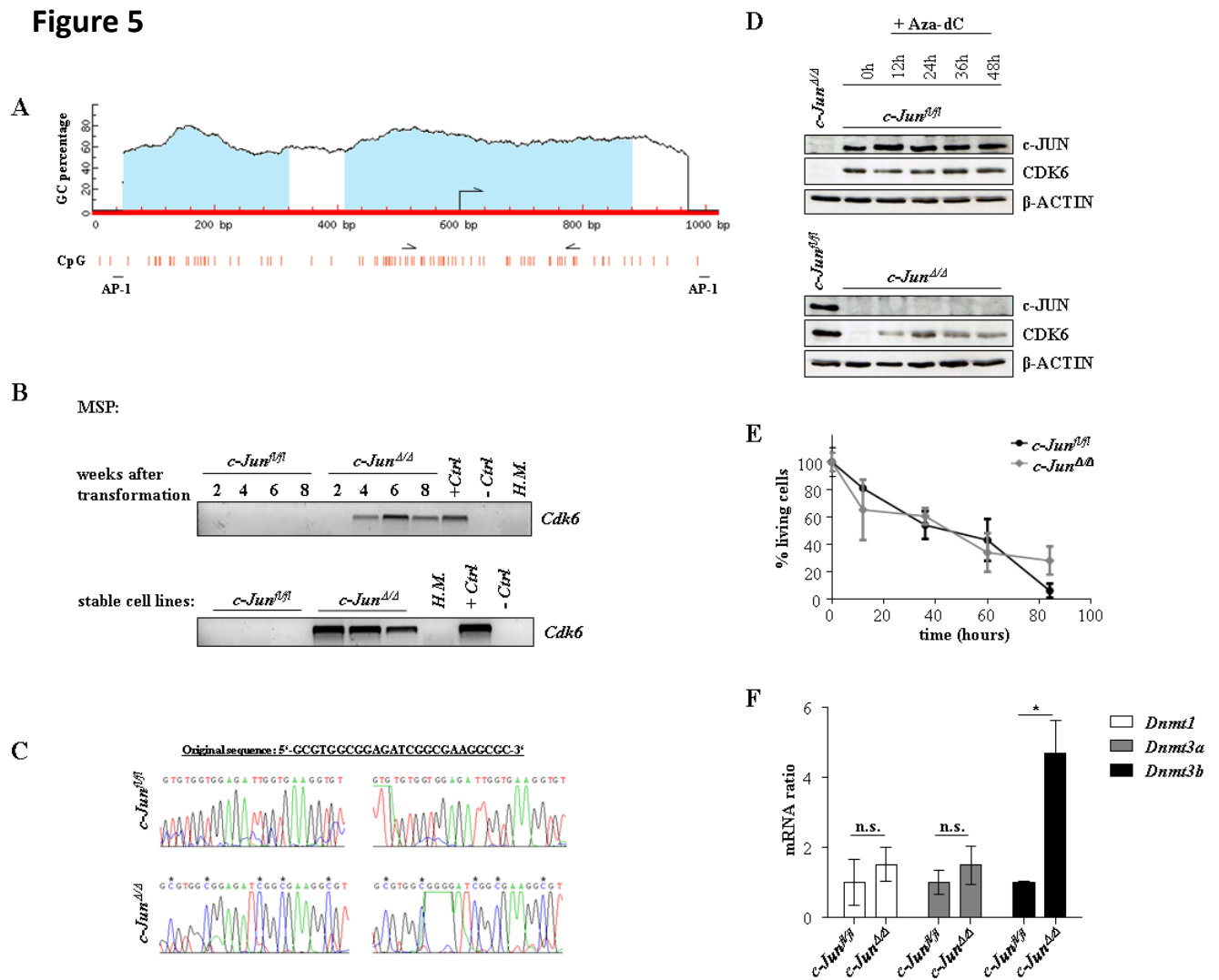


Figure 5: Methylation pattern in the 5' region of *Cdk6* in *c-Jun^{Δ/Δ}* cells. **A)** CpG island search and location of MSP primers in the *Cdk6* 5' region. A 1000 bp fragment which includes the transcription start site of *Cdk6* was analyzed for the presence of a CpG island using the MethPrimer program⁵⁰. Blue boxes indicate the presence of a CpG island, CpG sites are shown as orange vertical bars. MSP primer binding sites are shown as arrows. AP-1 binding sites are shown as black horizontal bars **B)** Hypermethylation in *c-Jun^{fl/fl}* and *c-Jun^{Δ/Δ}* cells after 2, 4, 6 and 8 weeks of *p185^{BCR-ABL}* transformation (**upper panel**) and in stable *c-Jun^{fl/fl}* and *c-Jun^{Δ/Δ}* cell lines (**lower panel**). A visible PCR product indicates the presence of methylated alleles. Abbreviations: H.M., bone marrow of a healthy mouse; +Ctrl (control for methylated samples); -Ctrl (control for unmethylated samples). **C)** Bisulphite sequencing of the *Cdk6* 5' region of *c-Jun^{fl/fl}* (**upper panel**) and *c-Jun^{Δ/Δ}* (**lower panel**) leukemic cells. The bisulphite sequence allows a positive display of 5-methyl cytosines in the gene promoter as unmethylated cytosines appear as thymines, while 5-methylcytosines appear as cytosines in the final sequence. One representative set of data is depicted. **D)** Immunoblot for c-JUN and CDK6 of *c-Jun^{Δ/Δ}* and *c-Jun^{fl/fl}* cells after 12, 24, 36 and 48 hours of Aza-dC treatment. β-ACTIN served as loading control. One representative set of data is depicted. **E)** Percentage of living cells 12, 36, 60 and 84 hours after Aza-dC treatment of *c-Jun^{Δ/Δ}* and *c-Jun^{fl/fl}* leukemic cells. Viability was analyzed by PI-staining. **F)** *Dnmt1*, *Dnmt3a* and *Dnmt3b* mRNA levels of *c-Jun^{fl/fl}* and *c-Jun^{Δ/Δ}* cells were analyzed by RT-PCR (n = 3; two tailed; *Dnmt3b*: p = 0.016). The fold change compared to *c-Jun^{fl/fl}* mRNA levels is shown. Results were normalized by comparison to their *Gapdh* mRNA expression.

Figure 6

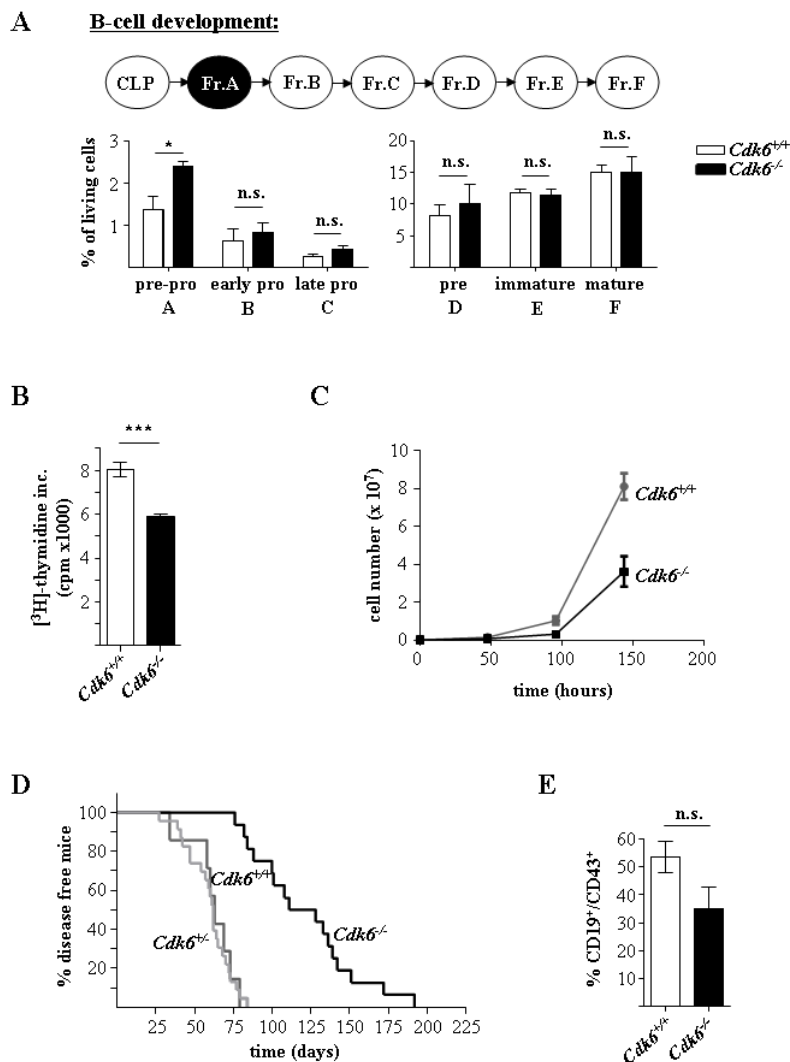


Figure 6: CDK6 advances *p185^{BCR-ABL}* induced leukemia. **A)** Percentages of B-cells of Fraction A-C in bone marrows (**left panel**) and Fraction D-F in spleens (**right panel**) of *Cdk6^{-/-}* mice compared to *Cdk6^{+/+}* mice were analyzed by FACS (n = 3; fraction A: two-tailed; p = 0.0379). **B)** [³H]-thymidine incorporation into fetal liver-derived *Cdk6^{+/+}* and *Cdk6^{-/-}* *p185^{BCR-ABL}*-transformed cell lines was measured (n = 6; two-tailed; p < 0.0001). **C)** 1 x 10⁵ cells of *p185^{BCR-ABL}*-transformed *Cdk6^{+/+}* (n = 3) and *Cdk6^{-/-}* (n = 3) were plated and total cell numbers were determined after 48, 96 and 144 hours. **D)** Injection of *Cdk6^{+/+}* (n = 7), *Cdk6^{+/-}* (n = 23) and *Cdk6^{-/-}* (n = 16) newborn mice with a replication-deficient Ab-MuLV encoding retrovirus resulted in B-lymphoid leukemia/lymphoma (mean survival 63 vs. 62 vs. 120 days in *Cdk6^{+/+}*, *Cdk6^{+/-}* and *Cdk6^{-/-}* mice, respectively; p < 0.0001 for *Cdk6^{+/+}* vs. *Cdk6^{-/-}*). **E)** The infiltration rates of CD19⁺/CD43⁺ cells in the spleens of diseased mice were analyzed by FACS (n = 6; two-tailed; p = 0.103).

Figure 7

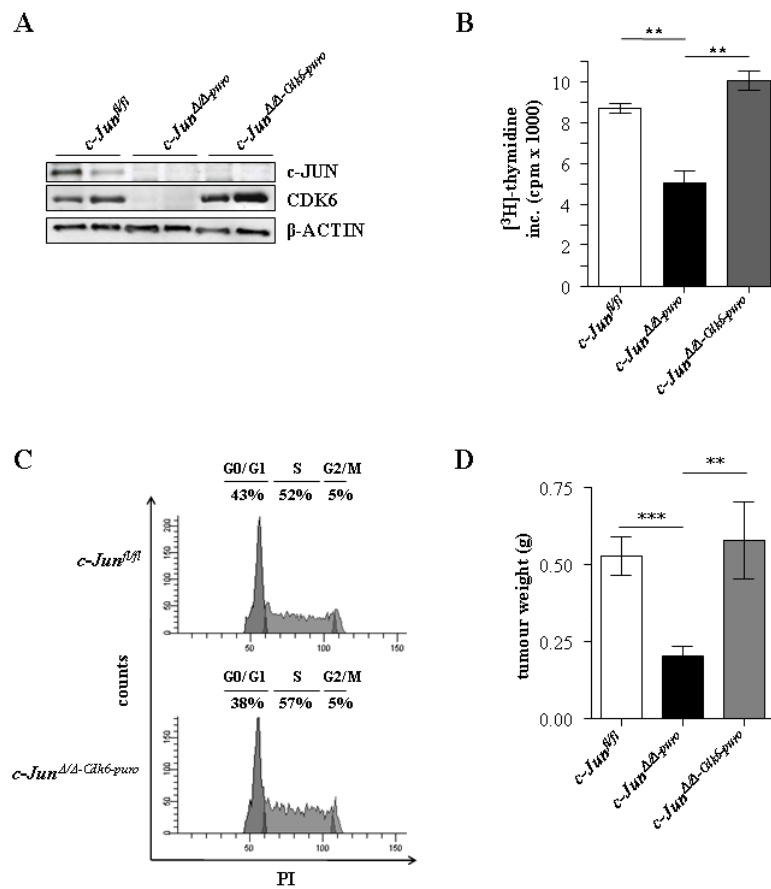


Figure 7: Re-expression of CDK6 in *c-Jun*^{Δ/Δ} cells rescues the proliferative defect. **A)** Immunoblot analysis of the enforced expression of CDK6 with a *pMSCV-Cdk6-puro* retrovirus in *c-Jun*^{Δ/Δ} cells. β-ACTIN served as loading control. **B)** [³H]-thymidine incorporation in *c-Jun*^{fl/fl}, *c-Jun*^{Δ/Δ-puro} and *c-Jun*^{Δ/Δ-Cdk6-puro} cell lines (n = 3; two-tailed; *c-Jun*^{fl/fl} vs. *c-Jun*^{Δ/Δ-puro} p = 0.0053; *c-Jun*^{Δ/Δ-puro} vs. *c-Jun*^{Δ/Δ-Cdk6-puro} p = 0.0029) **C)** Cell cycle profiles of *c-Jun*^{fl/fl} (n = 5) and *c-Jun*^{Δ/Δ-Cdk6-puro} (n = 4) cells, gated on living cells. One representative set of data is depicted. **D)** 1 × 10⁶ *c-Jun*^{fl/fl}, *c-Jun*^{Δ/Δ-puro} and *c-Jun*^{Δ/Δ-Cdk6-puro} *p185^{BCR-ABL}*-transformed cells were injected subcutaneously into *Nu/Nu* mice. 2 independent cell lines per each cell type were injected into mice (two-tailed; *c-Jun*^{fl/fl} (n = 17) vs. *c-Jun*^{Δ/Δ-puro} (n = 17) p < 0.0001; *c-Jun*^{Δ/Δ-puro} (n = 17) vs. *c-Jun*^{Δ/Δ-Cdk6-puro} (n = 11) p = 0.0017).

3.5 Leukemic challenge unmasks a requirement for PI3Kdelta in NK cell-mediated tumor surveillance.

Published in Blood. 2008 Dec 1;112(12):4655-64. Epub 2008 Aug 6.

I contributed to this project by generating NK cell cultures, by performing Western Blots, and by helping with the *in vivo* experiments.

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Leukemic challenge unmasks a requirement for PI3K δ in NK cell-mediated tumor surveillance

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Specific inhibitors of PI3K isoforms are currently evaluated for their therapeutic potential in leukemia. We found that *BCR/ABL*⁺ human leukemic cells express PI3K δ and therefore explored its impact on leukemia development. Using PI3K δ -deficient mice, we define a dual role of PI3K δ in leukemia. We observed a growth-promoting effect in tumor cells and an essential function in natural killer (NK) cell-mediated tumor surveillance: Abelson-transformed PI3K δ -deficient cells induced leukemia in RAG2-deficient mice

with an increased latency, indicating that PI3K δ accelerated leukemia progression in vivo. However, the absence of PI3K δ also affected NK cell-mediated tumor surveillance. PI3K δ -deficient NK cells failed to lyse a large variety of target cells because of defective degranulation, as also documented by capacitance recordings. Accordingly, transplanted leukemic cells killed PI3K δ -deficient animals more rapidly. As a net effect, no difference in disease latency in vivo was detected if both leukemic cells and NK cells lack

PI3K δ . Other tumor models confirmed that PI3K δ -deficient mice succumbed more rapidly when challenged with T- or B-lymphoid leukemic or B16 melanoma cells. Thus, the action of PI3K δ in the NK compartment is as relevant to survival of the mice as the delayed tumor progression. This dual function must be taken into account when using PI3K δ inhibitors as antileukemic agents in clinical trials. (Blood. 2008;112:4655-4664)

Introduction

The 8 mammalian phosphoinositide 3-kinase (PI3K) isoforms have been classified based on their lipid specificity and structure. Class I PI3Ks form heterodimers consisting of a catalytic subunit (PI3K α , β , γ , δ) and a regulatory subunit (p85 α , p85 β , p55 α , p55 γ , and p50 α for the class IA forms; PI3K α , β , δ ; and p101 and p84 for PI3K γ , the class IB enzyme): upon stimulation, the heterodimers are recruited to the plasma membrane, where they generate the lipid second messenger PIP₃ (phosphatidylinositol 3,4,5-trisphosphate) by phosphorylating phosphatidylinositol-4,5-bisphosphate (PIP₂). PIP₃ provides a signal recognized by pleckstrin homology domains, which are present in a large number of downstream targets (including protein kinases such as Akt/protein kinase B and Bruton tyrosine kinase, guanine nucleotide exchange factors, GTPase-activating proteins, and adaptor molecules). Class IA catalytic subunits (PI3K α , β , δ) signal downstream of cytokine and tyrosine kinase receptors (eg, B-cell receptors) through binding of their Src-homology domain 2 (SH2)-containing regulatory subunits to phosphotyrosine residues in YxxM-containing motifs. The class IB PI3K γ is the only isoform which transmits signals downstream of heterotrimeric G protein-coupled receptors.

A constitutive activation of PI3Ks and its downstream target Akt has been found in human cancer and is linked to cell survival and proliferation.¹ Gain-of-function mutations of *PI3K α* are among the most frequent mutations identified so far and have been linked to cancer

progression in colon, breast, brain, and lung. The role of the other isoforms is less clear, although the expression of PI3K β , γ , and δ has also been shown to transform chicken embryo fibroblasts.²

The inhibitors LY294002 and wortmannin have been widely used to study the PI3K pathway. While these compounds are popular pharmacologic tools, their significance is limited by their lack of selectivity, which is predicted to cause global effects on all PI3K isoforms. In addition, there are known off-target effects on other signaling molecules (eg, inhibition of PLA₂).³ Accordingly, gene targeting was critical to specifically delineate the roles of individual PI3K isoforms in health and disease.⁴

The catalytic subunits PI3K α and PI3K β are widely expressed and gene deletion results in embryonic lethality.^{5,6} In contrast, PI3K γ and PI3K δ display a tissue specific expression: PI3K δ is predominantly found in hematopoietic cells, and the phenotype of *PI3K δ* ^{-/-} animals is dominated by defects in B-cell development and function.⁷⁻¹⁰ It was also shown that both PI3K γ and PI3K δ are required for T-cell development and thymocyte survival.¹¹ Very recently, the importance of PI3K δ in natural killer (NK) cells has also been appreciated.^{12,13}

The expression pattern of PI3K δ and its effects in B-lymphoid cells have raised hopes that specific inhibitors of PI3K δ may suppress the proliferation and survival of transformed hematopoietic cells. Indeed, high levels of PI3K δ have been found in

Submitted February 12, 2008; accepted July 9, 2008. Prepublished online as *Blood* First Edition paper, August 6, 2008; DOI 10.1182/blood-2008-02-139105.

An Inside *Blood* analysis of this article appears at the front of this issue.

The online version of this article contains a data supplement.

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leukemic cells from patients with acute myeloid leukemia (AML).¹⁴ Here, we explored the foundations of this conceptual framework by examining how the absence of *PI3K δ* affects leukemia formation in Abelson-induced B-cell transformation. This model is of clinical relevance in humans since the Abl kinase is constitutively activated by a chromosomal translocation (t(9;22). The resulting abnormal chromosome is referred to as Philadelphia chromosome: it encodes a fusion protein, BCR/ABL, which has constitutive kinase activity and is associated with chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL). Our experiments revealed a dual role of *PI3K δ* in Abelson-driven leukemia progression: the lack of *PI3K δ* inhibited disease progression by a direct, cell-autonomous effect in the tumor cells. However, in vivo, this effect was overridden, because *PI3K δ* deficiency significantly accelerated leukemia formation by disabling NK-cell function. The absence of *PI3K δ* severely impaired the ability of NK cells to undergo degranulation and to kill leukemic cells.

Methods

Statistical analysis

P values were calculated using an unpaired 2-tailed *t* test (mean values represent data $\pm$ SEM; all experiments were performed in triplicate, and 3 independent experiments were evaluated). Differences in Kaplan-Meier plots were analyzed using the log-rank test by GraphPad Prism software (San Diego, CA).

Mice and disease models

All mice were on a 129Sv/C57BL/6 background. To ensure appropriate controls, experiments were performed with littermates. *PI3K δ ^{-/-}* mice⁹ and *RAG2^{-/-}* mice¹⁵ were described previously. Two disease models were used: newborn mice were infected by intraperitoneal injection of 100 μ L replication-incompetent ecotropic retrovirus encoding for *v-abl*.¹⁶ For transplantation experiments, either 10⁶ cells from independently derived *PI3K δ ^{+/-}* and *PI3K δ ^{-/-}* leukemic cell lines, or 5 $\times$ 10⁵ EL4 (subclone) or 5 $\times$ 10⁵ E μ -myc-derived leukemic cells (in 100 μ L phosphate-buffered saline [PBS]) were injected via tail vein into sublethally irradiated recipient mice. E μ -myc cells (provided by C. Schuster, MUW, Vienna, Austria) were derived from E μ -myc transgenic mice.¹⁷ Injected mice were checked daily for onset of disease. Sick mice were killed and analyzed for organ weights, white blood cell counts, and the presence of leukemic cells in bone marrow, spleen, and blood. NK-cell depletion was performed using the NK1.1 antibody, which was produced using the hybridoma cell line PK136 (No. HB-191; ATCC, Manassas, VA). Briefly, cell culture supernatant was harvested after serum deprivation and precipitated using saturated ammonium sulfate solution. The pellet was resuspended in buffer (20 mM sodium phosphate; pH 7.0), and antibodies were purified by HiTrap Protein G-HP affinity columns (GE Healthcare, Little Chalfont, United Kingdom). Eluted antibody was desalted using PD-10 columns (GE Healthcare). The purity of the antibody solution was determined by denaturing SDS-PAGE. A total of 150 μ g purified antibody was injected intraperitoneally 3 days before transplantation of leukemic cells and subsequently every third day.

B16 melanoma cells (5 $\times$ 10⁵ in 0.1 mL PBS) were injected via tail vein into sublethally irradiated recipient mice. After 3 weeks, metastatic lung infiltration was evaluated. All animal experiments were carried out with 8- to 20-week-old littermate control mice in accordance with Austrian legal regulations.

Cell culture

Cell culture was performed as described by Szremska et al,¹⁶ Sexl et al,¹⁸ and Hoelbl et al.¹⁹

Details are provided in Document S1 (available on the Blood website; see the Supplemental Materials link at the top of the online article).

FACS and cell-cycle analysis

Cells were analyzed by fluorescence-activated cell sorting (FACS) using the BD FACS-Canto II FACS device and BD FACS Diva software (Becton Dickinson, Vienna, Austria) as described by Hoelbl et al.¹⁹ The following antibodies were used: (1) BD Biosciences-Pharmingen: CD19-FITC, CD43-PE, B220-PerCP, DX5-APC, CD3-PE, Ly49D (4E5), CD244 (2B4), CD107a-FITC, Mac-1-PerCP, CD27-PE, CD16, IFN- γ -PE, and H-2D^d-PE; (2) eBioscience (San Diego, CA): Ly49H(3D10), NKG2D-PE, and NKp46; (3) NK-cell ligands on tumor target cells: PanRae-1 and Mult-1 (R&D Systems, Minneapolis, MN).

To obtain cell-cycle profiles, cells were harvested in 0.5 mL hypotonic lysis solution (50 μ g/mL propidium iodide in 0.1% sodium citrate, 0.1% Triton X-100, 100 μ g/mL RNase) and analyzed by FACS.

Generation of NK-cell cultures

Freshly isolated splenocytes were incubated with DX5-coupled magnetic-activated cell sorting (MACS) beads (Miltenyi Biotec, Auburn, CA) and subjected to positive selection. NK-cell preparations were pooled from at least 5 animals for each genotype and expanded in vitro using medium containing 5000 U/mL recombinant human IL-2 for 10 days.^{20,21} Purity of NK-cell cultures was determined by FACS.

Since high amounts of IL-2 during NK-cell expansion might obscure NK-cell defects,²² NK cells were starved by deprivation of IL-2 for 12 hours prior to all experiments.

Cytotoxicity assays, semiquantitative aggregation assay, and degranulation assay

[⁵¹Cr]-release assay was used to monitor target cell lysis as described by Wei et al.²³ A total of 10⁴ target cells/well were mixed with NK-effector cells in triplicates and incubated for 4 hours at 37°C in IL-2-free medium in 96-well round-bottom microplates. Tumor target cells used included: YAC-1, RMA, RMA-S, RMA-Rac-1, B16, an NK-responsive EL4 subclone, E μ -myc-transformed cells, Daudi and Jurkat cells, and *v-abl*-transformed *PI3K δ ^{+/-}* and *PI3K δ ^{-/-}* cells. Table S1 and Figure S2E depict surface receptor expression of NK-cell ligands of these tumor target cells. Redirected antibody-dependent cellular cytotoxicity (R-ADCC) was performed as described by Caraux et al²⁴ and Zompi et al.²⁵ Briefly, IL-2-expanded NK cells were preincubated with the antibodies (20 μ g/mL) directed against activating NK-cell receptors. These included CD16, NKG2D, NKp46, Ly49D, Ly49H, CD244, NK1.1, and isotype-matched control rat anti-mouse I-A antibodies. Incubation was performed for 20 minutes at 37°C before the 4-hour [⁵¹Cr]-release assay in which FeR⁺-Daudi cells were used as target cells. Daudi cells are per se not recognized by NK cells in the absence of activating antibodies. In addition, NK-cell degranulation was quantified by FACS using surface expression of CD107a.²⁶ Stimuli used to induce degranulation included coincubation with various tumor target cells and activating antibodies (analog to R-ADCC). Ionomycin (A23187; Sigma-Aldrich, St Louis, MO) treatment (5 μ M) was used as a positive control, and isotype-matched rat anti-mouse I-A was used as a negative control. Degranulation was analyzed 4 hours after coincubation with target cells and 12 hours after antibody-induced stimulation.

Aggregate formation was tested in a semiquantitative NK-cell aggregation assay as described by Stoekl et al²⁷ and Caraux et al.²⁴ Aggregate formation efficiency was determined at various time points as indicated.

IC87114 was provided by ICOS (Bothell, WA),²⁸ and used at 5 μ M. DMSO controls were performed.

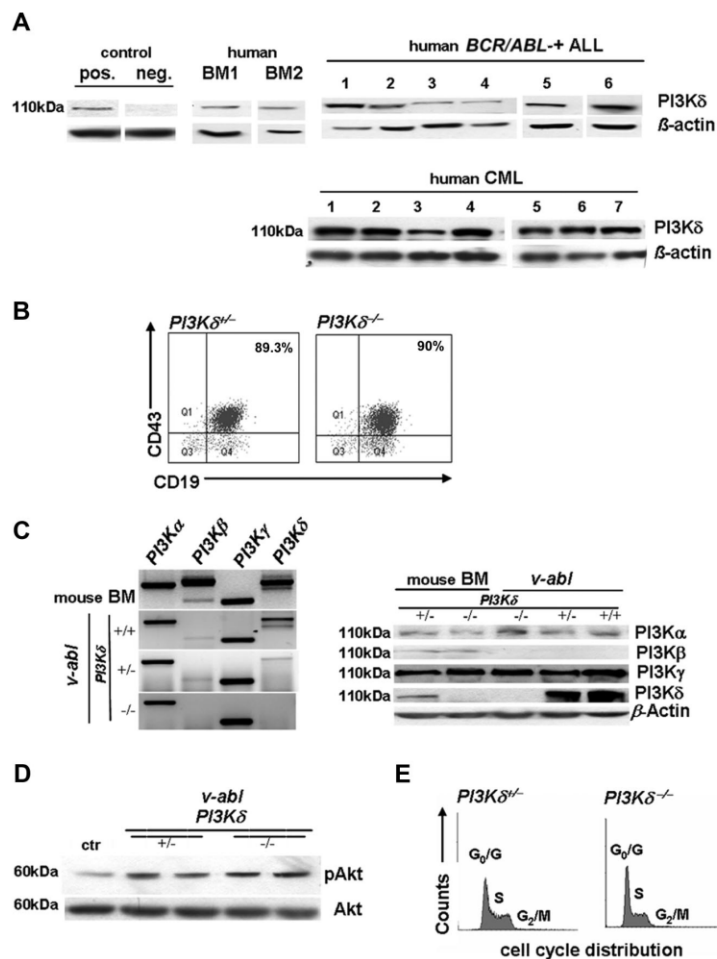
Confocal imaging

NK cells were handled according to standard protocols. Details are provided in Document S1.

Immunoblotting

Human leukemic cells were derived from the bone marrow of patients with CML (*n* = 7) or *BCR/ABL⁺* ALL (*n* = 6). Aspirated bone marrow cells were collected in heparin-containing syringes and subjected to isolation of

Figure 1. PI3K δ protein expression in *BCR/ABL*⁺ leukemia. (A) Immunoblotting for PI3K δ in cell extracts from 6 patients with *BCR/ABL*⁺ ALL and 7 patients with CML (right panel, top and bottom). All samples express PI3K δ . Healthy human bone marrow and naive murine PI3K δ ^{+/+} and PI3K δ ^{-/-} bone marrow preparations served as controls. (B) Typical example of FACS-based analysis of CD19 and CD43 surface expression of *v-abl*-transformed murine PI3K δ ^{+/+} and PI3K δ ^{-/-} cell lines (n = 6 for each genotype analyzed). (C) Representative RT-PCR analysis of PI3K α , β , γ , and δ isoform expression in freshly isolated bone marrow cells and in *v-abl*-transformed PI3K δ ^{+/+}, PI3K δ ^{+/+}, and PI3K δ ^{-/-} cell lines (left panel). Western blot analysis of PI3K α , β , δ , and PI3K γ in stable *v-abl*-transformed cell lines (right panel). MACS-purified pro-B cells derived from PI3K δ ^{+/+} and PI3K δ ^{-/-} bone marrow served as positive and negative controls, respectively. (D) Western blot analysis of phosphorylated Akt in *v-abl*-transformed PI3K δ ^{+/+} and PI3K δ ^{-/-} cell lines. NIH3T3 cells were used as control (ctr). (E) Cell-cycle profiles of *v-abl*-transformed PI3K δ ^{+/+} and PI3K δ ^{-/-} cell lines.



mononuclear cells (MNCs) using Ficoll. All patients gave informed consent in accordance with the Declaration of Helsinki before bone marrow puncture. MNCs were frozen in 90% FCS plus 10% DMSO and stored in liquid nitrogen. For immunoblots thawed human cells or 10^7 cells from established murine cell lines were lysed, processed as described previously,^{16,19} and probed either with antibodies specific for PI3K δ (sc-7176), β (sc-7175), α (sc-7174; all from Santa Cruz Biotechnology, Santa Cruz, CA), and PI3K γ ²⁹ (a generous gift from R. Wetzker, Jena, Germany) which were used as described.³⁰ Anti-pAkt (S473) and anti-Akt antibodies were from Cell Signaling Technology (Danvers, MA).

RNA isolation and RT-PCR analysis

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis are described in Document S1. Capacitance measurements are also described in detail in Document S1.

Results

PI3K δ expression in human and murine leukemic cells

PI3K δ was expressed in all leukemic cell samples derived from patients with *BCR/ABL*⁺ CML and ALL (Figure 1A). PI3K δ inhibitors are considered novel therapeutic options for the treat-

ment of hematopoietic malignancies. Hence, we studied the impact of PI3K δ for Abelson-induced leukemia progression. Murine PI3K δ ^{+/+} and PI3K δ ^{-/-} bone marrow cells were infected in vitro with a replication-incompetent ecotropic form of the Abelson virus (Ab-MuLV; *v-abl*). This procedure renders the cells independent of growth factors and allows them to form stable cell lines. Immortalized cell lines of each genotype were obtained from each bone marrow preparation. These cell lines consisted of B220⁺CD19⁺CD43⁺ cells (Figure 1B). Bone marrow cells express all 4 catalytic PI3K isoforms (α , β , γ , and δ). This pattern changed in the transformed cells that showed a predominant expression of PI3K α , γ , and δ . As expected, PI3K δ mRNA and protein was absent in the cell lines derived from PI3K δ ^{-/-} bone marrow (Figure 1C). Moreover, *v-abl*-transformed cells lost the expression of PI3K β —this was detectable both at the mRNA and protein levels (Figure 1C) and confirmed in human patient samples (E.Z., unpublished observations, December 2007). However, the lack of PI3K δ did not translate into changes in the phosphorylation of Akt, a major downstream signaling molecule (Figure 1D). Similarly, we failed to detect any alterations in the proliferative or apoptotic response of the PI3K δ ^{-/-} compared with PI3K δ ^{+/+} cell lines. Cell-cycle distribution, growth characteristics, apoptotic responses

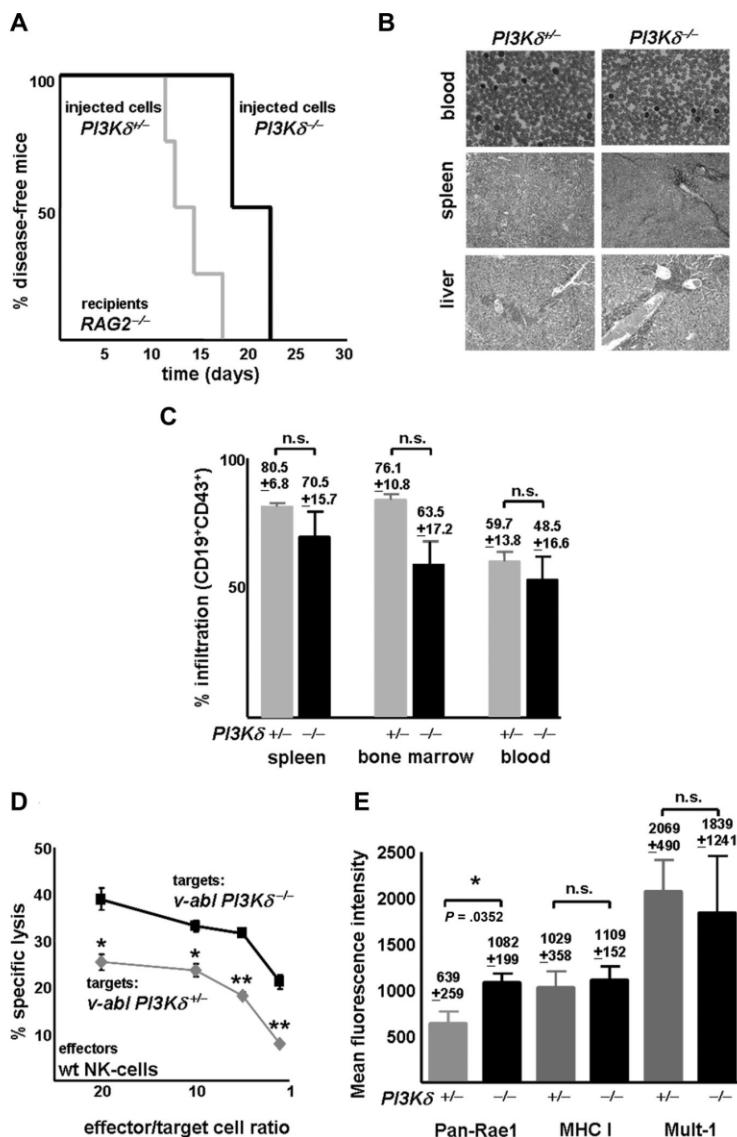


Figure 2. Abelson-transformed $PI3K\delta^{-/-}$ cells induce leukemia in mice with an increased latency. (A) Kaplan-Meier plot of $RAG2^{-/-}$ mice after transplantation of 10^6 transformed cells (3 independently derived cell lines per genotype were injected into $n = 8$ for $PI3K\delta^{+/+}$ and $n = 6$ for $PI3K\delta^{-/-}$). Mice that had been injected with transformed $PI3K\delta^{-/-}$ cells developed leukemia significantly later as determined by a log-rank test (median survival, 13 vs 20 days; $P = .005$). (B) H&E stains of blood smears (top), spleens (middle), and livers (bottom) from $RAG2^{-/-}$ mice after injection of $v-abl$ -transformed cells (magnification, $\times 100$, Zeiss AxioImager 21 [Jena, Germany], $10\times$ objective, NA 0.25, air; camera: Pixelink Color, 1600×1200 ; software: PixelINK Capture 3.0). (C) Spleen, bone marrow, and blood were analyzed for infiltration with $CD19^+CD43^+$ leukemic cells by FACS. Tissue infiltration was slightly lower in mice that had received $PI3K\delta^{-/-}$ cells without reaching statistical significance (data represent means $\pm$ SEM). (D) [^{51}Cr]-release assay using IL-2-expanded wt NK cells as effectors and Abelson-transformed leukemic cells as targets. $PI3K\delta^{-/-}$ leukemic cells were significantly better eradicated by wt NK cells than $PI3K\delta^{+/+}$ leukemic target cells at any effector-target cell ratio tested (for ratios 20:1, 10:1, 5:1, and 2:1, $P = .02$, $P = .02$, $P = .003$, and $P = .006$ as determined in an unpaired 2-tailed t test). (E) Quantification of pan-Rae-1, Mult-1, and MHC I expression by FACS of in vitro-derived Abelson-transformed cell lines. $PI3K\delta^{-/-}$ leukemic cells showed a significantly higher surface expression of pan-Rae1 when compared with $PI3K\delta^{+/+}$ leukemic cells (mean fluorescence intensity [MFI] of 1082 ± 199 vs 639 ± 259 ; $P = .035$ in an unpaired 2-tailed t test; $n = 4$ for each genotype). n.s., indicates $P > .05$; * $P < .05$; and ** $P < .01$.

to UV irradiation, and serum withdrawal were comparable in several individually derived cell lines (Figure 1E; data not shown). These experiments supported the conclusion that $PI3K\delta$ was not a prerequisite for the transformation of B cells by the Abelson oncogene and that lack of $PI3K\delta$ did not impair cell proliferation nor alter apoptotic responses of Abelson-transformed cells in vitro.

Abelson-transformed $PI3K\delta^{-/-}$ cells induce leukemia in mice with an increased latency

To study the behavior of the Abelson-transformed cell lines in vivo, we injected 10^6 cells of 3 individually derived $PI3K\delta^{+/+}$ and $PI3K\delta^{-/-}$ cell lines via tail vein into $RAG2^{-/-}$ animals ($n = 8$ for $PI3K\delta^{+/+}$ and $n = 6$ for $PI3K\delta^{-/-}$). As depicted in Figure 2A, mice that had received $PI3K\delta^{+/+}$ leukemic cells succumbed to disease with a significantly faster time course than mice that had received

$PI3K\delta^{-/-}$ leukemic cells (median survival, 13 days vs 20 days; $P = .005$). The phenotype of the disease induced by $PI3K\delta^{-/-}$ or $PI3K\delta^{+/+}$ leukemic cells was comparable (Figure 2B,C). Based on the fact that no significant differences between $PI3K\delta^{+/+}$ and $PI3K\delta^{-/-}$ leukemic cells were detectable in vitro but became apparent in vivo, we reasoned that an altered interaction with the immune system might account for this discrepancy. Abelson-transformed cells are mainly recognized and eradicated by NK cells, which are the only lymphoid compartment present in $RAG2^{-/-}$ mice. We therefore tested whether the increased disease latency upon injection of $PI3K\delta^{-/-}$ leukemic cells resulted from an altered interaction with NK cells. Indeed, $PI3K\delta^{-/-}$ leukemic cells were significantly better lysed by wild-type (wt) NK cells than $PI3K\delta^{+/+}$ cells (Figure 2D). NK cells recognize tumor targets via distinct surface receptors; the importance of the NKG2D receptor

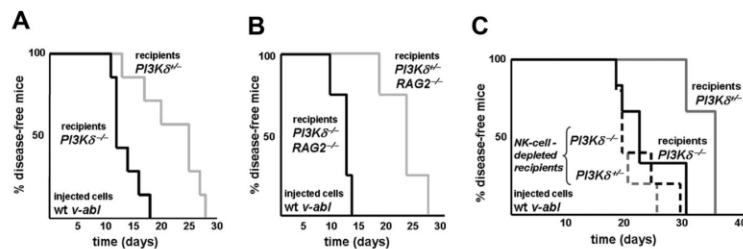


Figure 3. Mice deficient for $PI3K\delta$ develop leukemia significantly faster. (A) Kaplan-Meier plot of $PI3K\delta^{+/-}$ and $PI3K\delta^{-/-}$ recipient animals after injection of wt $v-abl$ -transformed cells. $PI3K\delta^{-/-}$ recipient mice developed disease significantly earlier when compared with their $PI3K\delta^{+/-}$ littermate controls (log-rank test; $P = .003$; $n = 7$ for each group; 3 independently derived cell lines were injected). (B) Kaplan-Meier plot of $PI3K\delta^{+/-}$ and $PI3K\delta^{-/-}$ recipients on a $RAG2^{-/-}$ background after injection of wt $v-abl$ -transformed cells. Again, $PI3K\delta^{-/-}$ recipients succumbed significantly earlier to disease than $PI3K\delta^{+/-}$ littermate controls (log-rank test; $P = .007$; $n = 4$ for each group). (C) Kaplan-Meier plot of 10 $PI3K\delta^{+/-}$ and 10 $PI3K\delta^{-/-}$ recipient animals after injection of wt $v-abl$ -transformed cells. A total of 5 animals of each group received anti-NK1.1 antibody to deplete NK cells (dashed lines). In the absence of NK-cell depletion, $PI3K\delta^{-/-}$ recipient mice again developed disease significantly earlier when compared with their $PI3K\delta^{+/-}$ littermate controls (solid lines; log-rank test; $P = .03$). This difference was lost upon NK-cell depletion (dashed lines; log-rank test; $P = .61$; $n = 5$ for each group).

in tumor surveillance has recently been highlighted by the generation of NKG2D-deficient animals.³¹ Interestingly, $PI3K\delta^{-/-}$ leukemic cells showed significantly higher expression of the NKG2D ligand Rae-1 (Figure 2E).

Impaired lysis of leukemic cells by $PI3K\delta^{-/-}$ NK cells in vivo

The experiments summarized in Figure 2 documented that loss of $PI3K\delta$ delays tumor progression in vivo. Based on these findings, specific inhibitors of $PI3K\delta$ ought to be useful in a clinical setting. However, $PI3K\delta$ is also present in immune cells which are capable of eliminating tumor cells. An inhibitor of $PI3K\delta$ thus may have an impact on immunologic surveillance. This was separately examined by injecting 3 independently derived wt Abelson-transformed leukemic cell lines into $PI3K\delta^{+/-}$ and $PI3K\delta^{-/-}$ recipient mice ($n = 7$ for each group). Whereas $PI3K\delta^{-/-}$ recipient mice succumbed to the disease rapidly (median survival, 12 days), $PI3K\delta^{+/-}$ animals survived significantly longer (median survival, 25 days; $P = .003$; Figure 3A). This effect was attributable to differences in NK cell-mediated tumor surveillance: experiments in $RAG2^{-/-}PI3K\delta^{-/-}$ and $RAG2^{-/-}PI3K\delta^{+/-}$ recipient animals recapitulated the results obtained with $PI3K\delta^{+/-}$ and $PI3K\delta^{-/-}$ mice (Figure 3B). Leukemia development occurred with a significantly shortened latency in $RAG2^{-/-}PI3K\delta^{-/-}$ animals when compared with $RAG2^{-/-}PI3K\delta^{+/-}$ recipient mice (median survival, 12 days vs 23 days; $P = .007$). Antibody-mediated NK-cell depletion further confirmed the central role of NK cells (Figure 3C): injection of wt leukemic cell lines resulted in a significant difference in disease latency between $PI3K\delta^{+/-}$ and $PI3K\delta^{-/-}$ recipient mice (median survival, 35 days for $PI3K\delta^{+/-}$ vs 22 days for $PI3K\delta^{-/-}$ mice; $P = .03$) that was completely abolished by NK-cell depletion (median survival, 19 days; $P = .61$). These experiments verified the key role of $PI3K\delta$ for NK cell-mediated lysis of leukemic tumor targets in vivo.

Cell-intrinsic roles of $PI3K\delta$ in leukemic cell and NK cell: the net effect

It was difficult to predict the net effect, which would result in vivo from the absence of $PI3K\delta$ in both the leukemic cells and the NK cells. The impaired ability of $PI3K\delta^{-/-}$ cells to form leukemia might be counteracted by the reduced ability of $PI3K\delta^{-/-}$ NK cells to combat leukemia. Hence, disease latency may remain unaffected because of the opposite effects of $PI3K\delta$ deficiency in the tumor cells (favoring increased latency) and NK cells (promoting decreased latency). This question appears of particular relevance in view of the development of $PI3K\delta$ kinase inhibitors. To test whether impaired transformation or reduced immunologic surveillance would prevail, we injected Abelson-transformed $PI3K\delta^{+/-}$

leukemic cells into $PI3K\delta^{+/-}$ recipient animals. Similarly, we injected $PI3K\delta^{-/-}$ leukemic cells into $PI3K\delta^{-/-}$ littermate animals. A total of 3 independently derived Abelson-transformed leukemic cell lines of each genotype were used, and the experiments are summarized in Figure 4A. No significant difference was detected (mean survival time, 19 vs 19.5 days; $P = .95$). In addition, we used a second disease model that closely mimics leukemia development in humans. Newborn $PI3K\delta^{+/-}$ and $PI3K\delta^{-/-}$ mice were challenged with a single exposure to Ab-MuLV. This procedure induces the development of a slowly evolving mono- or oligoclonal leukemia; the leukemic cells progressively infiltrate bone marrow, spleen, liver, and lymph nodes. Again, as depicted in Figure 4B, we failed to see any significant alterations in latency or

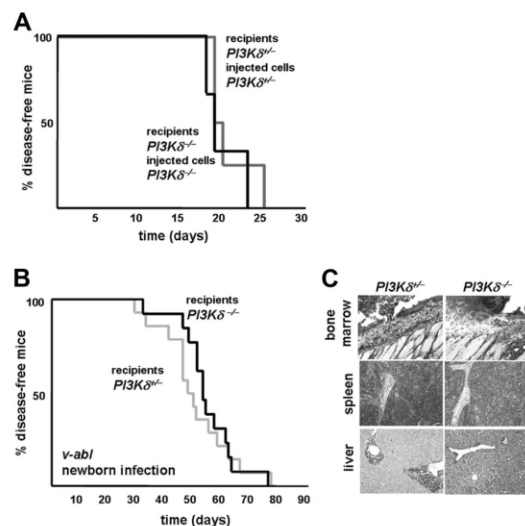


Figure 4. Net effect of $PI3K\delta$ deficiency for leukemia development. (A) Kaplan-Meier plot of $PI3K\delta^{+/-}$ recipients after injection of $PI3K\delta^{+/-}$ $v-abl$ -transformed cells and of $PI3K\delta^{-/-}$ recipients after injection of $PI3K\delta^{-/-}$ $v-abl$ -transformed cells. Matching genotypes of recipient animals with the genotype of the injected cell line abolished any differences in leukemia onset and progression (log-rank test; $P = .95$). (B) Accordingly, upon $v-abl$ infection of newborn $PI3K\delta^{+/-}$ and $PI3K\delta^{-/-}$ recipients, no significant difference in survival kinetics was detected (log-rank test; $P = 0.7$). No differences were observed between $PI3K\delta^{+/-}$ and $PI3K\delta^{-/-}$ control animals, as verified in an independent experiment (data not shown). (C) H&E-stained histologic sections of infiltrated bone marrow (top row), spleen (middle row), and liver (bottom row) of $PI3K\delta^{+/-}$ and $PI3K\delta^{-/-}$ animals that had been challenged with $v-abl$ at the newborn age (magnification, $\times 100$, Zeiss AxioImager 21, $10\times$ objective, NA 0.25, air, camera: Pixelink Color, 1600×1200 ; software: PixelINK Capture 3.0).

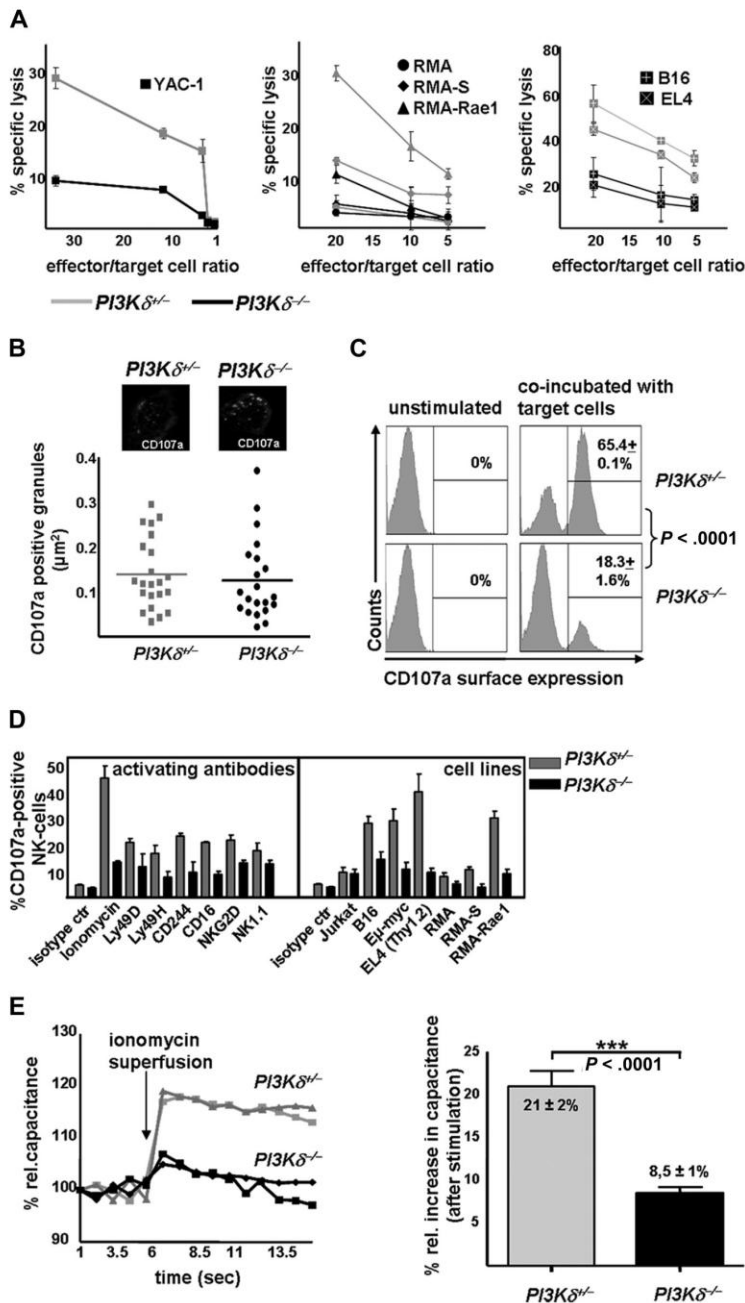


Figure 5. $PI3K\delta^{-/-}$ NK cells fail to efficiently lyse leukemic target cells. (A) The cytolytic function of IL-2-expanded $PI3K\delta^{+/+}$ and $PI3K\delta^{-/-}$ NK cells was assessed by ^{51}Cr -release assay using various cell lines as target cells as indicated. $PI3K\delta^{-/-}$ NK cells showed consistently impaired specific lysis when compared with $PI3K\delta^{+/+}$ NK cells, regardless of the target cell line used. The complete table of all target lines including statistics is provided in Table S3. (B) Top panels: typical confocal images of CD107a $^{+}$ granules of $PI3K\delta^{+/+}$ and $PI3K\delta^{-/-}$ NK cells are given. Bottom panel: quantification of granule numbers. Confocal images of CD107a $^{+}$ granules were acquired in the z-stack mode on a Zeiss LSM510 confocal microscope with a 100 $\times$ oil-immersion objective (NA 1.3) with the 488-nm line of the Ar laser and a LP 505-nm emission filter. About 24 slices were acquired with a distance of 0.3 μm between the slices. Images were imported into ImageJ software, and maximum projection images were calculated. Stained vesicles were selected by auto-thresholding, followed by quantification and analysis of all selected regions. Horizontal lines indicate data mean. (C) A FACS-based degranulation assay measuring surface expression of the late endosomal marker CD107a was performed. FACS histograms illustrate the percentage of $PI3K\delta^{+/+}$ (top row) and $PI3K\delta^{-/-}$ (bottom row) NK cells expressing CD107a under basal conditions (left panels) and after stimulation by co-incubation with *v-abl*-transformed target cells. Significantly more $PI3K\delta^{+/+}$ NK cells were stimulated to express CD107a on their surface compared with $PI3K\delta^{-/-}$ NK cells (data mean $\pm$ SEM). (D) Bar graphs depicting different stimuli used to induce degranulation in $PI3K\delta^{+/+}$ and $PI3K\delta^{-/-}$ NK cells: none of the target cells or specific antibodies induced degranulation of $PI3K\delta^{-/-}$ NK cells. (E) Electrophysiological capacitance measurements were performed and confirmed the defect of $PI3K\delta^{-/-}$ NK cells in Ca^{2+} -dependent exocytosis/degranulation. Electrophysiological capacitance measurement relies on recording the passive current, which has to be injected into the cell to keep a constant voltage and this corresponds to the size (surface area) of a cell: the bigger the cell, the more current has to be injected. In vitro-expanded murine NK cells are round cells (means of absolute capacitance of unstimulated $PI3K\delta^{+/+}$ NK cells, 12.4 ± 5.4 pF; of $PI3K\delta^{-/-}$ NK cells, 13.73 ± 5.9 pF). Two typical examples of the exocytotic response under the superfusion with calcimycin are illustrated (left panel). The mean increase in relative capacitance after superfusion of $PI3K\delta^{+/+}$ and $PI3K\delta^{-/-}$ NK cells was compared (mean increase in relative capacitance for $PI3K\delta^{+/+}$ NK cells: $21\% \pm 2\%$; for $PI3K\delta^{-/-}$ NK cells: $8.5\% \pm 1\%$; $P < .001$; $n = 10$ for each genotype; the experiment was repeated with 3 independent NK-cell preparations). The increase in relative capacitance was obtained by comparing the peak increase in relative capacitance after superfusion with calcimycin with resting cell capacitance under superfusion with control solution (right panel). Error bars represent SEM. n.s. indicates $P > .05$; * $P < .05$; and ** $P < .01$.

incidence in this disease model (mean survival time, 49 vs 53 days; $P = .7$). Regardless of the expression of $PI3K\delta$, all mice died of leukemia within 80 days. The phenotype of the disease was comparable (Figure 4C).

$PI3K\delta^{-/-}$ NK cells have an impaired cytolytic ability

Abelson-transformed leukemic cells are mainly subject to surveillance by NK cells as shown previously.^{21,32} To understand why the deletion of $PI3K\delta$ impaired NK-cell function and consequently the

clearance of leukemic cells in vivo, we isolated NK/NKT cells for further characterization. We did not detect any obvious differences in NK-cell numbers and functional subsets (Figure S1) and in the expression of cell-surface markers on primary and IL-2-expanded NK cells regardless of the genotype (Table S2). Differences were evident in cytotoxicity assays against target cells (Figure 5A). Regardless of the nature of the target cells, $PI3K\delta^{-/-}$ NK cells were significantly less capable to lyse these when compared with $PI3K\delta^{+/+}$ NK cells. We used target cells recognized by various

different pathways, including Abelson-transformed cells (see Table S3 for a detailed summary of [^{51}Cr]- and FACS-based quantification). These experiments pointed at a common defect unrelated to a distinct activation pathway. The presence of a general defect was further supported by experiments using R-ADCC. Preincubation with activating antibodies stimulated NK-cell cytotoxicity toward Daudi cells that are per se not recognized by NK cells. Regardless of the receptor stimulated, $PI3K\delta^{-/-}$ NK cells were never considerably activated (Figure S2; Table S3), which indicates that $PI3K\delta$ is a key element of a common lytic pathway.

One major mechanism in NK cell-mediated tumor clearance is the lytic granule secretory pathway. To efficiently kill target cells, the NK-cell releases the content of its lytic granules by fusion with the plasma membrane. During this process, CD107a (LAMP-1), a component of lysosomal membranes, is integrated into the plasma membrane (degranulation). We first verified the presence of CD107a $^{+}$ lytic granules by confocal microscopy in $PI3K\delta^{-/-}$ NK cells. We failed to detect any significant differences in number, shape, or size of CD107a $^{+}$ granules (Figure 5B). Similarly, effector molecules such as perforin and granzymes were expressed at comparable amounts (Figure S1C). However, FACS-based degranulation assays revealed that coincubation of $PI3K\delta^{+/+}$ and $PI3K\delta^{-/-}$ NK cells with wt $v\text{-abl}$ target cells resulted in a 4-fold increase of CD107a expression on the surface of $PI3K\delta^{+/+}$ NK cells compared with $PI3K\delta^{-/-}$ NK cells (Figure 5C right panels). Similar results were obtained with various other experimental stimuli (Figure 5D), including coincubation with 7 target cell lines and stimulation of 6 distinct activating receptors by specific antibodies.

It is evident from Figure 5D that the response to the different stimuli was variable; however, $PI3K\delta^{-/-}$ NK cells were always less responsive. The notable exception was challenge with Jurkat cells (compare bars 3 and 4 in the right panel of Figure 5D); these, however, are killed by a FAS-dependent pathway rather than by NK-cell granule exocytosis.³³ Degranulation is ultimately triggered by a rise in intracellular Ca^{2+} . It is also evident from Figure 5D that the defect in $PI3K\delta^{-/-}$ NK cells affected a very late step because the difference was readily detected upon challenging cells with the calcium ionophore ionomycin (compare bars 3 and 4 in the left panel of Figure 5D). This observation again indicates that $PI3K\delta$ is a key molecule involved in the ultimate common degranulation trigger regardless of the upstream stimulus (ie, at a very distal level independent from any target cell contact or activating receptor). This conjecture was rigorously tested by directly monitoring degranulation in real time: the fusion of the membrane of the lytic granules with the outer cell membrane leads to a subtle increase in cell size that can be measured by recording NK-cell capacitance in a whole-cell patch clamp configuration. Single NK cells were superfused with a Ca^{2+} ionophore (ionomycin), which triggers degranulation, and the increase in cell capacitance was recorded. Two typical examples of the exocytotic response under the superfusion with ionomycin are illustrated for $PI3K\delta^{+/+}$ and $PI3K\delta^{-/-}$ NK cells in Figure 5E. In $PI3K\delta^{+/+}$ NK cells, superfusion with ionomycin resulted in a mean increase in relative capacitance of 21% plus or minus 2% compared with 8.5% plus or minus 1% for $PI3K\delta^{-/-}$ NK cells ($P < .001$). Hence, $PI3K\delta^{+/+}$ NK cells showed a significantly higher increase in cell-surface area compared with $PI3K\delta^{-/-}$ NK cells. These results prove a central role for $PI3K\delta$ at a very distal step in the process of degranulation.

It is conceivable that additional upstream defects in $PI3K\delta^{-/-}$ NK cells impair the ability of cells to engage their targets. Accordingly, we assessed the ability of NK cells to form aggregates with target cells. Both, $PI3K\delta^{+/+}$ and $PI3K\delta^{-/-}$ NK cells form

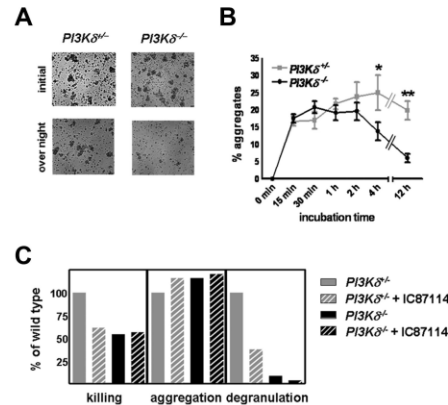


Figure 6. $PI3K\delta$ is dispensable for the initial formation of aggregates with leukemic target cells, but is indispensable for the maintenance of aggregates and for degranulation. (A) Initial aggregate formation (top panels) and maintenance of aggregates (bottom panels) of $PI3K\delta^{+/+}$ and $PI3K\delta^{-/-}$ NK cells with wt $v\text{-abl}$ target cells was analyzed by light microscopy (magnification: top panel, $\times 100$; bottom panel, $\times 430$; one example for each genotype is depicted, obtained with a Zeiss Axiovert 135, 10 $\times$ Ph1 [NA 0.25], camera: Cool Snap HQ [Ottobrunn, Germany], software: Metamorph 4.0 [Visitron, Puchheim, Germany]). (B) quantified by FACS, and observed over time. Error bars represent SEM (* $P < .05$; ** $P < .01$). (C) A selective inhibitor of $PI3K\delta$ was used to describe the requirement for $PI3K\delta$ for killing, aggregate formation, and degranulation of NK cells when incubated with leukemic target cells. As normalized to $PI3K\delta^{+/+}$ NK cells, $PI3K\delta$ inhibition severely impaired killing (61.3% of $PI3K\delta^{+/+}$ NK cells) and degranulation (37.7% of $PI3K\delta^{+/+}$ NK cells), whereas initial aggregate formation was not affected (115% of $PI3K\delta^{+/+}$ NK cells).

aggregates initially at comparable numbers (Figure 6A top panel). However, after 4 hours, aggregates formed by $PI3K\delta^{-/-}$ NK cells were apparently less stable because of the decline in numbers (Figure 6B); this effect was even more pronounced after 12 hours (Figure 6A bottom panels, 6B). These results were confirmed with a selective inhibitor of $PI3K\delta$: aggregate formation by $PI3K\delta^{+/+}$ NK cells was not impaired in the presence of a selective $PI3K\delta$ inhibitor, but degranulation and killing of target cells was reduced (Figure 6C). This pharmacologic approach provided independent confirmation for our conclusion that $PI3K\delta$ was indispensable for degranulation and killing of target cells.

$PI3K\delta^{-/-}$ mice are highly susceptible to tumor development

Additional tumor models were chosen that expressed significant levels of $PI3K\delta$, because these qualified as suitable candidates for a $PI3K\delta$ -targeted therapy (Figure 7; data not shown). A total of 5×10^5 cells of the murine T lymphoma cell line EL4 were injected into $PI3K\delta^{+/+}$ and $PI3K\delta^{-/-}$ recipient mice. These subsequently developed leukemia with spleno- and hepatomegaly. The $PI3K\delta^{-/-}$ recipient mice succumbed to the disease rapidly (median survival, 17 days on the $RAG2^{+/+}$ background; 16.5 days on $RAG2^{-/-}$ background). In contrast, the $PI3K\delta^{+/+}$ animals survived the leukemic challenge significantly longer (median survival, 30 days and 20 days on the $RAG2^{+/+}$ [$P = .045$] and $RAG2^{-/-}$ [$P = .039$] background, respectively) (Figure 7A; data not shown). Similarly, a cell line derived from an E μ -myc transgenic mouse, which represents a model for human Burkitt lymphoma, was injected intravenously into $PI3K\delta^{+/+}$, $PI3K\delta^{-/-}$, $RAG2^{-/-}PI3K\delta^{+/+}$, and $RAG2^{-/-}PI3K\delta^{-/-}$ mice. The onset of disease was rapid in $PI3K\delta^{-/-}$ animals, with a median survival of 25.5 and 13 days on $RAG2^{+/+}$ (Figure 7B) and $RAG2^{-/-}$ (not shown) backgrounds, respectively. Again, the $PI3K\delta^{+/+}$ animals survived significantly longer (median survival of 45 days on $RAG2^{+/+}$ background; $P = .039$; Figure 7B; median survival of 31 days on $RAG2^{-/-}$ background; $P = .022$; data

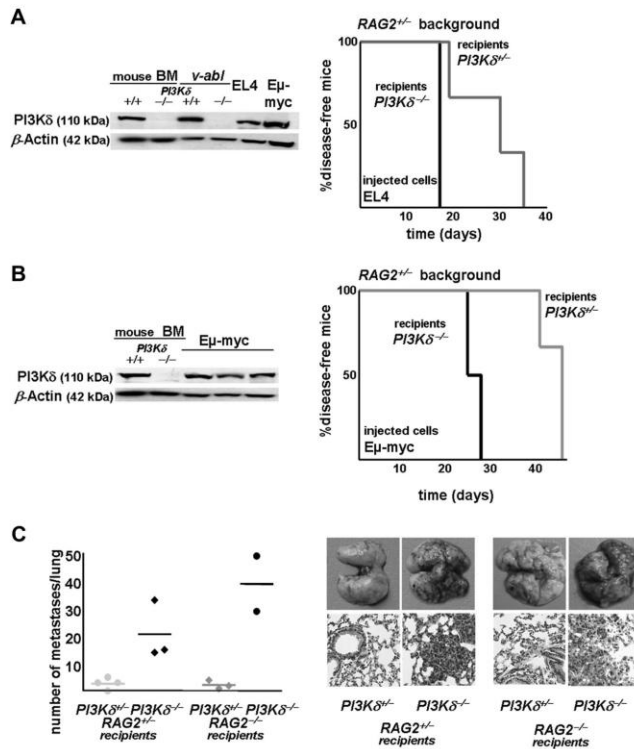


Figure 7. PI3Kδ^{-/-} mice in additional hematologic and nonhematologic tumor models. (A) PI3Kδ protein expression in murine leukemic cell lines. Naive murine PI3Kδ^{+/+} and PI3Kδ^{-/-} bone marrow preparations served as controls. Kaplan-Meier plot after injection of EL4 cells intravenously into PI3Kδ^{+/+}-RAG2^{+/+} and PI3Kδ^{-/-}-RAG2^{+/+} recipients (log-rank test; $P = .045$). (B) PI3Kδ protein is expressed in Eμ-myc-derived leukemic cells. Upon injection of Eμ-myc-derived cells, PI3Kδ^{-/-}-RAG2^{+/+} recipients succumbed to disease significantly faster than PI3Kδ^{+/+}-RAG2^{+/+} littermate controls (log-rank test; $P = .039$). (C) B16 melanoma cells were injected intravenously and numbers of metastatic infiltrates per lung were counted under the binocular microscope. RAG2^{+/+} PI3Kδ^{-/-} recipient mice showed a significantly higher number of metastatic infiltrates than RAG2^{+/+} PI3Kδ^{+/+} ($P = .005$), and this was also true on the RAG2^{-/-} background ($P = .015$). Horizontal lines indicate data mean. Thus, increased tumor formation in PI3Kδ^{-/-} mice is not restricted to lymphoid malignancies. Formation of lung metastasis in littermate controls (top panels; photographs, digital camera, Canon EOS 300D; bottom panels: H&E-stained histologic sections of lungs; magnification, $\times 20$ Zeiss AxioImager 71; see Figure 2B).

not shown). Finally, we challenged our concept in an independent, nonhematologic tumor model by injecting the melanoma cell line B16 intravenously in PI3Kδ^{+/+}, PI3Kδ^{-/-}, RAG2^{+/+} PI3Kδ^{+/+}, and RAG2^{-/-} PI3Kδ^{-/-} littermate controls (Figure 7C). B16 cells have been reported to express PI3Kδ³⁴ and are known to be under the immunologic control of NK cell-mediated clearance.³⁵ At 3 weeks after the injection of the B16 cells, the metastatic infiltrates in lungs of recipient mice were compared. At this time point, the PI3Kδ^{-/-} animals were severely affected and showed significant infiltration of tumor cells in the lungs, regardless of whether they were on a wt or a RAG2^{-/-} background. Despite the low animal numbers, the differences clearly met the criteria of being statistically significant ($P = .005$ for wt and $P = .015$ for RAG2^{-/-} background; Figure 7C). The difference is also emphasized by hematoxylin and eosin (H&E) stains of sections of the affected lungs. Thus, PI3Kδ regulates NK cell-mediated tumor surveillance regardless of the origin of the tumor cell.

Discussion

It has been appreciated in the past that signaling via PI3K was important for growth and survival of transformed cells.¹ The PI3Kδ isoform has recently been established as important subform for leukemia progression in AML.¹⁴ We therefore investigated whether PI3Kδ is also expressed in human BCR/ABL-transformed cells and found a consistent expression in both BCR/ABL⁺ CML and ALL

leukemic cells. Thus, PI3Kδ inhibition would also qualify as potential treatment option for these disease entities. Our data show that in *v-abl*-induced murine leukemia, the presence of PI3Kδ indeed accelerated leukemia progression *in vivo*. Hence, the absence of PI3Kδ obviously cannot be compensated for by any other of the isoforms expressed. We noted a striking difference between the *in vitro* and the *in vivo* situation: *in vitro*, absence of PI3Kδ did not impair transformation, immortalization, and growth of tumor cells. In contrast *in vivo*, the absence of PI3Kδ clearly impaired leukemia progression. Our observations are consistent with the interpretation that differences in NK cell-mediated clearance are responsible for the differences in disease latency. wt NK cells lyse PI3Kδ^{-/-} leukemic cells significantly better than their PI3Kδ^{+/+} leukemic counterparts. Leukemia surveillance is accomplished—at least in part—by NK cells.^{21,32} NK cells recognize tumor cells via receptors on their cell surface (reviewed by Lanier³⁶). The NKG2D receptor plays a key role in mediating tumor cell lysis by binding to Rae1 on the target cell. Indeed, Rae1 is expressed at significantly higher levels on PI3Kδ^{-/-} cells. Regardless of the underlying mechanism, these previous experiments and our current observations would conceptually validate PI3Ks, specifically the PI3Kδ isoform, as a target in leukemia. However, our current experiments and previous reports highlight the role of the immune system in determining the natural course of a tumor disease.^{21,32,37-39} Here, we observed that PI3Kδ deficiency resulted in a major impairment of NK-cell function. Because NK

cells are required to eliminate leukemic cells of B-cell origin, the clearance of tumor cells was hampered; this shaped the course of the disease, obviating any benefit arising from the lack of *PI3Kδ* in the tumor cells. This fact is highlighted by the experiments in *RAG2^{-/-}PI3Kδ^{-/-}* mice. These animals rely on NK cells as their sole means of eliminating tumors: as predicted, the decreased latency in leukemia development was recapitulated in *RAG2^{-/-}PI3Kδ^{-/-}* mice. Conversely, antibody-mediated NK-cell depletion abolished any difference in survival between *PI3Kδ^{+/-}* and *PI3Kδ^{-/-}* mice. In summary, these data unequivocally document the importance of NK cells for immune-surveillance of *v-abl*-induced leukemia and the absolute requirement for *PI3Kδ* in this process.

Phosphatidyl inositol phosphates are known regulators of exocytosis. Individual lipids apparently regulate distinct steps in different vesicle populations; priming of neurotransmitter vesicles, for instance, is contingent on the formation of phosphatidyl inositol-3-phosphate (PtdIns3P).⁴⁰ Very recently, Garçon et al showed that *PI3Kδ* is the main *PI3K* isoform responsible for accumulation of PIP3 at the immune synapse.⁴¹ We now show that even though *PI3Kδ* is dispensable for the initial formation of the synapse, it is required to maintain the integrity of the synapse over time. In line with Jiang et al,⁴²⁻⁴⁴ who proposed that *PI3Ks* trigger cytotoxicity through sequential activation of the small G-protein Rac1, the p21-activated kinase 1 (PAK1), MEK, and ERK1/2, we here show that efficient degranulation of NK cells is absolutely dependent on *PI3Kδ*. This conclusion is based on 2 independent sets of experiments, namely the externalization of the granule membrane constituent CD107a and the change in membrane capacitance associated with exocytosis. The first approach allowed verifying the general nature of the secretion defect (ie, target cells elicited less externalization of granule content). The measurements of membrane capacitance clearly identified a deficit in a late step. Membrane capacitance is proportional to the surface area of a given cell and thus affords real-time recordings of fusion events on a single-cell level.^{45,46} *PI3Kδ^{+/-}* and *PI3Kδ^{-/-}* NK cells did not differ in membrane capacitance under basal conditions. However, during Ca^{2+} -triggered exocytosis, the surface area of *PI3Kδ^{+/-}* NK cells increased on average by 21%; in contrast, there was only a modest increase (on average by about 8%) in *PI3Kδ^{-/-}* NK cells. A general defect on exocytosis is furthermore suggested by the defect in IFN- γ secretion that might also contribute to the reduced cytolytic capacity of *PI3Kδ*-deficient NK cells (Figure S2). Degranulation in other cell types seems also to depend on *PI3Kδ*. We have evidence that CD8⁺ cells also require *PI3Kδ* for degranulation (E.Z., O.S., unpublished observations, August 2007). Others have recently reported an impairment of exocytosis in mast cells upon *PI3Kδ* inhibition or deficiency.⁴⁷

Our observations define a new key role for *PI3Kδ* in NK-cell function and tumor surveillance. The general importance of our

findings is underscored by the fact that comparable results were obtained upon transplantation of E μ -myc-derived leukemic cells and a subclone of the T-lymphoma cell line EL4. These leukemia models were chosen since both transformed cell lines express significant amounts of *PI3Kδ* and therefore also qualify as potential targets for *PI3Kδ* inhibitors. Besides hematopoietic cells, melanoma cells express *PI3Kδ*.³⁴ Consistent with our concept, surveillance of the murine melanoma cell line B16 was also impaired in *PI3Kδ*-deficient animals.

The repercussion of impaired NK cell-mediated tumor surveillance is worth considering: inhibition of *PI3Kδ* has been proposed to be an interesting option in the treatment of leukemia and our data do—in part—support this concept. However, our data clearly identify the possible source of a confounding effect. At the current stage, inhibition of *PI3Kδ* may still be a viable strategy because the dose-range required to block NK-cell functions is not necessarily the same as that causing inhibition of leukemia progression. In addition, *PI3Kδ* inhibitors might still represent a useful therapeutic option in patients where the leukemic cells have escaped detection by the immune system. However, our data clearly send a cautionary message: when evaluating the overall benefit of *PI3Kδ* inhibitors, the planning of clinical studies will have to consider both the inhibitory effects on NK cells and on tumor cells.

Acknowledgments

We thank K. H. Hilber, X. König, and B. Nürnberg for help with capacitance measurements and discussions. We are grateful to the staff of the Biomedical Research Institute (MUW) for taking care of mice and to J. N. Ihle for providing *PI3Kδ^{-/-}* mice. Selective *PI3Kδ* inhibitor IC87114 was generously provided by ICOS (Bothell, WA).

This work was supported by the Austrian Nationalbank (OeNP-11132), the Austrian Science Fund (SFB-28-10, SFB-F18-6), the Gen-AU Project DRAGON, and the Austrian Academy of Sciences (DOC-fForte fellowship to O.S.).

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

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CONCLUSIONS

NK cells present a lymphoid subset of immune cells mediating innate immunity. Moreover, NK cells shape and modulate the outcome of the adaptive immune response by producing cytokines such as IFN- γ and TNF- α . Recent evidence revealed that NK cells possess features of the adaptive immune system, including learning and memory (1). This functional pleiotropy led to the concept of distinct NK cell subsets with defined roles where diverse effector functions are achieved through NK cell diversification (2).

During the last decade, most studies concentrated on disclosing the mechanism of target cell recognition and described signaling pathways leading to NK cell effector functions. The complex process of NK cell differentiation occurring at various distinct tissue sites remains largely unknown as well as the transcriptional machinery behind. Several transcription factors have been implicated in NK cell development, particularly from the iNK cell stage on. Very recently, E4BP4 has been identified as key transcription factor that is selectively and critically required for early NK cell development (3). Transcription factors control cytokine responsiveness of NK cell precursors. Thereby, the cytokine IL-15 plays a crucial role in NK cell development even though the detailed mechanism by which IL-15 operates has not been elucidated.

Until now, one important limitation to study the role of transcription factors in NK cell biology has been the lack of a NK cell specific Cre line. The loxP Cre system is widely used to overcome the problems of a straight knock out mouse that often results in a severe phenotype, which precludes assigning the function of a target protein to a certain cell type.

The main project of my PhD study (see section 3.1), was to generate a novel mouse line that expresses the Cre recombinase under the control of the *Ncr1* promotor. This mouse line expresses the Cre recombinase exclusively in NK cells without affecting other lymphoid cells and allowed me to define the essential role of the transcription factor STAT5 for NK cells development and viability.

The transcription factor STAT5, activated by IL-2, IL-7 and IL-15, is an important regulator of B and T cell development (4-6). I could show that deletion of STAT5 in NK cells impaired NK cell development in the bone marrow. Any toxic effects of the Cre recombinase expression *per se* was ruled out since I performed a comprehensive analysis of NK cell development and effector functions of *Ncr1^{iCre}* mice *in vitro* as well as *in vivo*. Whereas *Ncr1^{iCre}* mice showed an unaltered NK cell development and function, I demonstrated a severe reduction of NK cells in the periphery of *Stat5^{f/f} Ncr1^{iCre}* mice. NCR1 becomes expressed at the iNK cell stage in the bone marrow. *Ncr1* induced expression of Cre recombinase and subsequent deletion of *Stat5* coincides with the disappearance of developing NK cells. At the iNK cell stage, the precursor cells in the bone marrow become greatly reduced in number. Real time PCR verified that the few NK cells remaining in the periphery embody escapers since they show unaltered *Stat5* mRNA levels. This data suggests that STAT5 is absolutely required for NK cell survival. Moreover, I infected purified splenic NK cells with an adenovirus encoding for the Cre recombinase thereby deleting *Stat5 in vitro*. Again, the deletion of *Stat5* led to the disappearance of NK cells confirming my observation *in vivo*. The fact that STAT5 regulates antiapoptotic genes such as *Bcl-x_L* and *Bcl-2* in B and T cells supports the theory of STAT5 being a critical survival factor for NK cells. However, I observed an accumulation of NKPs in the bone marrow of *Stat5^{f/f} Ncr1^{iCre}* mice. This is in line with the more immature NK cell phenotype observed in the remaining bone marrow NKs. These data suggest an additional role for STAT5, since the accumulation alludes to a developmental block at the NKP stage and indicates an important role for STAT5 in the transition to the iNK cell stage. For this transition, STAT5 might thereby be downstream of IL-15, which has been shown to be an essential fuel driving the differentiation from NKPs to the iNK cell stage. STAT5 may regulate either directly or indirectly E4BP4 determining the fate of NKPs in the bone marrow. Further studies are required to unravel the transcriptional network regulating NK cell differentiation.

The importance of NK cells in tumor surveillance has been previously shown *in vitro* as well as *in vivo* (7, 8). In a model using a B16F10 melanoma cell line, which is cleared by NK cells, I failed to observe functional NK cells in *Stat5^{f/f} Ncr1^{iCre}* mice, as expected considering my previous data. The severe reduction of NK cells, however, did not impact on the tumor surveillance of an adenomacarcinoma cell line called MC38 which is mediated by CD8⁺ T cells. Therefore, in this experimental setting, the absence of NK cells did not impair the adaptive arm of the immune system. It has been shown that STAT5 is essential for leukemic initiation and maintenance (9, 10). STAT5 might therefore be an attractive therapeutic target. However, the inhibition of STAT5

would be associated with a strong decrease in NK cell numbers and a severe reduction of NK cell mediated tumors. It will therefore be of great importance to evaluate which malignant diseases are subjected to NK cell mediated tumor surveillance.

During my PhD studies, I also contributed to a study investigating the roles of TRAIL and NK cells in tumor surveillance against ovarian cancer (see section 3.2). Activated NK cells express tumor necrosis factor related apoptosis inducing ligand (TRAIL) that recognizes TRAIL receptors on the target membrane (e.g. of a transformed cell) and induces apoptotic cell death. We could show that the combination of AD5-10 (TRAIL2 agonistic antibody) and carboplatin (a chemotherapy drug) induced an additive antitumor activity. Moreover, we demonstrated that depletion of NK cells from mice bearing tumors resulted in increased tumor growth and abolished the cytotoxic effect of AD5-10 (11). In summary, the data obtained in this study demonstrates an important role for NK cell mediated immunosurveillance in ovarian cancer by using death domain receptor mediated apoptosis. Therefore, the administration of STAT5 inhibitors would have negative consequences for the host NK cell mediated immunosurveillance in ovarian cancer.

Another study, to which I contributed, investigated the role of NK cells in leukemogenesis (see section 3.5). It has previously been shown that leukemia surveillance is at least partially mediated by NK cells (12, 13). We demonstrated that the intracellular signal transducer enzyme called phosphoinositide 3-kinase δ (PI3K δ) is involved in Abelson induced leukemia since the presence of PI3K δ accelerated leukemia progression *in vivo*. We also discovered that PI3K δ in NK cells is required for efficient degranulation of NK cells and thereby NK cell mediated leukemia surveillance. Transplantation studies revealed that PI3K $\delta^{-/-}$ mice succumbed more rapidly to cancer and are tumor prone. This study demonstrates a dual role of PI3K δ in Abelson induced leukemia progression that should be taken into consideration when developing a PI3K δ inhibitor as therapeutic strategy. Firstly, disease progression is inhibited upon PI3K δ deficiency by a tumor cell intrinsic effect. Secondly, lack of PI3K δ leads to impaired NK cell function and therefore to a higher tumor susceptibility.

As NK cells are capable to recognize and eradicate some types of leukemia and solid tumors very efficiently it will be of major importance to evaluate which malignant diseases are cleared by NK cells and which molecular inhibitor should be applied to use the therapeutic potential of NK cells.

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Selected Meetings

09/2010	12 th Meeting of the Society for Natural Immunity. Dubrovnik. Oral Presentation: A novel <i>Ncr1</i> -Cre mouse reveals the essential role of STAT5 for NK cell survival and development.
11/2009	EFIS-EJI Natural Killer Cell Symposium. Freiburg. Poster Presentation: Generation and analysis of transgenic mice expressing CRE recombinase specifically in NK cells. Eckelhart E., Casanova C., Sexl V.
05/2008	4. PhD Symposium MUW. Poster Presentation: The role of JAK2 in BCR-ABL induced leukemia. Eckelhart E., Hölbl A., Kovacic B. and Sexl V.
06/2007	3. PhD Symposium MUW. Poster Presentation: Generation of transgenic mice expressing CRE recombinase specifically in NK cells. Eckelhart E., Sexl V., Casanova E.

Scientific Publications

Eckelhart E, Warsch W, Zebedin E, Simma O, Stoiber D, Kolbe K, Rüllicke T, Mueller M, Casanova E and Sexl V.

A novel *Ncr1*-Cre mouse reveals the essential role of STAT5 for NK cell survival and development. Manuscript in revision (Blood)

Warsch W, Kollmann K, **Eckelhart E**, Fajmann S, Cerny-Reiterer S, Hölbl A, Gleixner K, Dworzak M, Sillaber C, Egger G, Valent P, Moriggl R and Sexl V.

STAT5 is an important mediator of imatinib resistance in Abelson-induced leukemia. Manuscript in Revision (Blood)

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