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The role of type I interferon in v-abl induced leukemia

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Für meine Eltern

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Abstract

The most important type I interferons (IFNs) are IFN-α and IFN-β, which are mainly produced after a viral infection and bind to a type II cytokine receptor, consisting of an IFNAR1 and IFNAR2 receptor chain. Type I IFNs have been shown to bridge the innate and the adaptive immune response by activating especially dentritic cells (DCs). Recently, IFNs have also been identified as critical coordinators of the interaction between the immune system and tumor cells.

In this study we investigated the impact of type I IFN signaling on B cell leukemia in mice. Our tumor model is the Abelson-induced leukemia. The human correlate of this disease is the bcr-abl induced chronic myeloid leukemia (CML) and acute lymphoid leukemia (ALL). Our laboratory has shown previously that IFNAR1-/- and IFN-β-/- mice succumb to B-lymphoid leukemia significantly faster than wild type controls after challenge with the Abelson oncogene. In addition, transplantation of Abelson transformed IFNAR1 and IFN-β knock out bone marrow cell lines into Rag2-/- mice lead to a faster progression of B-lymphoid leukemia than wt v-abl transformed cells.

Based on these findings we addressed the question whether the observed phenotype was due to a tumor cell intrinsic effect or due to a defective immune response. To do so, we crossed IFNAR1$^{fl/fl}$ mice with CD19cre+ mice to delete the IFNAR1 receptor chain of the type I IFN receptor specifically in B-lymphocytes. We infected newborn IFNAR1$^{fl/fl}$CD19cre+ mice and their wild type littermates with A-MuLV and found that IFNAR1$^{fl/fl}$CD19cre+ mice disease slightly faster than IFNAR1$^{fl/fl}$CD19cre- mice. This suggests that the faster progression of B-lymphoid leukemia is due to a tumor cell intrinsic effect. However, we could not confirm the hypothesis that wild type animals are less prone to develop Abelson-induced leukemia because wild type tumor cells produce constitutive low levels of IFN-β and thereby decelerate their own proliferation. When wt and IFNAR1-/- v-abl transformed cells were transplanted into Rag2-/-γ_c-/- mice both groups of recipient mice succumbed to leukemia with comparable latency. This finding indicates that impaired type I IFN signaling also influences Nk cell effector functions. During this study we also stroke a new path to elucidate the role of type I IFN signaling in v-abl induced leukemia. We started to analyze the impact of type I IFN signaling in angiogenesis and could show that tumor cells deficient in type I IFN signaling have an increased VEGF expression level and lead to faster migration of endothelial cells in scratch assays.

Taken together, this work demonstrates the importance of type I IFN signaling in Abelson-induced leukemia. The obtained data suggest that the combination of a tumor cell intrinsic effect and a defect in immune response is responsible for higher susceptibility to Abelson-induced leukemia in mice with impaired type I IFN signaling.

Zusammenfassung

Die wichtigsten Typ I Interferone sind IFN-α und IFN-β, welche hauptsächlich nach Virusinfektionen produziert werden und an den Typ II Zytokinrezeptor binden, der aus einer IFNAR1 und einer IFNAR2 Rezeptorkette besteht. Darüber hinaus wurde gezeigt, dass Typ I Interferone die angeborene und die adaptive Immunantwort verbinden, vor allem in dem sie dentritische Zellen aktivieren. Erst kürzlich hat man herausgefunden, dass Interferone wichtige Koordinatoren in der Interaktion von Immunsystem und Tumorzellen sind.

In dieser Studie haben wir den Einfluss von Typ I Interferon signaling auf B-Zell Leukämie in Mäusen untersucht. Das humane Korrelat dazu ist der replikationsinkompetente Abelson Mausleukämie Virus (A-MuLV). In unserem Labor wurde gezeigt, dass IFNAR1-/- und IFN-β-/- Mäuse signifikant schneller an B-lymphoider Leukämie sterben als die wildtyp Kontrollen nach einer einer Virusinfektion von neugeborenen Mäusen. Außerdem führt die Injektion von v-abl transformierten IFNAR1 und IFN-β knock out Knochenmarkzelllinien in Rag2-/- Mäuse zu einem schnelleren Fortschritt der B-lymphoiden Leukämie, als die Injektion von wildtyp v-abl transformierten Zellen.

Basierend auf diesen Erkenntnissen, haben wir uns die Fragen gestellt ob der beobachtete Phänotyp auf Grund eines Tumorzell intrinsischen Effektes oder auf Grund eines Defektes der Immunantwort herbeigeführt wird. Um diese Fragen zu beantworten, haben wir IFNAR1$^{fl/fl}$ Mäuse mit CD19cre+ Mäuse gekreuzt, um die IFNAR1 Rezeptorkette vom Typ I Interferonrezeptor speziell auf B-Lymphozyten zu deletieren. Wir haben neugeborene IFNAR1$^{fl/fl}$CD19cre+ Mäusen und ihren Wildtyp Wurfgeschwister mit A-MuLV infiziert und herausgefunden, dass IFNAR1$^{fl/fl}$CD19cre+ Mäuse etwas früher sterben als IFNAR1$^{fl/fl}$CD19cre- Mäuse. Dies deutet darauf hin, dass der schnellere Fortschritt der B-Zell Leukämie auf einem Tumorzell intrinsischen Effekt basiert. Jedoch konnten wir die Hypothese, dass wt Tiere weniger anfällig sind eine Abelson-induzierte Leukämie zu entwickeln, weil wt Tumorzellen ein konstitutives geringes Level an INF-β produzieren, nicht bestätigen. Transplantationsversuche mit Rag2-/-γ_c-/- Mäusen als Empfänger, haben gezeigt, dass Mäuse, injiziert mit wt Tumorzellen vergleichbar schnell an B-Zell Leukämie gestorben sind, als Mäuse die mit Knockout Tumorzellen injiziert wurden. Dieses Ergebnis liefert den Hinweis, dass beeinträchtigtes Typ I Interferon signaling die Effktor- Funktionen von Nk Zellen beeinflusst. Während dieser Studie haben wir außerdem einen neuen Weg eingeschlagen um die Rolle von Typ I Interferon signaling während einer v-abl induzierten

Leukämie aufzuklären. Wir haben begonnen die Auswirkung von Typ I Interferonen auf die Angiogenese zu analysieren und konnten zeigen, dass Tumorzellen mit fehlerhaften Typ I Interferon signaling verstärkt VEGF expremieren und dazu führen, dass Endothelzellen schneller zusammen wachsen in einem Scratch assay.

Zusammenfassend zeigt diese Arbeit die Wichtigkeit von Typ I Interferon signaling in der Tumorentstehung. Unsere Daten liefern einen Hinweis darauf, dass die Kombination eines Tumorzell intrinsischen Effektes und eines Defektes in der Immunantwort vielleicht verantwortlich ist für die höhere Anfälligkeit für Abelson-induzierte Leukämie in Mäusen mit beeinträchtigtem Typ I Interferon signaling.

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1. Introduction

1.1. Type I Interferons

50 years ago, interferons (IFNs) were originally described as soluble secreted molecules capable of interfering with viral infections (Aboagye-Mathiesen *et al.,* Isaacs *et al.*, 1957). IFNs are pleiotropic cytokines with important pro-inflammatory functions needed in the defense against bacterial and viral infections. Moreover, it has been realized that type I IFNs play a crucial role in promoting anti-tumor effects. The importance of type I IFNs in cancer immunosurveillance, immune homeostasis and immuno suppression has been shown previously and is well documented.

Up to now, several different IFNs have been described. They are divided into two main classes: type I IFN and type II IFN. IFN-γ is the only member of type II IFNs, whereas several type I IFNs have been discovered, including IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ξ and IFN-ω (Pestka *et al.*, 2004).

IFN-α is a family of approximately twenty structurally related proteins encoded by different genes. IFN-β is a single protein. All virally infected cells produce type I IFNs. IFN-α/β bind to the same receptor, called type I IFN receptor, and induce similar biologic responses. The receptor belongs to the type II cytokine receptor family, which consists of two heterologous polypeptides that are called IFNAR1 and IFNAR2. Binding of IFN-α/β activates the JAK/STAT pathway, which in turn activates transcription of the respective target genes (Kotenko *et al.*, 2003).

Two different ways are known how virally infected cells can produce type I IFNs: 1. Specific pathogen-associated molecular patterns expressed by viruses and microbes are recognized by Toll-like receptors (TLRs) (Tanabe *et al.*, 2003) (Akira *et al.*, 2003), which is also called the TLR-dependent pathway. 2. The TLR-independent pathway is mediated by RNA helicases called retinoic acid inducible gene-I (RIG-I) and melanoma differentiation associated gene 5 (Mda5) (Yoneyama *et al.*, 2005, Rothenfusser *et al.*, 2005) which recognize cytoplasmic viral RNA.

Figure 1 demonstrates the complex signaling pathways, which are activated upon a viral infection leading to the transcription of IFN-α/β. Either the TLR-dependent or TLR-independent recognition pathway activates IFN response factor (IRF)- kinases (IKK related

kinases) by phosphorylation of the transcription factors IRF-3 and IRF-7. In the early phase of an infection, only the constitutively expressed IRF-3 resides in the cytoplasm, which enters the nucleus after phoshorylation. In the nucleus, IRF-3 associates with co-activators and binds to IRF binding elements (or PRDI elements) within the IFN-β promoter. The binding leads to the expression of IFN-β, which in turn triggers the transcription of the transcription factor IRF-7. The IRF-7 gene contains ISRE sites (IFN stimulated response element) that respond to the IFN stimulated gene factor 3 (ISGF3), which is a tetrameric transcriptional activator consisting of signal transducer and activator of transcription 1 (STAT1), STAT2 and IRF9. This ISGF3-dependent IRF-7 transcription amplifies transcription of IFN-α and IFN-β genes followed by enhanced production of IFN-α and IFN-β (Marie *et al.*, 1998, Sato *et al.*, 1998). Thus it is called the positive feedback regulation of IFNs.

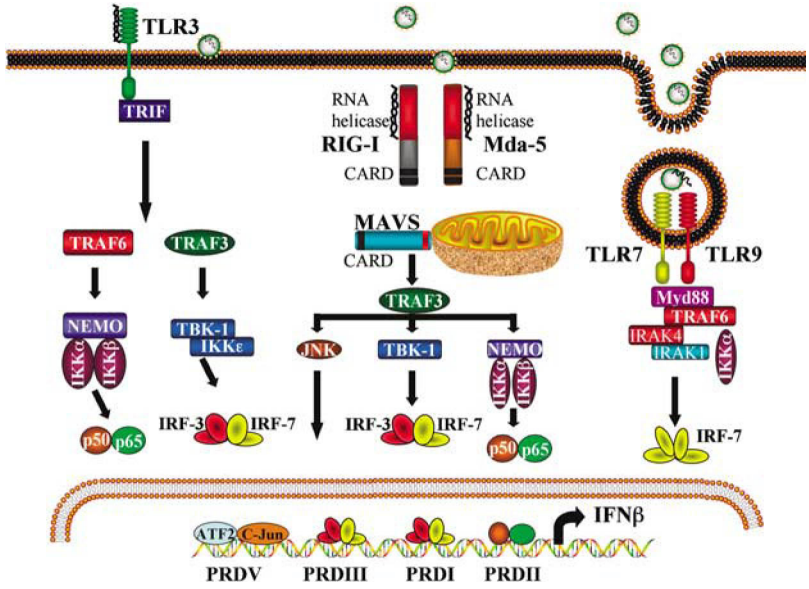

Figure 1. Summary of recognition pathways and downstream signaling leading to IFN-β production. Either TLRs (TLR3, TLR7 and TLR9) or cytoplasmic helicases (RIG-I and Mda5) are activated after a viral infection, which leads to the activation of several kinases and finally to the phosphorylation of transcription factors belonging to the IRF family (IRF-3 and IRF-7). Phosphorylated IRFs enter the nucleus and activate IFNβ transcription. (Figure taken from Oncogene, Hiscott et al., 2006).

Deonarain and his colleagues showed that mice deficient in the *IFN-β* gene were highly susceptible to a more aggressive tumor growth. It has been also shown that IFN-β-/- mice have reduced levels of circulating mature B and myeloid lineage cells. Moreover it was reported that in IFN-β deficient mice not only virally induced IFN-β, but also IFN-α production is abolished (Deonarain *et al.*, 2003) (Erlandsson *et al.*, 1998), indicating that the expression of IFN-α genes is at least partially dependent on IFN-β expression. The studies

revealed that IFN-α can be induced by IFN-β, but IFN-β cannot be induced by IFN-α (Asano *et al.*, 1990).

1.2. Type I IFN and the JAK/STAT Pathway

One very well studied pathway by which cytokines transduce signals and activate transcription of target genes involves kinases called Janus kinases (JAKs) and transcription factors called signal transducers and activators of transcription (STATS). The JAK/STAT pathway is important for cell-development, cell-differentiation and host defense mechanisms. The discovery of the JAK/STAT pathway resulted from biochemical and genetic analyses of IFN signaling (Darnell *et al.*, 1994).

Up to now there are four different JAKs known (JAK1, JAK2, JAK3, TYK2) that are named after the two-headed Roman god because of the presence of two kinase domains – only one of which is active. JAKs are non-receptor tyrosine kinases loosely attached to the cytoplasmatic domains of their respective cytokine receptors. Upon ligand binding, two receptor molecules are brought together and the receptor-associated JAKs are activated by transphosphorylation, and they phosphorylate tyrosine residues in the cytoplasmic domain of the receptors. Cytosolic STAT proteins bind via their Src homology 2 (SH2) region to the phosphotyrosine residues of the receptor and get activated. The SH2 domain of one STAT can now bind the phosphotyrosine residue of another STAT and the dimer dissociates from the receptor. As a result, STAT dimers migrate to the nucleus, where they bind promotor regions of cytokine-responsive genes and activate transcription. Janus kinases are structurally divided into an N-terminal Ferm domain that binds to the respective cytokine receptor chain, followed by a SH2 domain, a catalytically inactive pseudokinase domain and a C-terminal tyrosine kinase domain (O'Shea *et al.*, 2002) (Imada *et al.*, 2000), as shown in Figure 2.

So far there are 7 different STAT proteins known (STAT1-STAT4, STAT5A, STAT5B, STAT6). STAT1 and STAT2 were the first members identified as mediators of the cellular response to IFNs. Each STAT protein responds to different extracellular stimuli and leads to another gene transcription, but they share a characteristic structure. The N-terminus is important for STAT dimerisation, followed by a coiled-coil (cc) domain that is involved in protein-protein interactions. STATs bind to the DNA in the nucleus via their DNA-binding domain (DBD) and to cytokine receptors and other STAT proteins, respectively via their SH2 domain. As shown in Figure 2B STATs have a conserved single tyrosine residue that is the site of phosphorylation after activation. The C-terminus is called the transactivation domain (TAD) and plays an important role in transcriptional activation (Reich *et al.*, 2006).

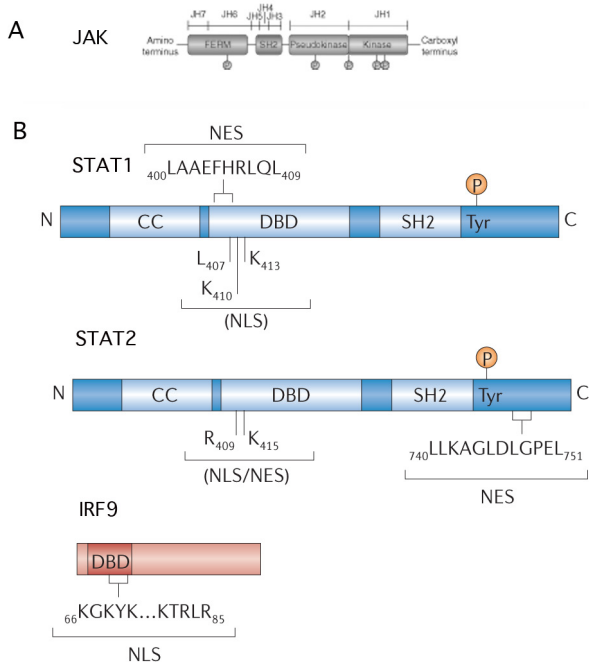

Figure 2. Proteins involved in type I IFN signaling. (A) JAK consists of a N-terminal FERM domain followed by a SH2 domain and 2 kinase domains (Yamaoka et al., 2004) **(B)** The structure of STAT1 and STAT2 shows characteristic coiled- coil domains, followed by a DNA-binding domain and a SH2 domain. Together with IRF9 they form the ISGF3 complex that enters the nucleus (Nancy C. Reich, Ling Liu, 2006).

As mentioned above, IFN-α and IFN-β are produced upon viral infections. In order to mediate an early innate immune response, the cytokines bind to the type I interferon receptor that is composed of IFNAR1 and IFNAR2. IFNAR1 is associated with TYK2 and IFNAR2 with JAK1 that phosphorylate STAT1 and STAT2. Phosphorylated STAT2 and STAT1 proteins form STAT1/STAT2 heterodimers and dissociate from the receptor. Binding of the transcription factor IRF9 in the cytosol enables them to enter the nucleus as the ISGF3 complex. In the nucleus, the ISGF3 complex binds to IFN stimulated response elements (ISRE) and activates transcription of target genes, which are called IFN stimulated genes (ISGs) (Dunn *et al.*, 2006). In addition STAT1 homodimers are also formed and enter the nucleus, where they bind to promoters at the IFNγ-activated site (GAS) (Decker *et al.*, 1991) (shown in Figure 3).

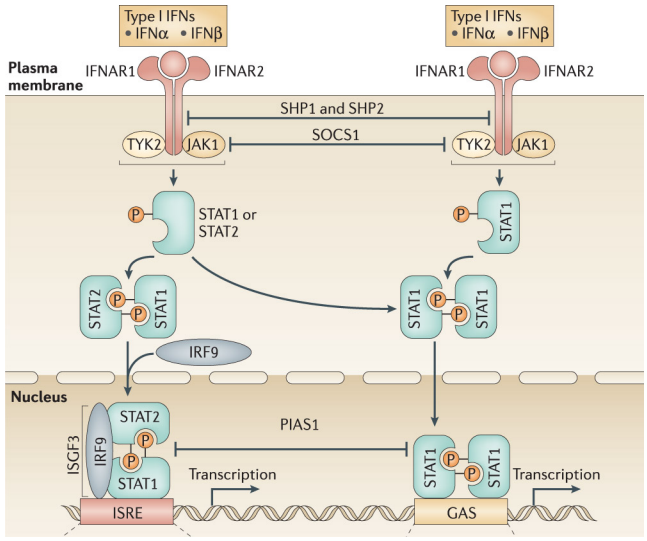

Figure 3. Scheme of type I IFN signaling. Binding of IFN-α/β leads to receptor dimerisation and phosphorylation of JAKs, which in turn phosphorylate STAT1 and STAT2. Phosphorylated STAT proteins form heterodimers and bind IRF9, forming the ISGF3 complex. The ISGF3 complex further enters the nucleus and binds to ISRE elements thereby starting gene transcription. In addition STAT1 homodimers also form which enter the nucleus and bind to promoters at the IFNγ-activated site (GAS) (This figure was taken and modified from Dunn et al., 2006).

Binding of ISGF3 leads to transcription of IRF-3 and IRF-7 maintaining the auotcrine positive feedback loop as mentioned above. Different genes involved in dentritic cell activation, T-cell survival, natural killer-cell activation, chemokine expression, lymph-node retention and anti-proliferative and anti-viral effects are expressed following type I IFN signaling. Binding of STAT1 homodimers to GAS sites is crucial for IRF-1 expression as well as the expression of different genes involved in MHC class I and class II regulation, chemokine expression, T_{reg}-cell inhibition, T_H1-cell differentiation, CTL activation and differentiation and antiproliferative effects (Dunn *et al.*, 2006).

Several controlling mechanisms of type I IFN signaling are involved in appropiate signal transduction: (1.) SHP-mediated de-phosphorylation of the receptor complex, including JAKs and STATs, (2.) SOCS mediated inhibition of the JAKs, (3.) PIAS mediated inhibition of STATs and (4.) proteasomal degradation of the JAKs are also required for proper type I IFN signaling (also shown in Figure 3) (Dunn *et al.*, 2006).

During this study we used IFNAR1-/- and IFN-β-/- mice, impaired in different steps of type I IFN siganling. Different studies have shown that IFNAR1-/- as well as IFN-β-/- mice were extremely susceptible to different types of viral infections. In IFN-β deficient mice not only

viral-induced IFN-β but also IFN-α production is abolished (Hwang *et al.*, 1995) (Muller *et al.*, 1994) (Erlandsson *et al.*, 1998).

1.3. Effects of Type I IFNs on the Immune System

As mentioned above one function of IFNs is to interfere with viral RNA or DNA and viral replication. There are two signalling ways how cells combat infections with a pathogen: (1) Infected cells produce type I IFN to protect non-infected neighbouring cells. The neighbouring cell that responds to IFN is therefore resistant to viral infection. These cells are further said to be in an "antiviral state". (2) Infected cells also combat in an autocrine way by inhibiting viral replication in the infected cell. Thus, the IFN system is a huge barrier against viral multiplication in the infected host (Abbas K., 2009).

It has been shown that IFN-α/β have also an immunemodulatory effect on essential effector cells in the innate immune system. They are important for the activation of natural killer (NK) cells, macrophages and dentritic cells (DCs). The identification of a unique subset of DCs, called plasmacytoid DCs that were found to produce huge amounts of IFNα/β, showed that IFNs are essential cytokines bridging the innate and adaptive immune systems (Takaoka *et al.*, 2006).

As outlined above, an autocrine positive feedback loop leads to massive synthesis of IFN-α/β after viral infection. Several groups have reported that there is evidence for a constitutive expression of IFN-α/β under normal physiological conditions, although at a very low level (Bocci *et al.*, 1985) (Tovey *et al.*, 1987). The presence of a constitutive low level of IFN was detected in mouse embryonic fibroblasts (MEFs), splenocytes and bone marrow cells and was described to respond very fast to rapid environmental changes, such as viral infection. It has become clear that weak IFN-α/β signaling plays a crucial role in IFN-γ and interleukin (IL)-6 response. It has also been shown that the constitutive expression of IFN-α/β plays an important role in maintaining the tyrosinephosphorylation of the IFNAR receptor chains which allows STAT proteins an efficient dimerization upon stimulation with IFN-α/β (Takaoka *et al.*, 2000),(Mitani *et al.*, 2001).

Thus constitutive, weak IFN-α/β signaling renders cells "ready to go" for cellular response and therefore also plays a role in the regulation of the adaptive immune response. Figure 4 demonstrates the hypothetic model of the "ready to go" or also called "revving-up" system regulated by low levels of IFN (Taniguchi *et al.*, 2001), (Takaoka *et al.*, 2006).

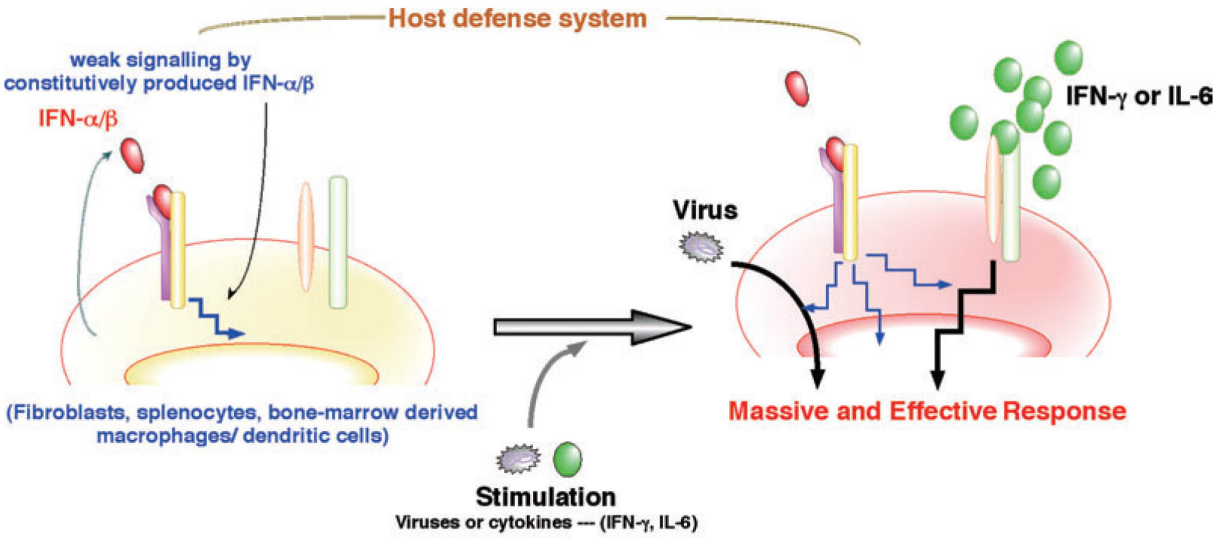

Figure 4. Model of the „revving-up system" regulated by weak IFN-α/β signaling. Constitutive expression of low levels of IFN-α/β enables cells to a fast response upon a viral stimulus. (This figure is taken and modified from Takaoka and Yanai, 2006)

1.4. Type I IFN and Cancer-Immunosurveillance

The immune system is not only able to distinguish between self and pathogen or self and non-self, but between self and transformed self. This concept resulted from studies with immunodeficient mice. Immunodeficient Rag2-/- mice, which completely lack natural killer T (NKT), T and B cells, developed more spontaneous tumors than their immunocompetent counterparts and enforced the hypothesis of cancer-immunosurveillance, which postulates that the immune system protects the host against the development of cancer. Cells of the innate and the adaptive immune system recognize transformed cells and eliminate them (Smyth *et al.*, 2006).

Further observations that tumors, derived from immunocompetent mice that had to combat an intact immune system, have a different immunogenic phenotype than tumors, derived from immunodeficient mice, lead to the model of cancer immunoediting (Dunn *et al.*, 2004). Many studies in mice and also humans indicated that the immune system can interfere with tumor progression, at least in part, by sculpting the immunogenic phenotype of tumors as they develop. The model of cancer immunoediting proposes that there are three phases of tumor initiation and further progression in a host. The first phase is equivalent with cancer immunosurveillance and is called elimination phase. The innate as well as the adaptive immune system recognize a transformed cell and eliminate it. But if some cells are not killed they proceed to the second or equilibrium phase. In this stage tumor cells remain in the host but are not able to disperse due to the immune pressure. But when tumor cells overcome the immune system they can proliferate which finally leads to tumor progression. Because tumor cells could escape the immune system, the third phase is called escape phase (Figure. 5) (Dunn *et al.*, 2004).

It has been shown recently that IFNs have a critical role in the elimination phase of cancer immunoediting. Both, type I IFNs and IFNγ, play important roles in promoting host antitumor immunity. It has been reported that neutralization of IFN-α/β with polyclonal antiserum enhances the growth of transplanted, syngeneic (genetically identical and immunologically compatible) tumors (Gresser *et al.*, 1983). Dunn and colleagues used models for tumor transplantation and primary tumor formation to show the critical function of endogenously produced IFN-α/β in cancer immunoediting. They found that endogenously produced IFN-α/β is required for rejection of highly immunogenic MCA sarcomas (MCA= methylcholantrene, a chemical carcinogen that induces sarcomas after subcutaneous

injection). Interestingly, IFNs do not mediate their antitumor activity on tumor cells but on hematopoietic cells of the host (Dunn *et al.*, 2005).

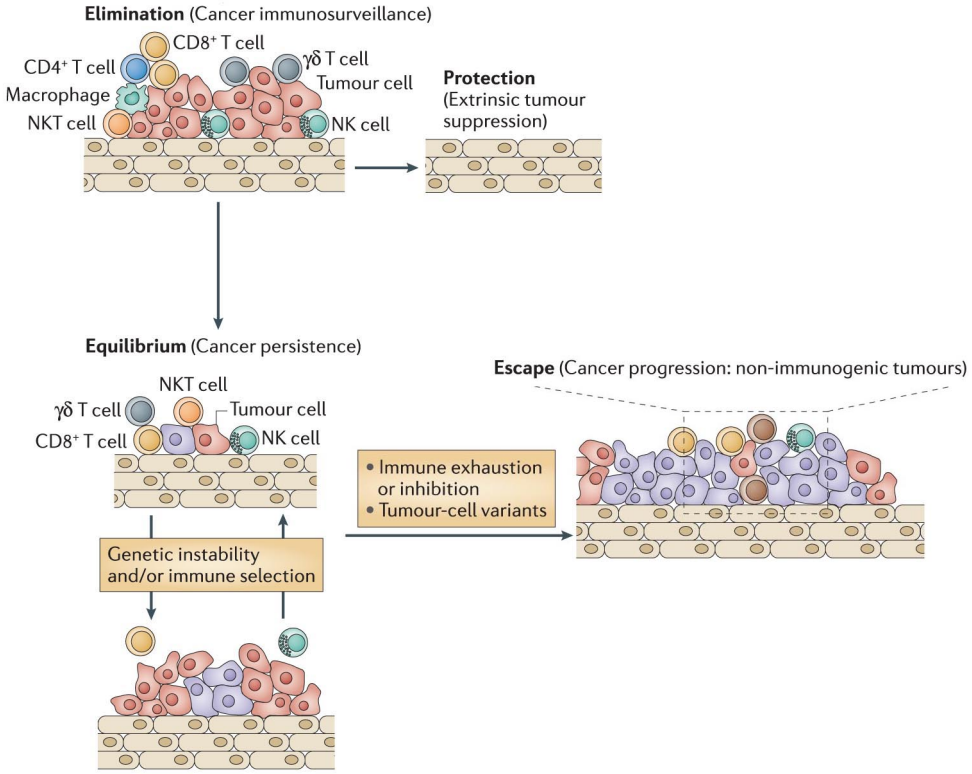

Figure. 5. Model of cancer immunoediting. There are 3 phases involved in cancer immunoediting: the first phase is called elimination phase. Transformed cells are recognized and killed by the innate and adaptive immune system. Cells that survive enter the equilibrium phase where they persist but cannot proliferate due to immune pressure. The third phase is called escape phase and starts when tumor cells overcome the immune system. (This figure is taken and modified from (Dunn *et al.*, 2006)).

Besides their important role in cancer immunosurveillance type I IFNs might play also an antitumorgenic role in the process of cell transformation. Type I IFNs were found to up-regulate the expression of the tumor suppressor protein p53 and IFN-β was reported to inhibit the *in vitro* transformation of wt mouse embryonic fibriblasts (MEFs) (Takaoka *et al.*, 2003).

1.5. Effects of Type I IFNs on Natural Killer (Nk) Cells

As outlined above, there is broad evidence that type I IFNs are essential in bridging the innate and the adaptive immune system. Interestingly, they do not only act on immune cells but also interfere with tumor cells. Type I IFNs have been found to inhibit the proliferation of tumor cells and to support the immune response by enhancing the cytolytic activity of Nk cells (Gutterman, 1994, Kirkwood, 2002).

Nk cells are part of the innate immune system and recognize infected, transformed or stressed cells. They are present mainly in blood and the spleen and are rare in other lymphoid organs. Nk cells have several activating and inhibiting cell surface receptors. The balance between activating and inhibiting signals regulates Nk cell activation. Inhibitory receptors bind to the major histocompatibility complex (MHC) class I molecules. Activating receptors bind various activating ligands expressed on target cells. Tumor cells often downregulate MHC class I expression to escape cytotoxic T lymphocytes (CTLs). However, downregulation of MHC class I on a target cell leads to its recognition by the Nk cell (Abbas K., 2009).

Type I IFN signaling has been shown to play a critical role in Nk cell regulation. Swann and his colleagues again used IFNAR deficient mice to demonstrate the essential role of type I IFNs in the regulation of Nk cell numbers, activation and antitumor activity. They could show that Nk cell numbers were diminished and the Nk cell mediated tumor response was decreased in IFNAR deficient mice (Swann *et al.*, 2007). As mentioned above, constitutive expression of low levels of IFN-α/β might be a source of IFN for maintaining the Nk cell population. Overall, further studies are required to elucidate the mechanism how type I IFNs influence Nk cell homeostasis.

1.6. Type I IFNs and Angiogenesis

Normal organ growth and development relies on blood supply that depends on endothelial cells, which form vascular walls. A new blood capillary forms by sprouting of an endothelial cell from the wall of an existing small vessel. This process is called angiogenesis and the role of angiogenesis in human tumor progression was originally postulated by Judah Folkman in 1971 (Folkman, 1971). Furthermore it has been recognized that inhibition of angiogenesis could be a useful means of solid tumor therapy. It has also been shown that angiogenesis might be involved in hematological malignancies, such as acute lymphoblastic and myeloid leukemias (ALL and AML) (Bauvois *et al.*, 2002) or B-chronic lymphocytic leukemia (B-CLL) (Kini *et al.*, 2000). Since the important role of the vascular endothelial growth factor (VEGF) in angiogenesis has been realized, there are also studies that demonstrate that VEGF triggers growth, survival and progression of leukemic cells. These observations suggest that angiogenesis is not only crucial in solid tumor formation but also in the pathogenesis of hematologic malignancies and metastatic spread through the lymphatic vasculature (Podar *et al.*, 2005) as illustrated in Figure 6.

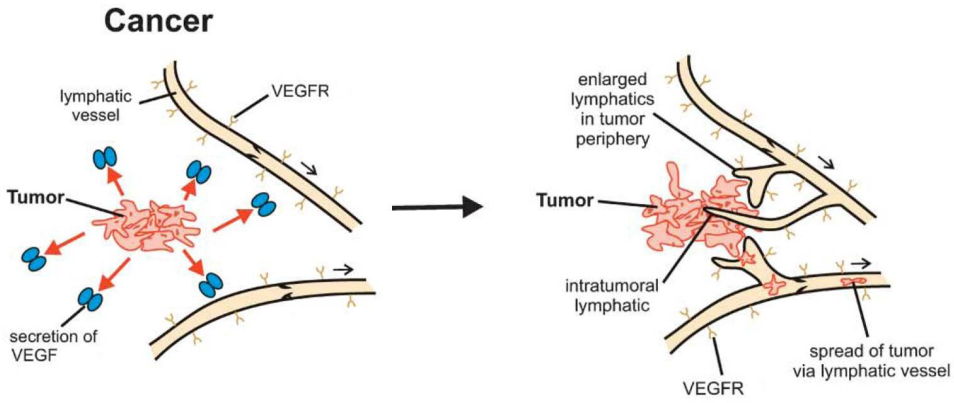

Figure 6. Schematic overview of lymphangiogenesis involved in tumor formation and metastastatic spread. Tumor cells secrete VEGF that binds to corresponding VEGF receptors on lymphatic vessels, which leads to the formation of new vessels that support tumor growth and spreading of tumor cells. (This picture was taken and modified from (Achen, 2005).

During the last several years a number of molecular tools helped to identify different pro-angiogenic and anti-angiogenic factors that either stimulate or inhibit proliferation of endothelial cells. The shift in balance between the production of anti-angiogenic and pro-angiogenic factors is called an angiogenic switch and is crucial in promoting tumor growth (Hanahan *et al.*, 1996). O'Reilly and his team discovered the inhibitors of angiogenesis angiostatin and endostatin. Injection of these inhibitors into mice showed that solid tumor growth and formation of metastasis was suppressed (O'Reilly *et al.*, 1994b, O'Reilly *et al.*, 1994a, O'Reilly *et al.*, 1997).

Even though little is known about type I IFNs and its impact on leukemia it is published that tumor cells treated with IFN-α/β *in vitro* and in the mouse model system *in vivo* were significantly less competent to initiate angiogenesis. This effect results from the anti-proliferative function of type I IFNs (fewer tumor cells initiate less angiogenesis) and they are supposed to inhibit the production and release of pro-angiogenic factors from the tumor cell or lymphocytes (Sidky *et al.*, 1987). Other studies have demonstrated the anti-angiogenic function of type I IFNs by inhibiting the production of the angiogenic growth factor basic fibroblast growth factor (bFGF) (Singh *et al.*, 1995), vascular endothelial growth factor (VEGF) (Rosewicz *et al.*, 2004) and interleukin-8 (IL-8) in human melanoma cells (Singh *et al.*, 1996). Type I IFNs inhibit angiogenesis by preventing endothelial cell differentiation. IFN-β treated endothelial cells are able to form new, small vessels but fail to form a complex capillary-like network (Taylor *et al.*, 2008). Furthermore matrix metalloproteinase-9 (MMP-9) has been identified as an important factor in angiogenesis. MMP-9 is capable of degrading type IV and V collagens, laminin and fibronectin, which are major components of the extracellular matrix (ECM). Proteolytic degradation of the ECM is also an important step in angiogenesis. A study on B-CLL patient samples revealed that type I IFNs suppress the production of MMP-9, indicating the importance of type I IFNs in inhibiting tumor angiogenesis (Bauvois *et al.*, 2002). Furthermore it has been reported that human bcr-abl$^+$ cells, derived from CML patients, as well as murine cells, transfected with the p185, p210 and p230 BCR-ABL tyrosine kinase versions, secrete angiogenic factors (VEGF, IL-8, HGF and FGF-2) and MMPs (MMP-9 and MMP-2) (Janowska-Wieczorek *et al.*, 2002). Moreover, leukemic cells express the pro-angiogenic factor VEGF as well as the VEGF receptor, which results in the generation of an autocrine loop that supports the proliferation and survival of leukemic cells (Kay *et al.*, 2002, Dias *et al.*, 2000, Wegiel *et al.*, 2009).

1.7. Abelson Induced Leukemia

The ABL family of proteins encodes tightly regulated non-receptor tyrosine kinases. One member is the ABL protein. Usually the cellular ABL tyrosine kinase (c-ABL) activity is tightly regulated and cannot transform fibroblasts and hematopoietic cells, even when it is over-expressed (Kruh *et al.*, 1986, Wang *et al.*, 1984). The c-ABL protein can be localized in the nucleus and in the cytoplasm. It has been postulated that c-ABL serves as cell growth promoter as well as cell growth inhibitor, depending on various circumstances. Van Etten and co-workers have shown that c-ABL on one hand stimulates the transcription of S-phase genes, while on the other hand c-ABL has been described to inhibit cell growth in the G1 phase of the cell cycle. There is also evidence that c-ABL modulates cell response to genotoxic stress as well as it influences the cytoskeleton and neuronal development (Van Etten, 1999).

Abl-deficient mice are variably affected. They are smaller, with abnormal head and eye development and often die perinatal (Ren, 2005). Mutations in the *abl* gene lead to a constitutively active ABL tyrosine kinase that is mostly cytosolic and always associated with transformation (Rosenberg *et al.*, 1988). ABL oncoproteins include v-abl (which is retrovirally transduced) and the translocation products BCR-ABL and TEL-ABL. Those oncoproteins are known to activate several different signaling pathways constitutively, including Ras, Myc, PI3-kinase and the JAK-STAT pathway (Danial *et al.*, 2000).

In our study we used the replication incompetent mouse pathogenic Abelson retrovirus, which is reported to induce a slowly progressing murine leukemia with lymphoblastic infiltrations of the lymph nodes, bone marrow and spleen (Rabstein *et al.*, 1971).

1.7.1. The BCR-ABL Fusion

The generation of a BCR-ABL fusion protein leads to malignant outcomes in humans. Acute lymphoblastic leukemia (ALL) and chronic myeloid leukemia (CML) are the consequences of reciprocal or balanced translocation between the 5' fragment of the *bcr* gene, located on chromosome 22 and the 3' portion of the *abl* gene, located on chromosome 9. This translocation leads to a shortend chromosome 22, which results in the so-called Philadelphia chromosome (Nowell *et al.*, 1960) shown in Figure 7. The *bcr-abl* fusion gene encodes a constitutively active tyrosine kinase that is involved in different signaling pathways as mentioned above.

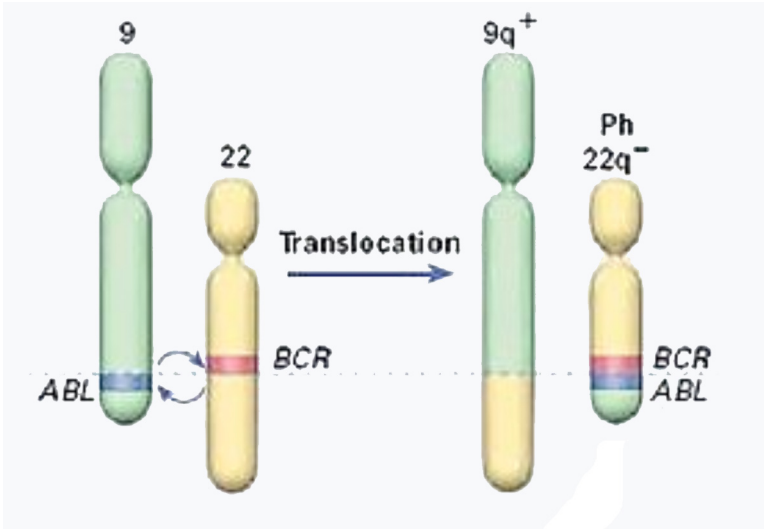

Figure 7. Overview of the occurring reciprocal translocation of chromosome 9 and chromosome 22. The translocation of the *abl* gene, located at 9q34, and the bcr gene, located at 22q11, yields in the formation of the Philadelphia chromosome (Ph22q-). The translocation 9q+ has no known phenotype. (This picture was taken and modified from http://a0.vox.com/6a00d10a7a748d8bfa00e398a3cdf80001-500pi)

The structures of the ABL tyrosine kinase and the BCR signaling protein have been studied extensively. It is known that there are two isoforms of ABL (human type 1a and 1b), which result from alternative splicing of Exon 1. As shown in Figure 8 isoform 1b has a myristoylation modification that can attach the ABL protein to a membrane protein. Futhermore, ABL contains tandem SRC homology 3 (SH3), SH2 and the tyrosine kinase domain. At the C-terminal end there are four prolin-rich SH3 binding sites (PPs), three nuclear localizations signals (NLS), one nuclear export signal (NES), a DNA binding domain (DBD) and an actin-binding domain located. The BCR protein contains a coiled-coiled (cc) oligomerization domain, a serine/theronin (S/T) kinase domain, a Dbl/CDC24 guanine-

nucleotide exchange factor homology (DH) domain, a calcium-dependent lipid binding site, a RAC guanosine triphosphate-activating protein (RAC-GAP), a binding site for growth-factor receptor bound protein 2 (GRB2) as well as for GRB10 and the ABL proteins. Different forms of the BCR-ABL protein with different molecular weight can be generated: p185 BCR-ABL, p210 BCR-ABL and p230 BCR-ABL. The three proteins are associated with ALL, CML and a milder form of CML (Ren, 2005).

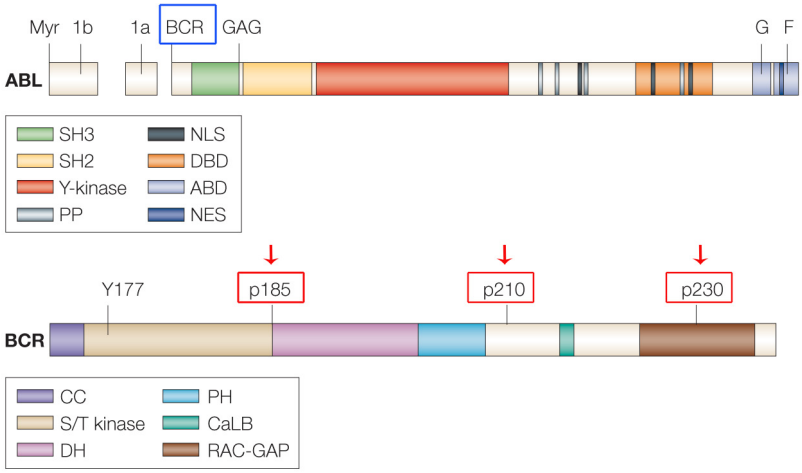

Figure 8. Structure of the ABL and BCR protein. ABL: The blue border indicates the fusion region with the *bcr* gene. Myr, myristoyl modification; SH3 and SH2 domain; tyrosine kinase domain; PP, proline rich SH3-binding domain; NLS, nuclear localization signal; DBD, DNA-binding domain; ABD, actin-binding domain; NES, nuclear export signal. **BCR:** Red borders indicate the different regions on the chromosome that can fuse with the *abl* gene, yielding in different BCR-ABL proteins. cc, coiled-coiled domain; S/T kinase, serine/threonin kinase; DH, Dbl/CDC24 guanine-nucleotide exchange factor homology; PH, pleckstrin homology domain; CaLB, calcium-dependent lipid binding site; RAC-GAP, RAC guanosine triphosphate-activating protein. This figure is modified and taken from (Ren, 2005).

As demonstrated in Figure 9 BCR-ABL proteins can form dimers or tetramers by binding of their cc domains and further trans-phosphorylate two tyrosine residues. Trans-phosphorylation initiates downstream leukemogenic signaling (Ren, 2005).

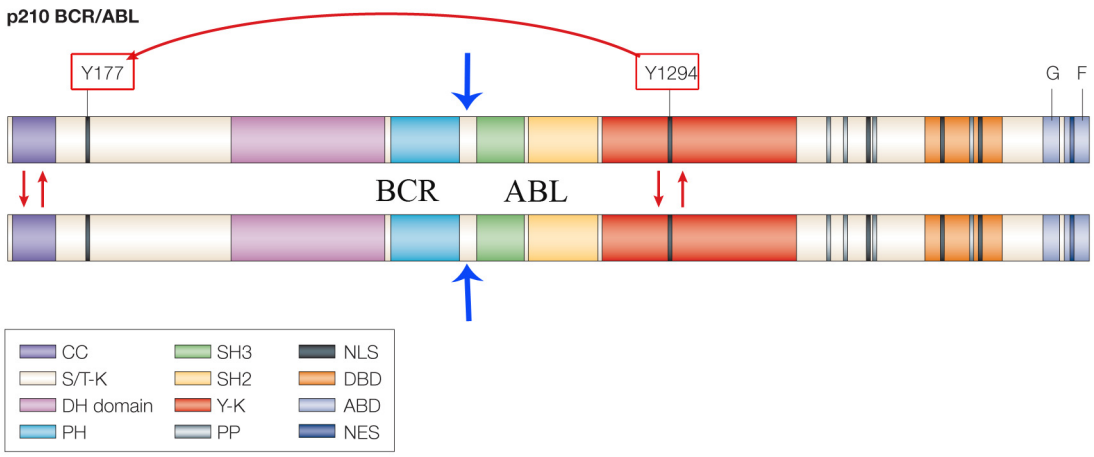

Figure 9. Structure of the BCR/ABL p210 fusion protein. BCR/ABL proteins form dimers or tetramers by interaction of their cc domains, followed by trans-autophosphorylation of two tyrosine residues indicated in the red border. Autophosphorylation leads to a constitutively activated tyrosine kinase function of the BCR/ABL fusion protein and triggers downstream signaling. Blue arrows indicate the fusion point of the *bcr* gene and the *abl* gene. This Figure was modified and taken from (Ren, 2005).

The expression of the BCR-ABL tyrosine kinase in self-renewing, hematopoietic stem cells is responsible for the onset of CML. CML progresses through 3 phases starting with an initial chronic phase. The chronic phase has a median duration of 3-4 years. In this phase there are fewer then 5% blasts in the blood and bone marrow but the expansion of the granulocytic cell type is characteristic. Most cases of CML are diagnosed at this phase and patients in the chronic phase of CML have a good prognosis after therapeutic actions. In the following accelerated phase the number of blasts increases up to 15% and this phase can last weeks to month. Often an additional abnormality leads to the onset of the third phase of CML, called the acute blastic phase or blast crisis that is comparable to the clinical pattern of ALL and in which more than 30% of cells are blasts. At this point, CML has become an aggressive fast growing leukemia, which results in a poor prognosis for patients.

1.7.2. The Abelson virus (A-MuLV)

In 1070 Abelson and Rabstein observed in an infection experiment with Moloney murine leukemia virus (Mo-MuLV), a virus with well-characterized thymus tropism (Moloney, 1960), one mouse that developed an unusual lymphosarcoma. Thereby they isolated an acute transforming retrovirus, named Abelson Murine Leukemia Virus (A-MuLV) that was capable of inducing multiple tumors of the lymph nodes with bone marrow infiltrations in the mouse (Abelson *et al.*, 1970, Rabstein *et al.*, 1971). Further studies demonstrated that the virus was able to transform fibroblasts (Scher *et al.*, 1975) and also cells derived from lymphoid tissues with 10-100-fold greater efficiency *in vitro* (Rosenberg *et al.*, 1975).

Studies concerning the molecular structure of A-MuLV revealed that it originates from the recombination of the Mo-MuLV with the murine *c-abl* gene generating a fusion gene containing Mo-MuLV *gag* and a truncated version of the endogenous murine *c-abl* gene that was named *v-abl* (Shore *et al.*, 2002). Figure 10 demonstrates a schematic overview of the viral transduction event.

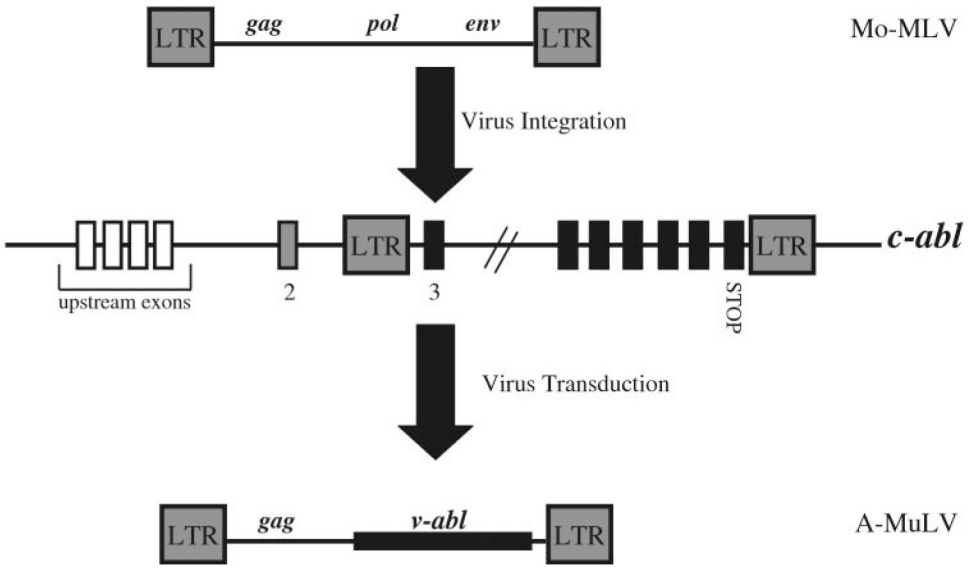

Figure 10. Schematic diagram of the integration of the Mo-MuLV *gag* into the murine *c-abl* gene. The replication competent murine leukemia virus Mo-MuLV integrates into Exon 3 of the endogenous murine *c-abl* gene yielding in the replication incompetent A-MuLV encoding *v-abl*. This picture was taken from (Shore *et al.*, 2002).

It has been shown that the size of the *v-abl* gene product can range between 90-160 kDa. $p120^{v\text{-}Abl}$ and $p160^{v\text{-}Abl}$ have been studied extensively and have been identified as plasma membrane-associated non-receptor tyrosine kinases. $p120^{v\text{-}Abl}$ was shown to phosphorylate itself as well as tyrosine residues of exogenous substrates. Infection of hematopoietic cells results in the abrogation of cytokine-dependent growth and the disability to differentiate in response to cytokines. (Shore *et al.*, 2002).

As mentioned above *in vitro* A-MuLV transfects pre-B cells and fibroblasts, whereas *in vivo* pro-B cells are the major target of A-MuLV (Witte, 1986). After transfection, pro-B cells are not able to differentiate, which results in the accumulation of immature B cells, which are characterized by B220, CD19 and CD43 cell surface markers, and further the development of B-lymphoid leukemia in mice.

1.8. Aim of this Master Thesis

The reciprocal translocation between the *abl* gene on chromosome 9 and the *bcr* gene on chromosome 22 yields a *bcr-abl* fusion gene that expresses a constitutive active tyrosine kinase. One of the activated pathways downstream of BCR-ABL is the JAK/STAT pathway, which is also activated by type I IFNs.

As outlined in the introduction, IFNs do not only play a crucial role in the defence against viral infections but were also shown to have a critical role in the elimination phase of cancer immunoediting. Several studies have demonstrated the importance of type I IFNs in promoting host anti-tumor immunity. The possible antitumor activity of type I IFNs in v-abl induced B-lymphoid leukemia has been investigated previously in our lab. In these studies, mice deficient for type I IFN signaling, namely IFNAR1-/- and IFN-β-/- mice, were used. IFNAR1-/- and IFN-β-/- bone marrow cell lines were retrovirally transformed with the v-abl oncogene and displayed no significant differences in proliferation and apoptosis *in vitro*. When newborn mice were infected with the Abelson virus IFNAR1 as well as IFN-β deficient mice succumbed to B-lymphoid leukemia significantly faster than their wt controls. Furthermore bone marrow transplantation of v-abl transformed cell lines into immuncompromised Rag2-/- mice revealed that lack of IFNAR1 and IFN-β significantly increased leukemia formation (Neugebauer, 2007).

The aim of this study was to clarify whether these previous observations are caused by tumor cell intrinsic (cell-autonomous) properties or by a defect in the immune system. Therefore, $IFNAR^{fl/fl}$CD19cre+ mice were used to delete the IFNAR1 chain of the type I IFN receptor specifically in B-lymphocytes. We performed newborn infections with $IFNAR^{fl/fl}$CD19cre+ mice and their wildtype littermates to test whether the lack of type I IFN signaling in B cells would lead to faster progression of B-lymphoid leukemia.

Furthermore, we wanted to investigate the basal level of IFN-β in v-abl transformed tumor cells. A constitutive low level of type I IFN has been detected in mouse embryonic fibroblasts (MEFs), splenocytes and bone marrow cells that enables cells to a very fast response to rapid environmental changes, such as viral infection. We therefore addressed the question whether v-abl transformed wt tumor cells might also produce a low level of IFN-β that inhibits leukemia progression and leads to the above-mentioned phenotype in transplantation experiments with Rag2-/- mice.

Another aim of this master thesis was to study the effect of type I IFNs on angiogenesis. We injected Rag2-/-γ_c-/- mice, which lack functional Nk, T and B cells, subcutaneously and looked for vascularisation. Rag2-/-γ_c-/- mice are particularly suited for injection experiments because cell autonomous properties are not affected by a functional immune system. Furthermore we analysed v-abl transformed cell lines for differences in expression levels of genes important in angiogenesis. Thus we hoped to identify genes affected by type I IFN signaling and to investigate the mechanism underlying the inhibitory function of type I IFNs in angiogenesis.

2. Experimental Procedures

2.1. Cell Culture

2.1.1. Culture conditions

All cell lines were kept at humidified 37°C and 5% CO_2. V-abl transformed cell lines were maintained in RPMI-1640 medium (PAA Laboratories) containing 10% heat inactivated FCS (PAA Laboratories), 100 U/ml penicillin/streptomycin (PAA Laboratories) and 5 µM beta-mercaptoethanol (Sigma). Raw cells were cultured in DMEM High Glucose (4.5g/l) medium (PAA Laboratories) containing 10% heat inactivated FCS (PAA Laboratories) and 100 U/ml penicillin/streptomycin (PAA Laboratories). For splitting RAW dissociation buffer (1.35 M KCl, 0.15 M sodium citrate, sterile H_2O) was used. 54C12 producer cells were cultured in DMEM High Glucose (4.5g/l) medium (PAA Laboratories) containing 10% heat inactivated FCS, 100 U/ml penicillin/streptomycin (PAA Laboratories) and 5 µM beta-mercaptoethanol (Sigma). Endothelial cells were cultured in RPMI-1640 medium (PAA Laboratories) containing 10% heat inactivated FCS (PAA Laboratories), 100 U/ml penicillin/streptomycin (PAA Laboratories) and 5 µM beta-mercaptoethanol (Sigma) mixed 1:1 with Epithelial Cell Growth Medium MV (PromoCell). For splitting Trypsin-EDTA (1x) (PAA Laboratories) was used.

For storage at -80 °C the stable cell lines were kept in 90% heat inactivated FCS (PAA Laboratories) enriched with 10% DMSO (Fluka).

2.1.2. Production of Abelson leukemia virus

For production of the ecotropic replication-deficient form of the Abelson retrovirus (A-MuLV), which encodes the v-abl oncogene, 54C12 cells were cultured on 10 cm cell culture dishes. Cells were grown in complete DMEM High Glucose medium until grown to 100 % confluency. Cells were centrifuged at 1200 rpm for 5 minutes and sowed in complete RPMI-1460 medium for one day. Supernatant was collected, centrifuged at 1200 rpm for 5 minutes and filtered sterilely through a 0.45 µM syringe filter (Abdool *et al.*). Viral supernatant was subsequently shock frozen in liquid nitrogen and stored at -80°C.

2.1.3. Stimulation of RAW 264.7 cells

For stimulation of RAW 264.7 cells with Lipopolisaccharid (LPS) (Sigma), cells were grown in 10 cm culture dishes until 90-100% confluency and stimulated with 100 ng/ml of LPS for one hour at 37°C/ 5% CO_2. Afterwards cells were harvested, centrifuged for 5 minutes at 1000 rpm and washed with phosphate buffered saline (PBS).

2.1.4. Scratch assay

$1x10^5$ endothelial cells were sowed on a 6-well culture dish in complete RPMI medium. After 24 hours medium was removed and a scratch through the well was performed with a yellow tip. Afterwards cells were washed carefully 2 times with PBS and covered with 2 ml of different supernatants.

For supernatant production $1x10^6$ v-abl transformed cells of different genotypes were sowed in complete RPMI medium on a 10 cm culture dish and collected after 24 hours by centrifugation at 1400 rpm for 4 minutes. Afterwards supernatant was filtered sterilely through a 0.45 µM syringe filter.

For identification of proliferation properties of endothelial cells grown in different supernatants pictures of cells were taken under a microscope (Nikon, Eclipse TS100; 10 times magnified) using a digital camera (Nikon, Coolpix P5000; Zoom F2.7) at time point 0h and time point 24h. To analyze proliferation differences a program (tScratch) was used that measures, compares and evaluates statistically open areas on culture dishes.

2.2. Flow cytrometric analysis (FACS)

For flow cytometry 50 µL of cell suspensions (~10^6 cells/ml) were incubated with 50 µL PBS, Fc-block and the B cell specific antibodies (listed in tab. X) on ice in the dark for 20 minutes. Cells were washed with PBS, centrifuged for 4 minutes at 1200 rpm and analyzed in a FACS Canto™ II (Becton Dickinson) with BD FACSDiva software.

Antibodies used for flow cytometry:

B cell staining BM	Name	Dilution	Source
	Fc-block	1:100	BD Pharmingen
	APC Cy7-conjugated CD19	1:50	BD Pharmingen
	PerCP-Cy5.5-conjugated B220	1:100	BD Pharmingen
	PE conjugated CD43	1:50	BD Pharmingen
	PE-Cy7 conjugated NK1.1	1:100	BD Pharmingen
	APC conjugated DX5	1:100	BD Pharmingen

Table 1. Antibodies used for flow cytometry.

2.3. DNA Methods

2.3.1. DNA isolation

For DNA isolation mouse tail tips were incubated in 500 µL tail prep buffer [50 mM Tris HCl pH=8,0, 100 mM NA_2EDTA, 100 mM NaCl, 1%SDS] and 12,5 µL proteinase K (0,5 mg/mL) either over night for 55 °C or for 2 hours at 55 °C 650 rpm shaking.

For protein precipitation 200 µL 5 M NaCl were added. After inverting the tubes they were centrifuged at 4 °C for15 minutes at 13600rpm.

450 µL of the supernatant were transferred to a new eppendorf tube and DNA was precipitated by adding 300 µL 2-propanol. Tubes were inverted and incubated at -20 °C for at least 5 minutes. Afterwards they were centrifuged again at 4 °C for 15 minutes at 13600 rpm. DNA pellets were washed with 1 mL 70% Ethanol at 4 °C for 10 minutes/13600 rpm. The supernatant was removed, the pellets were left to dry and dissolved in 150 µl H_2O for at least 2 hours shaking at 37 °C.

2.3.2. Reverse-transcriptase polymerase chain reaction (RT-PCR)

For definite genotyping of mice polymerase chain reaction was performed. Identification of the IFNAR floxed (fl) allele and the wt allele requires two different primer pairs but the

reactions can be performed at the same PCR program. For identification of the CD19 cre recombinase allele and the IFNAR fl/wt allele each reaction includes 20.5 µl H_2O, 2.5 µl 10x DreamTaq buffer (Fermentas), 0.25 µl dNTPs (10mM), 0.25 µl forward primer (10µM), 0.25µl reverse primer (10µM) and 0.25 µl DreamTaq DNA polymerase (Fermentas).

Gene	Primer sequence	PCR [no.(°C/s)]	Product size
IFNAR $^{fl/fl}$	5´-CAGGCCACTCTGCATTTCCTC-3´(F) 5´-CTTTTTGGATCGATCCATAACTTCG-3´(R)	4´94°C-40x[30´´94°C-30´´58°C-1´72°C]-10´72°C-4°C]	358 bp
IFNAR wt	5´-CAGGCCACTCTGCATTTCCTC-3´(F) 5´-CTTTTTGGATATCAAGAAAGCAAAT-3´(R)		358 bp
IFNAR Δ	5´-GGTTAAGCTCCTTGCTGCTATCTGG-3´(F) 5´-TTGGAGATGCAATCTGCTACTCAGC-3´(R)	4´94°C-40x[30´´94°C-30´´59°C-1´72°C]-10´72°C-4°C]	Δ 339 bp fl/fl 1160 bp
CRE	5´-CGGTCGATGCAACGAGTGATGAGG-3´(F) 5´-CCAGAGACGGAAATCCATCGCTCG-3´(R)	4´94°C-40x[30´´94°C-30´´55°C-1´30´´72°C]-10´72°C-4°C	500 bp

Table 2. Primer sequences used for genotyping of mice.

2.3.3. Electrophoresis

Agarose gel electrophoresis was performed in 1x TAE buffer (40mM Tris-acetat, 1mM NaEDTA (pH=8.3)) as running and preparation buffer. For visualization of DNA under UV light 6 µl of ethidiumbromid were added to 100 ml of 1.5%-2% agarose in 1x TAE. Samples were mixed with 5x Orange G loading dye and gels were run at 120V. GeneRuler 100bp DNA Ladder (Fermentas, #SM0241) was used as DNA marker.

2.3.4. RNA isolation

Total RNA was isolated from v-abl transformed cells using TRIzol reagent (Sigma). Cells were harvested from the culture dish and centrifuged for 5 minutes at 1000 rpm. Cell pellets were homogenized in 1 ml of TRI reagent and centrifuged for 10 minutes at 12000g at 4 °C. The supernatant was transferred into a new tube. After 5 minutes at room temperature 0.2 ml of chloroform were added and tubes were shaken vigorously. Supernatants were left to stand

for 15 minutes and centrifuged for 15 minutes at 12000g at 4 °C. The aqueous phase was transferred into a new tube and mixed with 0.5 ml of isopropanol. After 15 minutes at room temperature samples were centrifuged for 10 minutes at 12000g at 4 °C. The supernatant was discarded and RNA pellets were washed with 1 ml of 75% ethanol. Samples were centrifuged for 5 minutes at 12000 g at 4 °C. Afterwards the supernatant was removed and RNA pellets were left to air-dry for 10 minutes. RNA pellets were resuspended in 30 µl of RNAse free water (Fluka). Samples were shaken at 55 °C for 15 minutes.

2.3.5. DNA digest

For DNA digest RNA samples were incubated with 14.5 µl RNAse free water, 0.5 µl DNAse I recombinant, RNAse-free (Roche) and 5 µl 10x incubation buffer (Roche) for 20 minutes at room temperature. To stop the reaction 0.2 µl of 0.2 M EDTA (pH=8) were added and samples were incubated for 10 minutes at 75 °C. Finally RNA samples were stored at -80 °C.

2.3.6. Reverse transcriptase reaction

Total RNA was converted into cDNA using the Gene Amp RNA PCR Core Kit (Applied Biosystems). 3 µg RNA were mixed with MgCl2 solution (25mM), 10x PCR Buffer II, dATP (10mM), dTTP (10mM), dGTP (10mM), dCTP (10mM), Oligo d(T)16 (50 µM), RNAse Inhibitor (20U/µl), MuLV Reverse Transcriptase (50U/µl) and an appropriate volume of RNAse free water and after 10 minutes at room temperature samples were incubated at 42 °C for 60 minutes that followed 5 minutes at 99 °C. Samples were centrifuged and 30 µl of RNAse free water was added.

cDNA concentration was measured with a BioPhotometer 6131 (eppendorf). cDNA samples were stored at -20 °C.

2.3.7. Quantitative real-time PCR (qRT-PCR)

For measuring the relative genexpression quantitative real-time PCR (qRT-PCR) was performed. For the relative expression of IFNβ and VEGF different primer pairs were used but the reactions were performed at the same PCR program. Each reaction includes 13.6 µl H_2O, 2.5 µl 10x buffer advanced with Mg^{2+} (5 Prime), 2.25 µl DMSO, 0.5 µl dNTPs (10mM), Sybr green (diluted 1:100 in DMSO) and 0.1 µl Taq DNA polymerase (5 Prime). The SYBR green method was applied for determining concentrations of double stranded PCR products obtained after PCR using intron-spanning primers. The PCR cycle in which a given mRNA

specific fluorescent signal crossed an arbitrary threshold was defined as CT.cycle. The difference of a gene-specific CT value to that of an endogenous housekeeping gene (GAPDH) was called DCT value. Individual DCT for all genotypes were calculated and compared relatively to controls. The primer sequences were designed manually. The DNA sequences of primers used for qRT-PCR analysis are given in Table 3.

Each qRT-PCR was performed in triplicates.

Gene	Primer sequence	PCR [no.(°C/s)]	Product size
GAPDH	5´-AGAAGGTGGTGAAGCAGGCATC-3´ (Fw) 5´-CGGCATCGAAGGTGGAAGAGTG-3´(Rv)		100bp
IFNβ	5´-CACTGCAGCCTTTGACAGCCTTTG-3´(Fw) 5´-CCATAGGGATCTTGAAGTCCGC-3´(Rv)	1´94 °C- 40x[1´94 °C-30´´55 °C-30´´72 °C]-15´´95 °C-15´´60 °C-15´´95 °C	251bp
VEGF	5´-GCACAGCAGATGTGAATGCAG-3´(Fw) 5´-CGCTCTGAACAAGGCTCACA-3´(Rv)		73bp

Table 3. Primer sequences used for quantitative qRT-PCR.

2.3.8. Oligo GEArray Expression analysis for angiogenesis

For determination of expression and regulation of genes involved in angiogenesis an Oligo GEArray® System (SABiosciences) including an Oligo GEArray Reagent Kit was performed according to the user manual. 1 µg of isolated total RNA was transcribed into cRNA labeled with Biotin-16-UTP (Roche) using the TrueLabeling-AMP™ 2.0 kit. 3 µg of biotin-labeled cRNA were hybridized over night at 60 °C with the pre-hybridized array membrane. Next day the array membrane was washed several times with wash solution 1 (2x SSC, 1% SDS) and was solution 2 (0.1x SSC, 0.5% SDS) at 60 °C. Cooled array membranes were further blocked with GEAblocking Solution Q, washed with 1x Buffer F and Buffer G, respectively. RNA detection was achieved using CDP-Star chemiluminescent substrate on photosensitive films. GEArray Expression Analysis Suite 2.0 was used for analysis of the array membranes.

2.4. Protein analysis

2.4.1. Cell lysates

Cell pellets either from cultured cell lines or tumor samples were homogenized in an appropriate (400-600 µl) volume of Carin Lysis Buffer (20mM Tris-Base (pH= 8.0), 138 mM NaCl, 10 mM EDTA, 100 mM natriumfluorid, 1% NP40, 10% glycerol, 4 mM natriumorthovanadate (freshly added), 10 µl Complete Mini EDTA free Protease Inhibitor Cocktail ® as indicated by the manufacturer (Roche)), shock frozen in liquid nitrogen and stored at -80 °C.

2.4.2. Protein concentration

For the measurement of the protein concentration samples were unfrozen and centrifuged for 5 minutes at 13 600 rpm. Supernatant was used for protein measurement with BCA Protein Assay Reagent (Pierce Biotechnology) and immediately shock frozen in liquid nitrogen and stored at -80 °C. 1 ml Reagent A and 20 µl Reagent B of the BCA kit were mixed with the protein supernatant and incubated for 40 minutes at 36 °C. Protein concentration was measured at 562nm.

2.4.3. Electrophoresis

Protein samples were separated on 10% polyacrylamid gels in 1xSDS-PAGE running buffer (25 mM Tris-base, 192 mM glycine and 0.1% sodium dodecyl sulphate (SDS)) at 140V constant current per 0.75 mm mini-gel (Biorad). Supernatants were mixed with 4x laemmli buffer (250 mM Tris-base (pH=6.8), 8% SDS, 20% beta-mercaptoethanol, 43.4% glycerol), heated for 5 minutes at 95°C, centrifuged and well mixed before loading. As marker, PageRuler prestained protein ladder (#SM 0671, Fermentas) was used.

2.4.4. Western blotting

Proteins were blotted onto a nitrocellulose transfer membrane (PROTRAN®, Whatman®) at 0.4 A for 40 minutes (Transblot SD®; Biorad) per 0.75 mm mini-gel using electrotransfer buffer (25 mM Tris-base, 192 mM Glycin). All nitrocellulose membranes were blocked with 5% milk/ Tris-buffered saline with 0.1% Tween (TBS-T) (200 mM Tris-base, 1500 mM NaCl, pH=7.5) for 1 hour at room temperature. First antibodies were incubated in 3% BSA/TBS-T over night at 4 °C and second antibodies were incubated in 1% BSA/TBS-T for 1 hour at room temperature. Membranes were washed with TBS-T supplemented with 0.1%

SDS for 1 hour between the incubation steps. Protein detection was achieved by ECL™ (enhanced chemiluminiscence detection; Amersham Biosiences/GE Healthcare) on photosensitive films.

2.5. Histology

Spleens of diseased mice were fixed with 4% formaldehyde for 48 hours. Afterwards they were washed in PBS and embedded in paraffin. 5µm thick sections were cut and stained with Hematoxylin and Eosin (H&E) or with Periodic acid solution and Schiff reagent (PAS). Confocal images were taken using a Zeiss Axio Imager Z1 microscope.

2.6. Mice

2.6.1. Mouse strains

All mice were maintained at the Medical University of Vienna and the University of Veterinary Medicine of Vienna. Due to the compromised immune status of the knock out mice strains, they were maintained under pathogen free conditions in Micro-isolator ™ cages. Cages, food and water were sterilized and the mice were handled in laminar flow. The offspring was weaned at three weeks of age and for individual identification one ear of the animal was tagged. Tail-tip biopsy was performed for genotyping of the mice.

All mouse strains, which served as experimental model systems were on the C57BL/6 mouse background. Furthermore only littermates of the mouse strains were compared for experimental purpose among each other. All mouse experiments were carried out in accordance with protocols approved with the Austrian law.

For this study, the IFNAR$^{fl/fl}$ mouse strain was obtained from Ulrich Kalinke. IFNAR$^{fl/fl}$ mice were originally crossed with c-jun$^{fl/fl}$, CD19 cre + mice and Nk cre+ mice and its following offspring to breed IFNAR$^{fl/fl}$CD19cre + mice and IFNAR$^{fl/fl}$Nk cre+ mice. For further experiments IFNAR$^{fl/fl}$CD19 cre+ / Nkcre+ and IFNAR$^{fl/fl}$CD19 cre- / Nk cre- littermate controls were analysed.

Additionally, following mouse strains served as experimental model systems:

2.6.2. Rag2-/-γ_c -/- mice

In this study Rag2-/-γ_c -/- mice, which lack functional B-, T- and Nk-cells served as a leukemia mouse model system (Mazurier *et al*., 1999).

2.6.3. Injection of Abelson retrovirus (A-MuLV) into newborn mice

For newborn infection experiments, newborn mice were injected intraperitoneally and into the nuchal fold with 100 µl of replication incompetent ecotropic retrovirus (A-MuLV) encoding for v-abl. Mice were checked daily for disease onset. Diseased mice were sacrificed by cervical dislocation and blood samples were taken for blood smears, FACS analysis and cultivation of the cells. Spleens and lymph nodes were excised and analysed for their weight, FACS analysis and cultivation of the cells. For further histological analysis pathological sections of the spleen, liver and lymph nodes were taken and kept in 4% formaldehyde (Sigma) for 2 days and afterwards stored in PBS at 4°C.

The other part of the excised spleens and lymph nodes were pushed through a 70 µm cell-strainer (Abdool *et al.*) to obtain a single cell suspension. Subsequently bone marrow cells were isolated from femur and tibiae by flushing them with syringes. All cells suspensions were centrifuged for 5 minutes at 1 000 rpm. Supernatants were aspired and splenocytes were resuspended in erythrocyte lysis buffer (0.4 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM NaEDTA) to get rid of red blood cells. After 5-10 minutes centrifugation cells were centrifuged as before, supernatant aspired and all cell pellets were resuspended in 1 ml PBS. Single cell suspensions (10^6 cells/ml) were transferred to FACS tubes (Abdool *et al.*) for FACS analysis. Spenocytes, lymph node cells and bone marrow cells were maintained in RPMI-complete medium and observed for outgrowth of cells lines for further analysis.

2.6.4. Injection of Abelson transformed bone marrow B-cell lines into Rag2-/- γ_c-/- mice

For tail vein injection $1x10^5$ v-abl transformed cells were resuspended in 100 µl PBS and injected via tail vein into immuno-compromised Rag2-/-γ_c-/- mice. The recipients were checked daily for disease onset. Diseased mice were sacrificed by cervical dislocation and blood samples were taken for blood smears and FACS analysis. Spleens and lymph nodes were excised and analysed for their weight and FACS analysis. For further histological analysis pathological sections of the spleen, liver and lymph nodes were taken and kept in 4% formaldehyde (Sigma) for 2 days and afterwards stored in PBS at 4°C.

Furthermore the isolated organs were treated as described above to obtain single cell suspensions for FACS analysis.

2.7. Statistical analysis

For statistical analysis GraphPad Prism software was used. Statistics were calculated using an unpaired t-test (mean values represent data ± SEM). The log rank test was used to analyze differences in the Kaplan-Meier plot for statistical significance.

3. Results

3.1. Newborn infections of IFNAR1$^{fl/fl}$CD19cre+ and IFNAR1$^{fl/fl}$CD19cre- mice appear to result in faster progression of B-lymphoid leukemia in IFNAR1$^{fl/fl}$CD19cre+ mice

Previous data in our lab have shown that mice impaired in type I IFN signaling succumbed to B-lymphoid leukemia significantly earlier than their control animals. Since leukemia formation is a complex process between tumorigenic and immune cells, we addressed the question, whether the higher susceptibility to B-lymphoid leukemia is due to a tumor cell intrinsic effect or due to a defect in the immune defense. Therefore, we bred IFNAR1$^{fl/fl}$ mice with CD19cre+ mice, to delete the IFNAR1 receptor chain specifically in B-lymphocytes.

Due to the fact that newborn mice do not have a fully developed immune system, we challenged them with A-MuLV (as described in materials and methods) to induce a slowly developing leukemia. The infected mice were examined daily for signs of disease and diseased mice were sacrificed.

Interestingly, IFNAR1$^{fl/fl}$CD19cre+ mice (n=8) succumbed to B cell leukemia with shortened latency when compared to their control littermate IFNAR1$^{fl/fl}$CD19cre- mice (n=8), although it does not reach the criteria of statistical significance (log rank-test, n.s, $p<0.2458$) (Figure 11A).

As mentioned above, B-cell specific deletion of the IFNAR1 receptor chain was achieved by crossing IFNAR1$^{fl/fl}$ mice with mice expressing the cre recombinase under the B-cell specific CD19 promotor. To test for the deletion, splenocytes of the IFNAR1$^{fl/fl}$ CD19cre+ mice were cultured and DNA was isolated to perform PCR analysis. As shown in Figure 11B, the deletion of the floxed gene results in a 339bp fragment, whereas DNA of IFNAR1$^{fl/fl}$CD19cre- cells gives rise to an 1160bp fragment in gel electrophoresis.

As mentioned above, diseased mice were sacrificed and the isolated hematopoietic organs were analyzed carefully, including the determination of the spleen weight and lymph node weight. As shown in Figure 12A, IFNAR1$^{fl/fl}$CD19cre+ mice displayed significantly increased spleen weights compared to their IFNAR1$^{fl/fl}$CD19cre- littermate controls (unpaired t test, *,

$p < 0.0351$). Furthermore, we found that isolated lymph nodes displayed a slightly increased weight without reaching the criteria of being statistically significant (unpaired t test, $p < 0.05$).

In order to verify the leukemia of these mice, bone marrow cells, splenocytes, lymph node and peripheral blood cells were isolated and analyzed according to their surface marker expression. FACS analysis confirmed that these mice suffered from a B-lymphoid leukemia, as all hematopoietic organs were infiltrated with $B220^+$, $CD43^+$ and $CD19^+$ tumor cells (see Figure 12B and 13B). The expression levels of B-lymphoid markers varied among the individual examined mice. To elucidate the actual IFNAR1 expression on B cells we performed FACS analysis and showed that $IFNAR1^{fl/fl}$CD19cre- mice, which died later (e.g. mouse D11459, red peak in Figure13C) express more of the IFNAR1 receptor chain than mice that died earlier (e.g. mouse D11451, green peak in Figure 13C). We further characterized leukemic cells by doing Hematoxylin and Eosin (H&E) stainings of blood smears and histological sections of the spleen. As illustrated in Figure 13A1+A2 and Figure 12C1+C2 both genotypes did not show significant differences in white blood cell numbers and the infiltration of tumor cells.

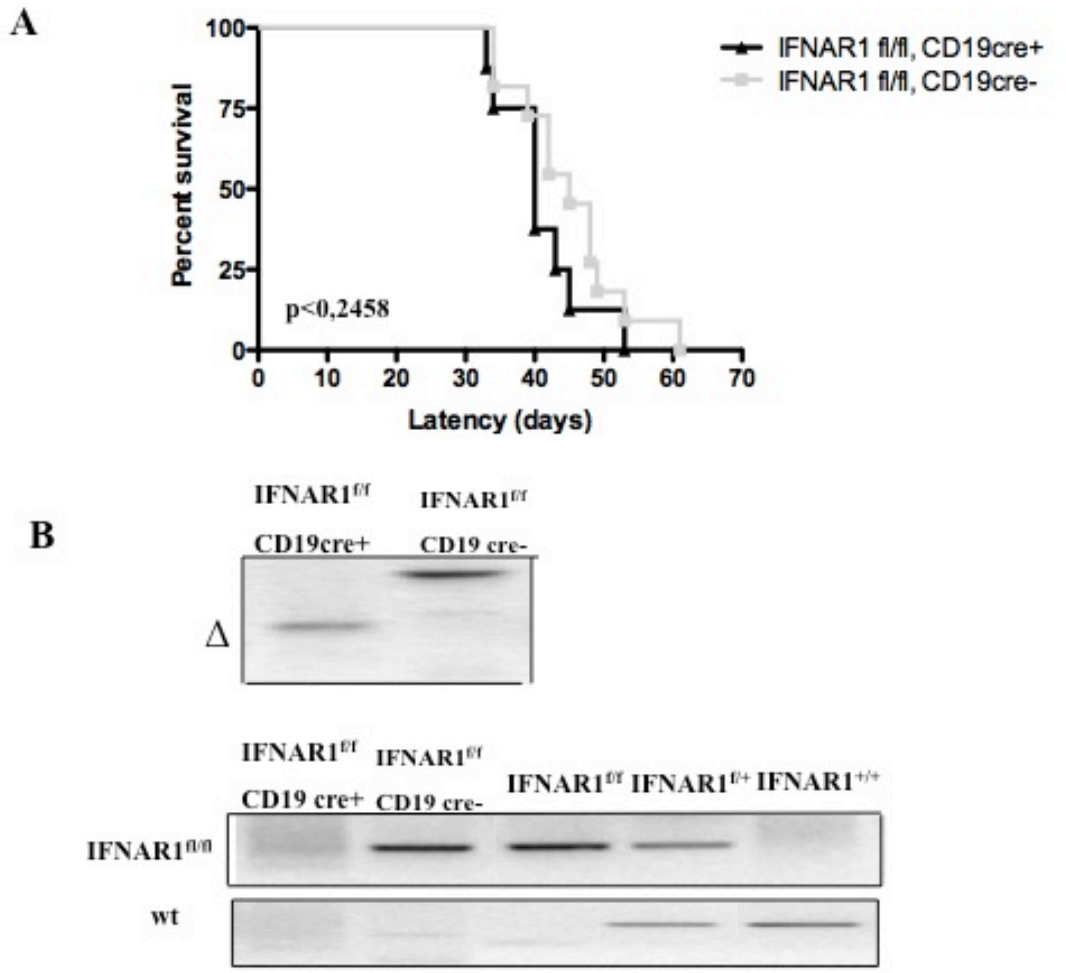

Figure 11. Challenge of newborn IFNAR1$^{fl/fl}$CD19 cre+ and IFNAR1$^{fl/fl}$CD19 cre- mice with the murine oncogene v-abl results in slightly earlier onset and progression of B-lymphoid leukemia. (A) The survival of newborn mice is depicted in the Kaplan-Meier plot (log rank test, n.s. p<0,2458). **(B)** PCR amplification of genomic DNA isolated from IFNAR1$^{fl/fl}$CD19cre+ cells resulted in a 339 bp fragment devoid of the loxP flanked fragment, whereas genomic DNA from IFNAR1$^{fl/fl}$CD19cre- cultured cells gave rise to a 1160 bp fragment. In order to ensure the deletion of the floxed IFNAR1 gene in diseased IFNAR1$^{fl/fl}$CD19cre+ mice, PCR was performed, amplifying the floxed gene in case of genomic DNA isolated from IFNAR1$^{fl/fl}$CD19cre- cells but not in case of IFNAR1$^{fl/fl}$CD19cre+ cells. Appropriate controls are also shown.

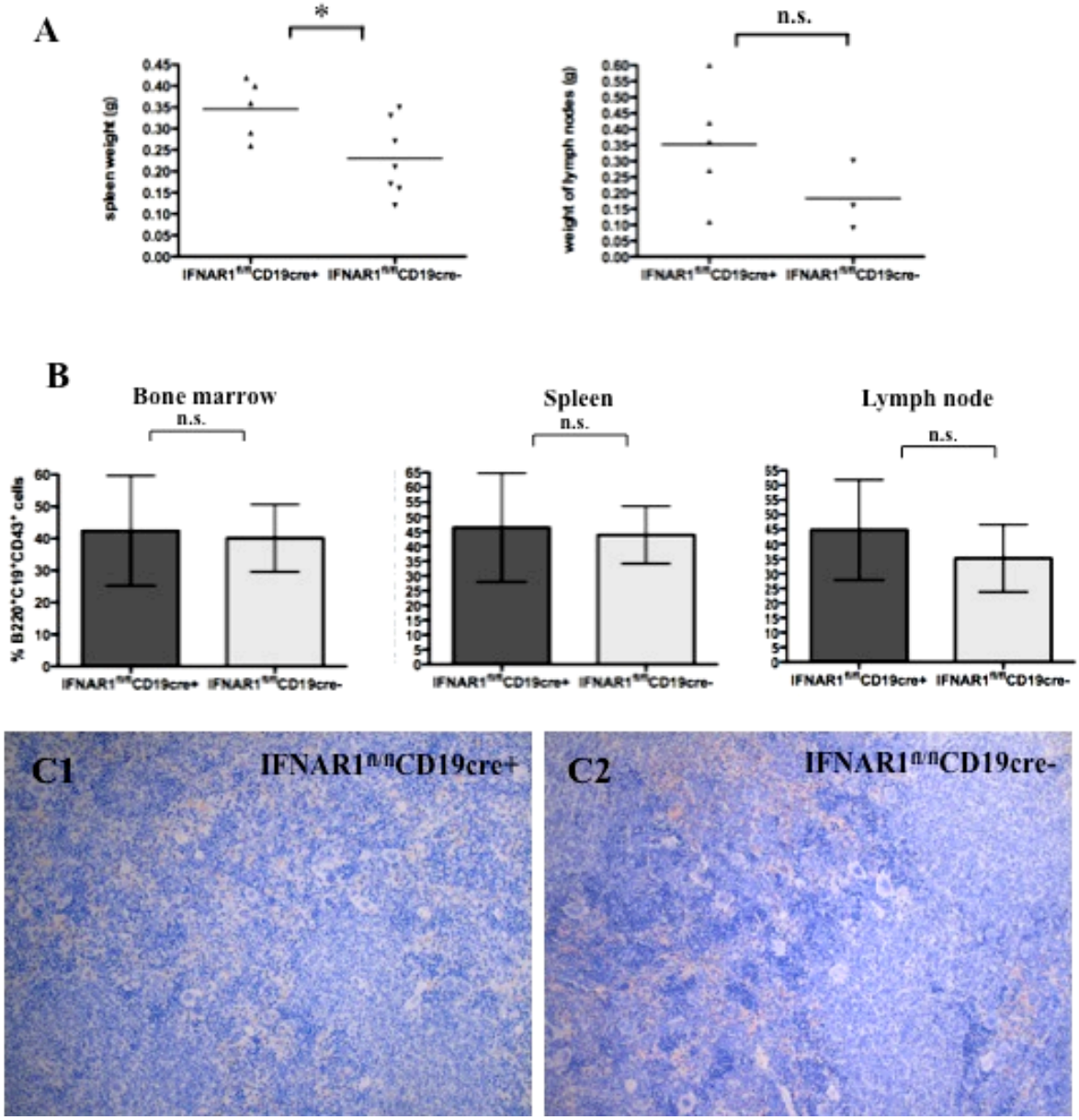

Figure 12 The infiltration of leukemic cells is comparable in IFNAR1$^{fl/fl}$CD19cre+ mice and IFNAR1$^{fl/fl}$CD19cre- mice. (A) Determination of the spleen weight of the indicated genotypes revealed a significantly increased spleen weight in IFNAR1$^{fl/fl}$CD19cre+ mice. No significant difference was observed in the lymph node weight. **(B)** Statistical analysis of B220^{+}CD19^{+}CD43^{+} cell populations showed no significant differences among both genotypes in bone marrow, spleen and lymph nodes. **(C)** Representative Hematoxylin and Eosin stained spleen sections of diseased mice are shown.

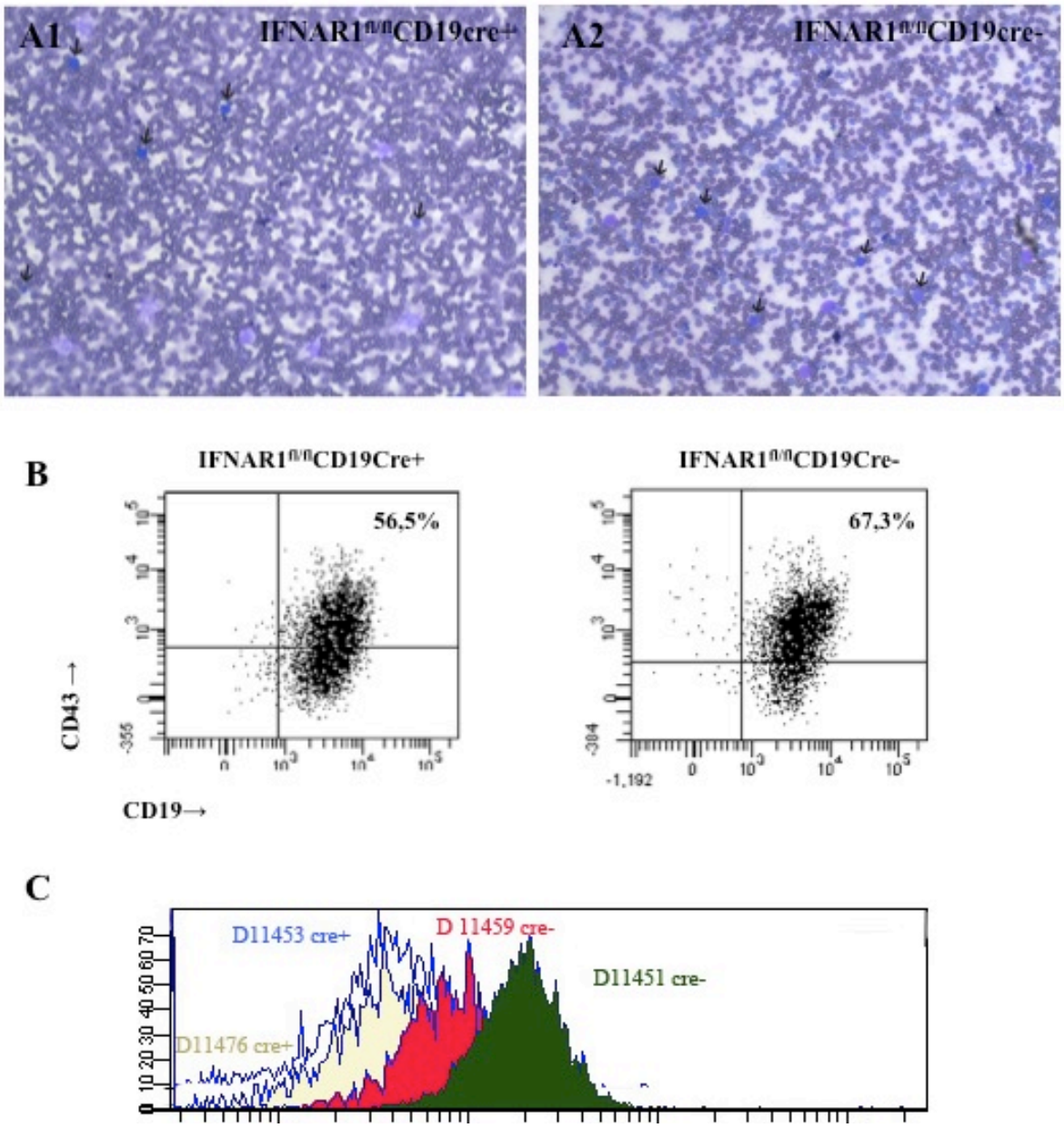

Figure 13. Characterization of leukemic cells derived from IFNAR1$^{fl/fl}$CD19cre+ and IFNAR1$^{fl/fl}$CD19cre- mice. (A1,2) Hematoxylin and Eosin (H&E) staining of blood smears showed no significant differences in white blood cell numbers among both genotypes. **(B)** Living cells were gated for the B220^{+} fraction and subsequently analyzed for surface expression of CD19^{+} and CD43^{+} B-cell markers. Percentages indicate CD19^{+}/CD43^{+} populations. One representative FACS plot for IFNAR1$^{fl/fl}$CD19cre+ and IFNAR1$^{fl/fl}$CD19cre- is shown. **(C)** FACS analysis for IFNAR1 receptor chain expression on B cells. IFNAR$^{fl/fl}$CD19cre- mice that died earlier expressed less of IFNAR1. Two representative histograms of each genotype are shown.

Taken together, the data obtained from the newborn infection experiment demonstrate that IFNAR1$^{fl/fl}$CD19cre+ mice are more prone to develop Abelson induced B cell leukemia, which indicates that the faster progression of B-lymphoid leukemia is due to a tumor cell intrinsic effect.

3.2. Wildtype tumor cells do not produce low levels of IFN-β *ex vivo* and *in vitro*

As mentioned above, previous data obtained from newborn infections and transplantation experiments in immuno-compromised mice showed that either infection with A-MuLV of mice with impaired type I IFN signaling or injection of v-abl transformed IFNAR1-/- and IFNβ-/- cells into Rag2-/- mice resulted in faster progression of B-lymphoid leukemia compared to the wild type. To elucidate the mechanism underlying the increased susceptibility of IFN-β-/- and IFNAR1-/- mice to B-lymphoid leukemia, we investigated whether this effect might be due to a tumor cell intrinsic effect or due to a defect in the immune system.

As outlined in the introduction, IFN-β has anti-tumor activity. It has been shown that IFN-β inhibits tumor growth. Therefore, it is possible that Abelson transformed cells also produce IFN-β and thereby limit their own growth. We therefore investigated the IFN-β levels from stable pro-B-cells transformed with v-abl. Different IFN-β levels in the tumor cells might be an explanation for the differences in latency in transplanted Rag2-/- animals.

To verify the level of IFN-β production of v-abl transformed pro B-cell lines *in vivo*, we injected 10^6 v-abl transformed wt and IFNβ-/- cells subcutaneously into Rag2-/-γ_c-/- mice. After 10 days, mice were sacrificed and solid tumors were isolated. Single cell suspension of tumor material was prepared and protein lysates were performed as described in materials and methods for Western blot analysis.

As outlined in the introduction production of IFN-β leads to JAK/STAT signaling and thereby phosphorylation of STAT2. Hence western blot analysis for phospho-STAT2 (p-STAT2) was performed. As illustrated in Figure 14A STAT2 was activated in isolated tumor samples injected with wt tumor cells, which indicates that the tumor cells produce type I IFNs that induce JAK/STAT signaling. But also tumor samples injected with IFN-β-/- tumor cells revealed p-STAT2 in Western blot analysis. This might be due to infiltrating cells that produce type I IFNs and therefore activate STAT2.

To test the level of IFN-β production of leukemic cell lines *in vitro*, we performed qRT-PCR analysis with v-abl transformed wt and IFN-β-/- cell lines. As shown in Figure 14B, there was no increased expression of IFN-β in v-abl transformed wt cell lines (n=4) compared to IFN-β-/- (n=2) and IFNAR1-/- (n=3) cell lines.

In conclusion, we could not detect constitutive expression of low levels of IFN-β neither *ex vivo* nor *in vitro*. Thus we could not prove our hypothesis that wt tumor cells produce IFN-β that delays tumor progression. The obtained results indicate that the longer latency of Rag2-/- mice injected with wt tumor cells is not due to their IFN-β production.

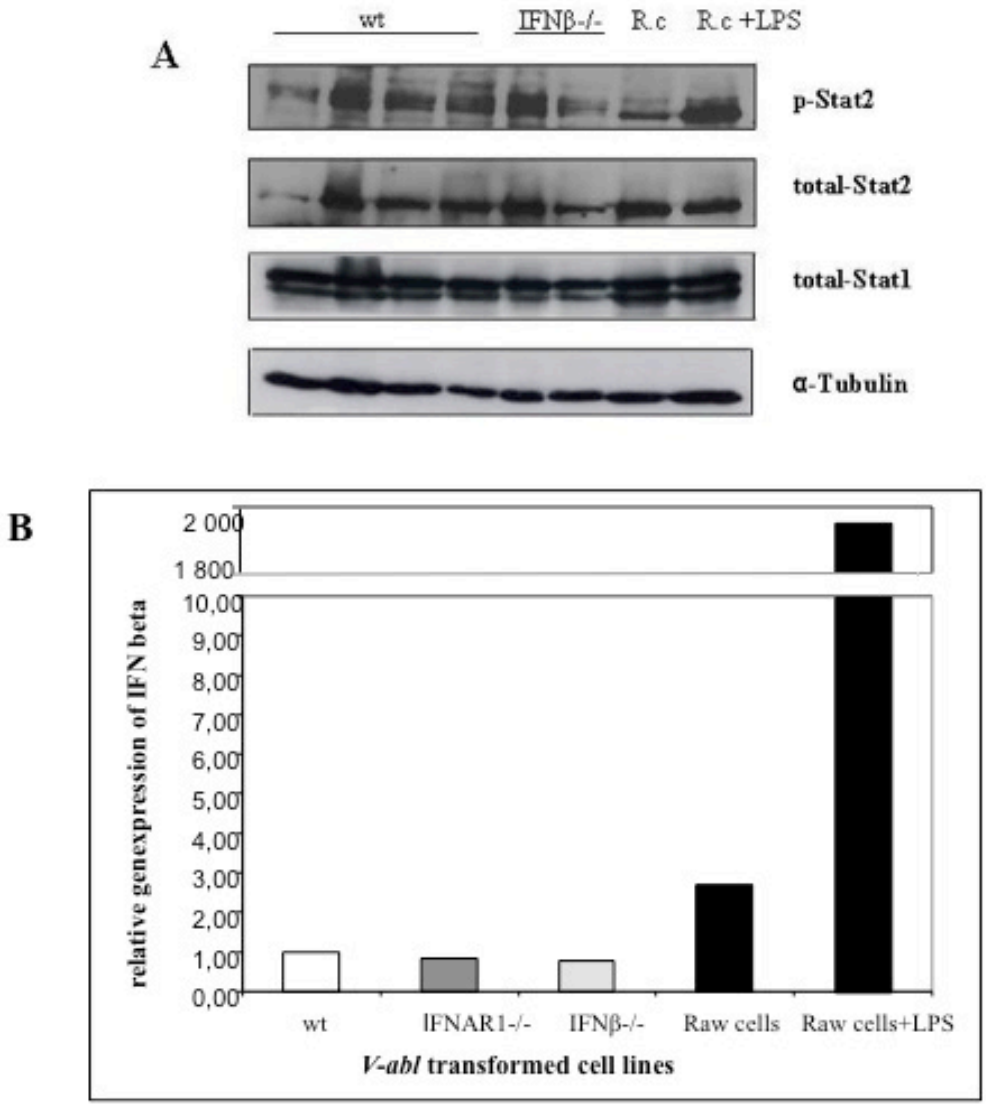

Figure 14. No difference in the IFN-β expression levels of tumor cells. (A) Rag2-/-γ_c-/- mice were injected subcutaneously with various *v-abl* transformed cell lines and solid tumors were isolated. Western blot analysis for phospho-Stat2 (p-Stat2) did not show any differences in the phosphorylation levels of Stat-2 between wt (n=4) derived and IFN-β-/- (n=2) derived tumors. Control immunoblots with anti-STAT2, anti-STAT1 and anti-α-Tubulin-antibodies are shown. **(B)** qRT-PCR for the *IFN-β* gene also demonstrated that there are no detectable levels of IFNβ in *v-abl* transformed wt (n=4) cell lines compared to IFNAR1-/- (n=3) and IFN-β-/- (n=2) cell lines. Raw 264.7 cells (R.c) and Raw 264.7 cells stimulated with LPS (R.c+LPS) were used as positive controls in both experiments.

3.3. V-abl transformed wt and IFNAR1-/- cells display comparable tumorigenicity in Rag2-/-γ_c-/- mice

Previous data in our lab have shown that Rag2-/- mice challenged with IFNAR1-/- or IFN-β-/- leukemic cell lines succumb to B-lymphoid leukemia significantly faster than Rag2-/- mice challenged with wt leukemic cell lines. As Rag2-/- mice lack B- and T-cells but not Nk cells, this effect might be due to recognition by Nk cells, which are important mediators in tumor surveillance. To test whether Nk cells play a role in our B cell leukemia model, we performed bone marrow transplantation of leukemic cells into Rag2-/-γ_c-/- mice. Rag2-/-γ_c-/- mice not only lack functional B-, and T-lymphocytes but also Nk cells (Mazurier *et al*., 1999). Hence, Rag2-/-γ_c-/- mice are particularly suited to study the onset of lymphoid leukemia because cell autonomous properties are not affected by a functional immune system.

To study the effect of v-abl transformed cell lines in immuno-compromised mice, 8 Rag2-/-γ_c-/- mice were challenged with 10^5 leukemic cells via tail vein injection. Two independently established wt and IFNAR1-/- cell lines were injected at least in two recipient mice. Prior to injection, the proliferation rate of the cell lines was determined by a ^{3}H-thymidine incorporation assay. The ^{3}H-thymidine incorporation experiment showed that the cell lines used were comparable in their proliferation rate *in vitro* (Figure 15A). Thus differences in the latency of injected mice is not due to a proliferative advantage. Furthermore cell lines were controlled for surface expression of B-lineage markers by FACS analysis before injection. All cell lines were gated for $B220^+$ and subsequently analyzed for $CD19^+$ and $CD43^+$ surface marker expression. In all cases we obtained homogenous populations of $B220^+CD19^+CD43^+$ B-lymphocytes (Figure 15B). Additionally, we confirmed the genotype of the recipient mice. Therefore, we analyzed one healthy wt, Rag2-/- and Rag2-/-γ_c-/- mouse, respectively, for the Nk cell surface markers NK1.1 and DX5. The wt, Rag2-/- and Rag2-/-γ_c-/- mice were sacrificed and the spleens were isolated to test for numbers of $CD3^-DX5^+NK1.1^+$ cells. As shown in Figure 15C only 0.1% of $CD3^-DX5^+NK1.1^+$ cells were detected in Rag2-/-γ_c-/- mice compared to 6.7% in wt mice and 64.4% in Rag2-/- mice.

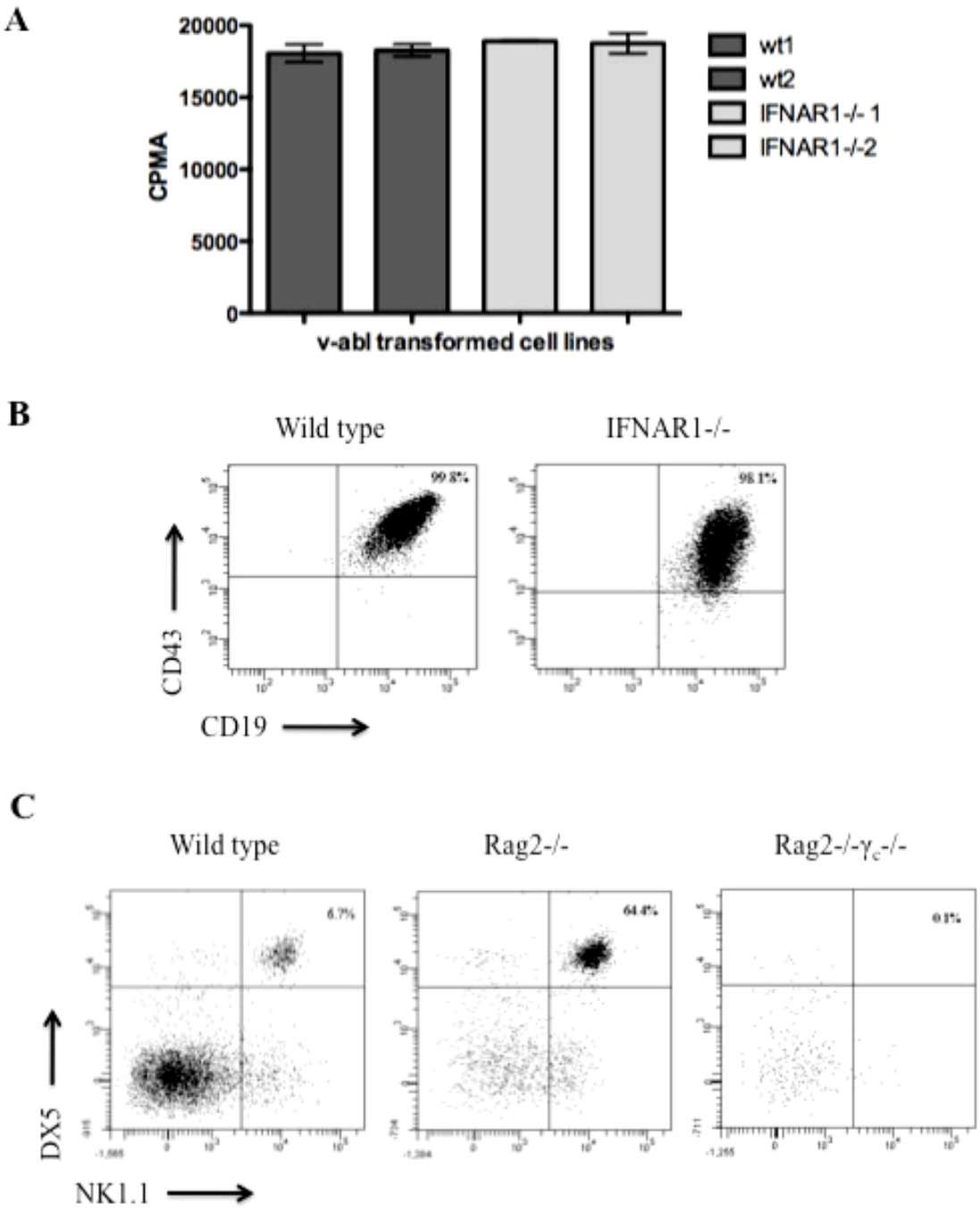

Figure 15. V-abl transformed cell lines were comparable in their proliferation rate and showed typical B cell markers. (A) A ^{3}H-thymidine incorporation assay was performed to determine the proliferation rate of six independently established cell lines. The proliferation among the cell lines was comparable. **(B)** FACS analysis of tumor cell lines. All used cell lines were gated for $B220^{+}$ cells and subsequently analyzed for surface expression of $CD19^{+}$ and $CD43^{+}$ B-cell markers. Characterization of the cell lines revealed a homogenous population of $B220^{+}CD19^{+}CD43^{+}$ B-lymphocytes. **(C)** FACS immuno phenotyping of healthy mice was performed to test for Nk cell numbers of Rag2-/-γ_c-/- mice. Splenocytes were gated for $CD3^{-}$ cells and subsequently analyzed for surface expression of $NK1.1^{+}$ and $DX5^{+}$. Only 0.1% of a $CD3^{-}NK1.1^{+}DX5^{+}$ cell population was detected in Rag2-/-γ_c-/- mice. Wt and Rag2-/- mice were used as controls.

Transplanted mice were examined daily for signs of disease. As soon as mice appeared sick they were sacrificed. After 16 days, the first Rag2-/-γ_c-/- mouse that had received v-abl transformed wt cells showed severe symptoms of leukemia. All recipients of v-abl transformed wt cells (n=4) died between day 16 and day 20 (mean survival: 17 days). Mice that received v-abl transformed IFNAR1-/- cells (n= 4) died between day 18 and day 27 (mean survival: 18.5 days). The disease latency of both groups was comparable as shown in Figure 16A (wt vs. IFNAR1-/- → log rank test, n.s. $p< 0.2286$).

In order to verify B-lymphoid leukemia single cell suspensions of bone marrow and spleen were analyzed according to the surface expression of B-lineage markers by FACS as described in materials and methods. All hematopoietic organs were infiltrated with $B220^+$, $CD43^+$ and $CD19^+$ tumor cells and were therefore classified as B-lymphoid tumors. The expression levels of B-lymphoid markers varied among the individual examined mice (Figure 16B).

Comparison of the spleen weights (Figure 16C) showed that spleens of Rag2-/-γ_c-/- mice injected with v-abl transformed wt cells had a slightly increased spleen weight without reaching the criteria of being statistically significant compared to mice injected with v-abl transformed IFNAR1-/- cells (wt → mean: 0.54 ± 0.047 g; IFNAR1-/- → mean: 0.48 ± 0.02 g).

These results indicate that type I IFN signaling is important for Nk cell mediated tumor surveillance. Similar results were obtained in three independently performed experiments.

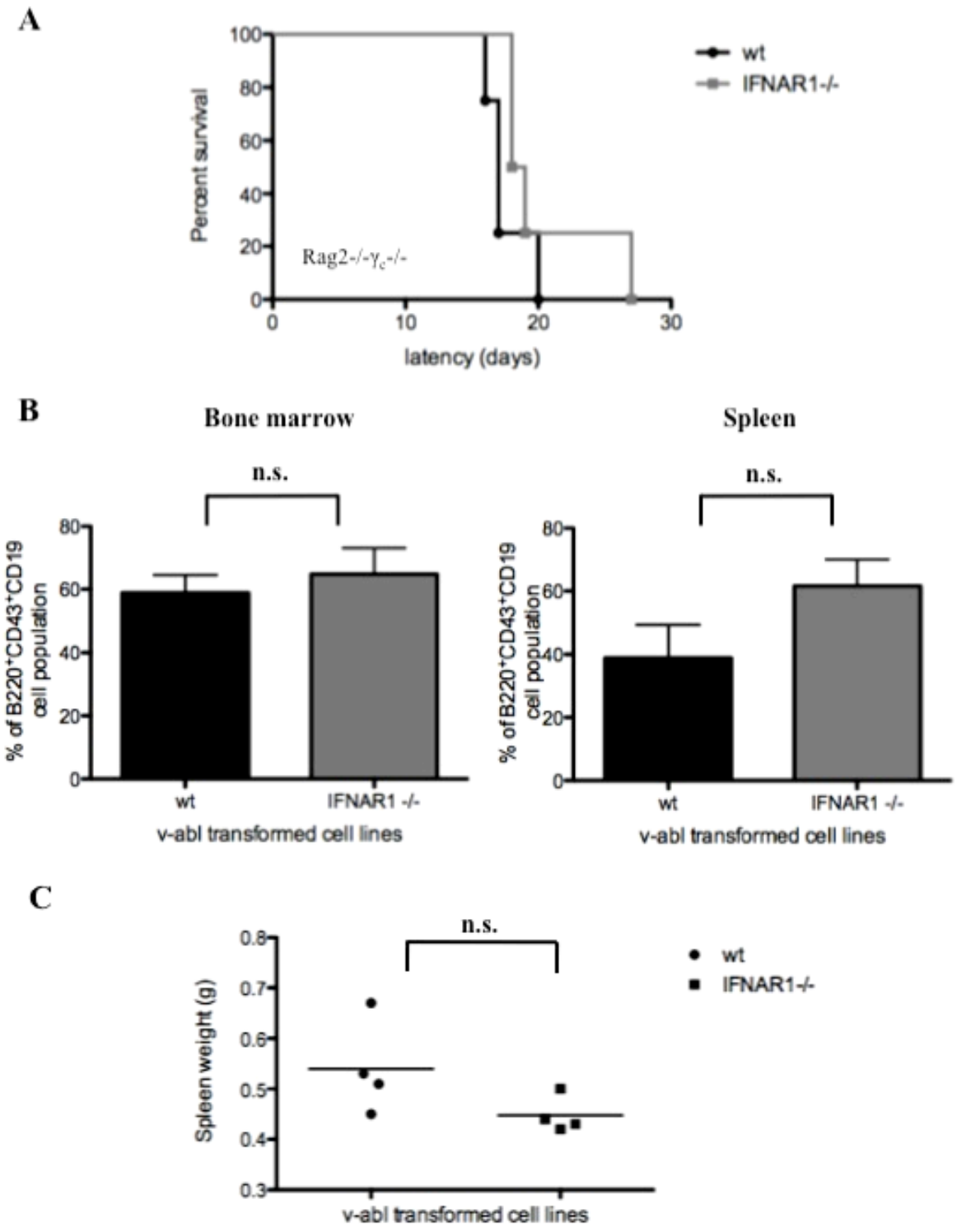

Figure 16. V-abl transformed wt and IFNAR1-/- bone marrow cells induce a comparably progressing leukemia in Rag2-/-γ_c-/- mice. (A) The survival of recipient mice is depicted in the Kaplan-Meier plot (wt vs. IFNAR1-/- → log rank test, n.s. $p< 0.2286$). **(B)** Statistical analysis of $B220^+CD19^+CD43^+$ cell populations showed no significant differences in bone marrow and the spleen. **(C)** Determination of the spleen weight of the indicated genotypes revealed no significant differences.

3.4. Type I IFN signaling plays a role in angiogenesis

As outlined in the introduction, several studies have demonstrated the anti-angiogenic function of IFNs. Thereby IFNs inhibit the production of angiogenic growth factors, such as basic fibroblast growth factor (bFGF) (Singh *et al.*, 1995) or vascular endothelial growth factor (VEGF) (Rosewicz *et al.*, 2004). Since it is known that angiogenesis is not only crucial in solid tumor formation but also in the pathogenesis of hematologic malignancies and metastatic spread throughout the lymphatic vasculature (Podar *et al.*, 2005), one aim of this master thesis was to investigate the role of type I IFN signaling in angiogenesis during v-abl induced leukemia progression.

When we injected v-abl transformed IFNAR1-/- and IFNβ-/- bone marrow cell lines subcutaneously into Rag2-/-γ_c-/- mice, we observed tumors, in which the blood supply was more efficient than in tumors evoked by wt leukemic cell lines (shown in Figure 17A). The increased vascularization of tumors deficient for IFN-β and IFNAR1 was confirmed by Periodic acid Schiff (PAS) staining of histological sections (Figure 17D). Thus we addressed the question whether our v-abl transformed bone marrow cell lines express VEGF. To respond to this question we performed qRT-PCR analysis, as described in materials and methods. As shown in Figure 17B the expression levels of VEGF-A seem to be more than five times increased in IFNβ (n=3) and IFNAR1 (n=2) deficient cell lines compared to wt controls (n=7). However, due to the high standard deviation, which indicates a high variance of expression levels among cell lines of the same genotype, data did not reach the criteria of being statistically significant (One-way ANOVA→ n.s., $p< 0.5276$).

In addition we performed scratch assays as described in materials and methods. As shown in Figure 17C epithelial cells cultured for 24 hours in the supernatant of v-abl transformed IFN-β-/- cell lines migrated significantly faster to close the scratch than the wt control (wt vs. IFN-β-/- → unpaired t test, *, $p< 0.015$). Migration of endothelial cells cultured in the supernatant of IFNAR1-/- tumor cell lines also yielded in faster disappearance of the scratch compared to the wt control but without being statistically significant (wt vs. IFNAR1-/- → unpaired t test, n.s., $p< 0.0613$).

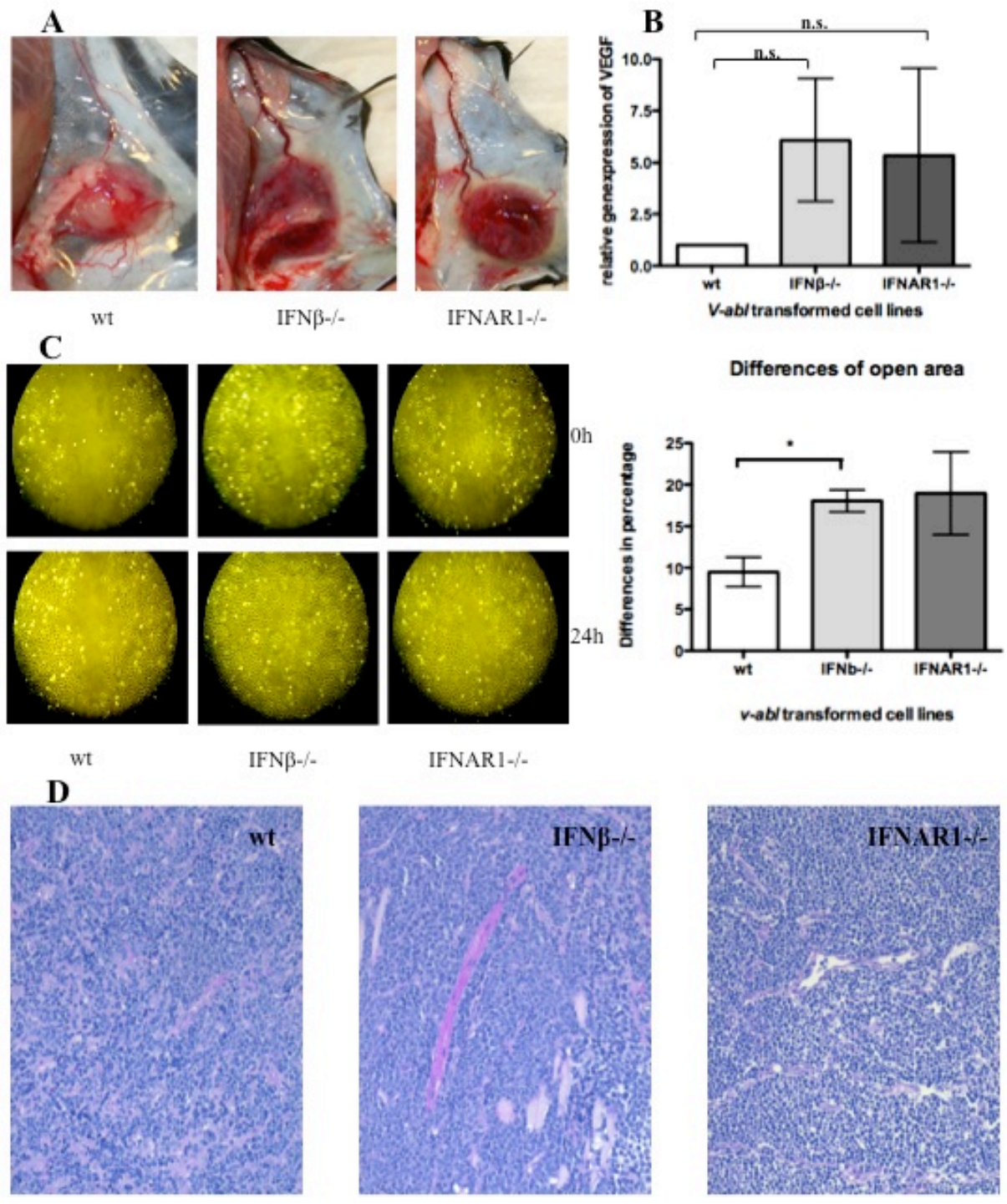

Figure 17. Type I IFNs display an inhibitory effect on angiogenesis. (A) S.c. injection of IFN-β-/- and IFNAR1-/- tumor cells into Rag2-/-γ_c-/- mice yielded in tumors with increased blood vessels compared to wt controls. **(B)** To test for the relative gene expression of VEGF-A *in vitro,* qRT-PCR was performed. **(C)** Scratch assays revealed that epithelial cells cultured for 24 hours in the supernatant of v-abl transformed IFN-β-/- and IFNAR1-/- cell lines migrated faster to close the scratch than the wt control. One experiment out of two is shown. Data are summarized in the right panel. **(D)** Histological Periodic acid-Schiff (Mes-Masson *et al.*) stainings of tumor samples confirm the increased vascularization in knock out tumors compared to wt tumors. One representative example of each genotype is shown.

To further analyze the impact of type I IFNs on angiogenesis in the Abelson tumor model we performed an Oligo GEArray for angiogenesis (see Figure 18) as described in materials and methods. Two independently established cell lines of each genotype were used. Table 4 and Table 5 summarize the genes up- and down-regulated in v-abl transformed knock out bone marrow cell lines compared to wt controls.

Abbrevation	Up-regulated genes	Fold difference	Abbrevation	Down-regulated genes	Fold difference
CCl 11	Small chemokine (C-C motif) ligand 11	2.2	Adra2b	Adrenergic receptor, alpha 2b	0.37
PDGF-B	Platelet derived growth factor, B polypeptide	33.4	Bai1	Brain-specific angiogenesis inhibitor 1	0.21
CxCl 1	Chemokine (C-X-C motif) ligand 1	51.8	Cxcl10	Chemokine (C-X-C motif) ligand 10	0.18
			Efna2	Ephrin A2	0.28
			Il12a	Interleukin 12A	0.47
			Ptgs1	Prostaglandin-endoperoxide synthase 1	0.64
			Ptn	Pleiotrophin	0.04
			Tgfb3	Transforming growth factor beta 3	0.22
			Tnf	Tumor necrosis factor	0.15
			Vegfb	Vascular endothelial growth factor B	0.15
			Vegfc	Vascular endothelial growth factor C	0.23

Table 4. Up- and down-regulated genes in v-abl transformed IFNAR1-/- bone marrow cell lines compared to wt controls. The mean of two different cell lines used is shown.

Abbrevation	Up-regulated genes	Fold difference	Abbrevation	Down-regulated genes	Fold difference
Adra2b	Adrenergic receptor, alpha 2b	1.82	Il12a	Interleukin 12A	0.53
Vegfc	Vascular endothelial growth factor C	15.73	Ptn	Pleiotrophin	0.61
			Tgfb3	Transforming growth factor beta 3	0.33

Table 5. Up-and down-regulated genes in v-abl transformed IFN-β-/- bone marrow cell lines. The mean of two different cell lines used is shown.

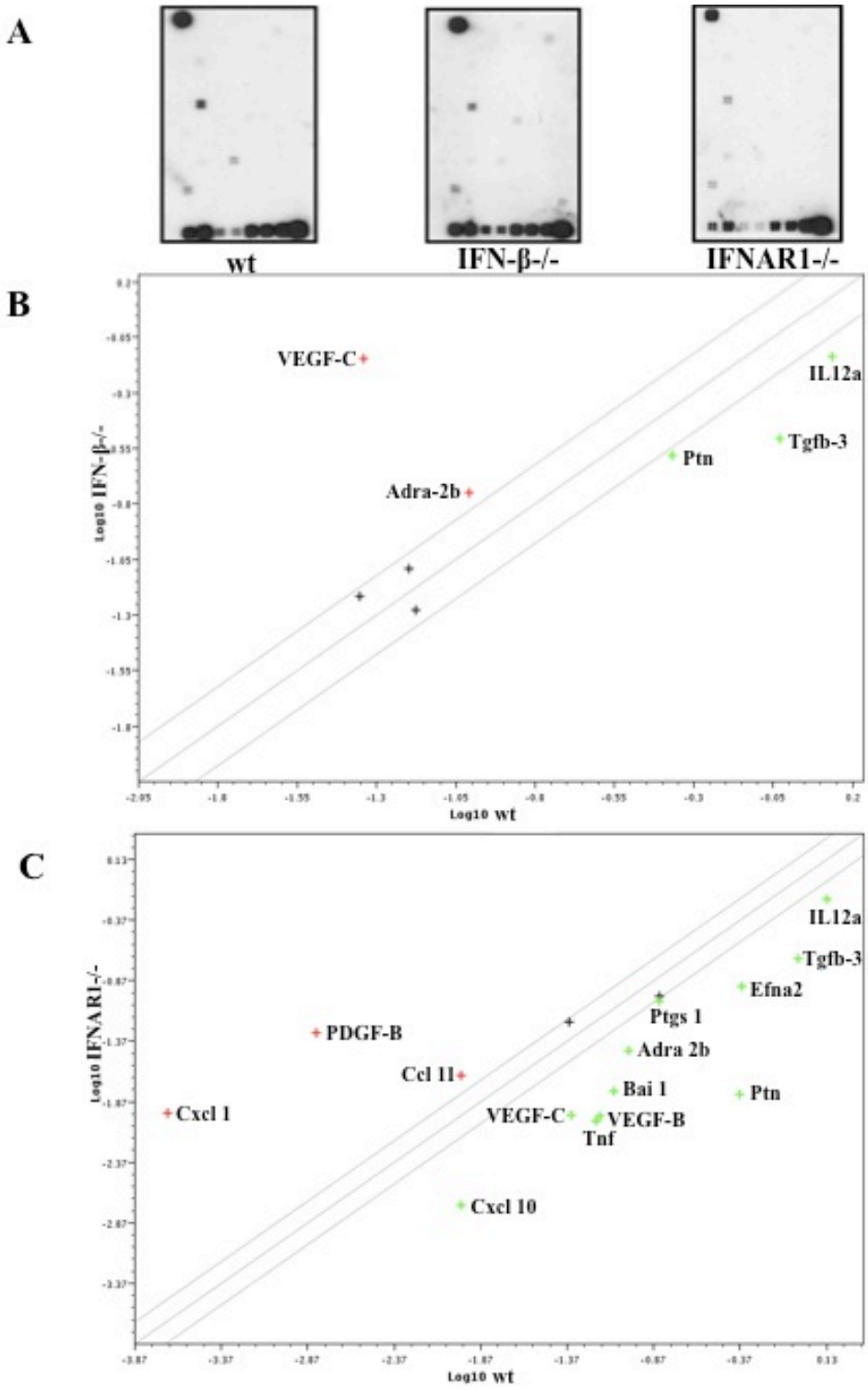

Figure 18. Oligo GEArray analysis revealed several genes involved in angiogenesis, which are up- and down-regulated in v-abl transformed IFN-β-/- and IFNAR1-/- bone marrow cell lines. (A) OligoGEArray membranes for each genotype were analysed using the GEArray expression analysis suite 2.0. **(B)** Scatter plot of IFN-β-/- cell lines compared to wt controls. **(C)** Scatter plot of IFNAR1-/- cell lines compared to wt controls. Up-regulated genes are indicated with red crosses and down-regulated genes are indicated with green crosses.

Oligo GEArray analysis shows that PDGF-B and CxCl1 was up-regulated in v-abl transformed IFNAR1-/- bone marrow cell lines. In IFN-β knock out tumor cell lines Vegfc was shown to be up-regulated. In case of IFNAR1 knock out tumor cell lines pleiotrophin was strongly down-regulated, however the expression levels of the genes listed above vary among the different cell lines of the same genotype. Therefore we compared each cell line alone to the wt control and found that in all the knock out tumor cell lines Il12a, pleiotrophin and Tgfb3 are consistently down-regulated. Thus it will be interesting to focus on these three genes in our further studies to test whether type I IFN signaling has an impact on them.

Taken together, all of the experiments we have done indicate that IFN-β has an anti-angiogenic effect and that type I IFN signaling has an inhibitory function in angiogenesis. Further experiments have to be done to identify genes affected by type I IFN signaling and the mechanism underlying the inhibitory function of type I IFNs in angiogenesis.

4. Discussion

As outlined in the introduction type I IFNs have been shown to play a crucial role in anti-viral defense as well as in cancer immunoediting. In case of viral infections of a host, type I IFNs are produced by infected cells and cells involved in the innate and adaptive immune response (Vilcek, 1996). Thus it was shown that IFNs play an important role in bridging the innate and the adaptive immune system (Swann *et al.*, 2007). In addition to their anti-viral activity it has been shown recently that IFNs have a critical role in the elimination phase of cancer immunoediting by promoting host antitumor immunity (Dunn *et al.*, 2006). The anti-proliferative nature of IFNs, as well as the fact that they are supposed to inhibit the production and release of pro-angiogenic factors from tumor cells or lymphocytes, makes them also possible inhibitors of angiogenesis (Sidky *et al.*, 1987).

In this study, we used v-abl induced B-lymphoid leukemia as a model system to mimic human leukemia formation in mice and to further analyze the impact of impaired type I IFN signaling on leukemia formation. It has been shown previously in our lab that IFNAR1-/- and IFN-β-/- mice injected with the Abelson Murine Leukemia Virus (A-MuLV) succumbed to B-lymphoid leukemia significantly faster than their wt controls. The obtained results prompted us to examine whether this phenotype was due to a direct tumor cell intrinsic effect or due to an indirect defect in the immune response. To do so, we used IFNAR1$^{fl/fl}$CD19cre+ mice to delete the IFNAR1 receptor chain of the type I IFN receptor specifically in B-lymphocytes. As described above, A-MuLV is a replication incompetent retrovirus that targets B-cells *in vivo*. Injection of newborn IFNAR1$^{fl/fl}$CD19cre+ mice and littermate controls with A-MuLV yielded in an accelerated B-lymphoid leukemia formation in IFNAR1$^{fl/fl}$CD19cre+ mice. However, the difference in latency did not reach the criteria of statistical significance. Inefficient deletion of the IFNAR1 chain in the IFNAR1$^{fl/fl}$CD19cre+ mice could be excluded by PCR of isolated tumor cells. Interestingly, when we tested for the expression of IFNAR1 on the tumor cells of the diseased mice by FACS analysis, we found a correlation between IFNAR1 expression levels and disease latency. IFNAR1$^{fl/fl}$CD19cre- mice that succumb to B cell leukemia with a longer latency display higher expression levels of IFNAR1 on their tumor cells than mice that died earlier. IFNAR1$^{fl/fl}$CD19cre+ mice consistently did not express IFNAR1 on the tumor cells. To confirm the correlation between disease latency and expression levels of IFNAR1 on leukemic cells, we certainly will have to transplant tumor cells expressing high or low levels of IFNAR1 or no IFNAR1 at all into wild type recipient

mice. If our assumptions are true, mice that receive tumor cells not expressing IFNAR1 again will lead to the faster progressing leukemia, whereas mice that are transplanted with tumor cells displaying high levels of IFNAR1 will be the last mice to die from leukemia.

Several groups have demonstrated that the presence of a constitutive low level of type I IFN in mouse embryonic fibroblasts (MEFs), splenocytes and bone marrow cells, plays a role in the regulation of the adaptive immune response (Bocci *et al.*, 1985);(Tovey *et al.*, 1987);(Taniguchi *et al.*, 2001). Previous data from our lab showed that Rag2-/- mice injected with v-abl transformed IFN-β-/- and IFNAR1-/- bone marrow cell lines succumb to B-lymphoid leukemia significantly faster than wt controls. We therefore hypothesized that v-abl transformed wt cells might also produce a constitutive low amount of IFN-β and thereby inhibit their own tumor progression. To verify low levels of IFN-β produced by v-abl transformed wt cells we did Western blot analysis for p-STAT2 of tumor samples derived from Rag2-/-γ_c-/- mice injected with v-abl transformed wt and IFN-β-/- bone marrow cell lines. STAT2 as one component of the transcription factor complex ISGF3 is exclusively phosphorylated upon type I IFN signaling. Therefore, detection of p-STAT2 would indicate type I IFN signaling as a response to IFN-β production. We could observe p-STAT2 in three of four wt tumor samples, however also in one IFN-β-/- tumor sample. This could be due to infiltrating cells that migrate into the tumor and produce type I IFNs. Alternatively, we tested for constitutive low levels of IFN in our v-abl transformed bone marrow cell lines *in vitro*. To do so, we did quantitative RT-PCR for the *IFNβ* gene. However, we failed to detect a constitutive low level of IFN-β directly in v-abl transformed wt cells. It is possible that these techniques were not sensitive enough to detect low levels of IFN-β produced by tumor cells. Taken together, so far our data do not support the hypothesis that the phenotype observed in the transplantation experiments mentioned above was due to IFN production by tumor cells.

An evolving tumor is constantly confronted with the immune system that can detect and eliminate transformed cells. To determine whether the phenotype is due to tumor cell intrinsic properties that lead to a defective immune response, transplantation experiments with immuno-deficient Rag2-/-γ_c-/- mice were performed. Due to the fact that those mice do not have any B-, T- and Nk cells, respectively, tumor cell autonomous properties are not affected by cellular mediators of the immune system. We could show that recipient mice injected with wt and IFNAR1-/- tumor cells succumbed to B-lymphoid leukemia with comparable latency. This finding suggests that type I IFN signaling is important in Nk cell activation and further

leukemia progression. However, to investigate the importance of type I IFN signaling for Nk cell mediated surveillance of Abelson-induced leukemia additional experiments need to be performed

As mentioned above IFNs have a crucial role in bridging the innate and the adaptive immune system, for example by enhancing effects on Nk cell activity (reviewed in Bellardelli F., 2002) In our laboratory it has been shown that Nk cells are the key mediators of tumor surveillance of Abelson induced leukemia (Stoiber *et al.*, 2004). To investigate whether deficiency in type I IFN signaling leads to a defect in Nk cell cytotoxicity we will cross IFNAR1$^{fl/fl}$ mice with NKcre+ mice (that were generated in our laboratory). With these mice will test whether Nk cell impaired in type I IFN signaling show differences in proliferation compared to wt Nk cells by ^{3}H-thymidin incorporation assays or CFSE labeling assays. In addition, defects in Nk cell effector functions could be elucidated by performing ^{51}chromium release assays to test the capacity of their cytotoxicity, as well as cytokine secretion such as IFN-γ.

During this study we stroke a new path to elucidate the role of type I IFN signaling in v-abl induced leukemia. We could show that v-abl transformed IFN-β and IFNAR1 knock out bone marrow cell lines had an increased expression level of VEGF compared to wt controls. Due to the fact that several studies have demonstrated that IFNs exhibit an anti-angiogenic function by inhibiting the production of angiogenic growth factors, such as basic fibroblast growth factor (bFGF) (Singh *et al.*, 1995) or vascular endothelial growth factor (VEGF) (Rosewicz *et al.*, 2004) we decided to extend our study to the effect of type I IFNs on angiogenesis. We could demonstrate that subcutaneous injection of v-abl transformed knock out bone marrow cell lines yielded in tumors with a better blood supply than wt controls in Rag2-/-γ_c-/- mice. These results indicate that type I IFN signaling is involved in the process of vascularization, which could be also confirmed by scratch assays. We further performed Oligo GEArrays for angiogenesis that resulted in a set of genes, which might be affected by impaired type I IFN signaling. We could show in IFN-β knock out tumor cell lines that Vegfc is strongly up-regulated, which recapitulates the data obtained by qRT-PCR. Even though we obtained various gene expression among the samples of the same genotype we detected that Il12a, pleiotrophin and Tgfb3 are consistently down-regulated in each cell line. As it has been shown that IL-12 is effective in antitumor response by suppressing the development of new blood vessels (Sorensen *et al.*, 2010), down-regulation of Il-12A in IFNAR1 and IFN-β knock

out cell lines is another hint that type I IFN signaling has a regulatory function in angiogenesis. Interestingly, pleiotrophin is reported to promote tumorgenesis and angiogenesis (Laaroubi *et al.*, 1994, Fang *et al.*, 1992). In addition it has been shown that Tgfb3 might play a protective role against tumorgenesis in skin, breast, oral and gastric mucosa (Laverty *et al.*, 2009). It is conceivable that functional type I IFN signaling is needed for up-regulation of pleiotrophin and Tgfb3 in tumor cells. We will have to perform additional qRT-PCR with more v-abl transformed bone marrow cell lines to confirm the obtained phenotype in this experiment. Furthermore we could inject knock out tumor cell lines subcutaneously into Rag2-/-γ_c-/- mice and inject Il12 into the tumor to study if additional Il12 can block vascularization and reverse the phenotype we observed during this master thesis.

Taken together this work demonstrates the importance of type I IFN signaling in tumor progression. The obtained data indicate that an interplay of a tumor cell intrinsic effect and a defect in immune response is responsible for the obtained phenotype during this study.

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6. Abbrevations

ALL	acute lymphoblastic leukemia
A-MuLV	Abelson induced mouse leukemia virus
BCR	breakpoint cluster region
bp	base piar
c-abl	cellular Abelson proto-oncogene
CML	chronic myeloid leukemia
DC	dentritic cell
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetaacid
FACS	flourescence activated cell sorting
FCS	fetal calf serum
FGF	fiboblast growth factor
GAS	interferon gamma activated sequence
IFN	interferon
IFNAR	interferon alpha/beta receptor
IFNAR1	interferon alpha/beta receptor chain 1
IFNAR2	interferon alpha/beta receptor chain 2
IFN-α	interferon alpha
IFN-β	interferon beta
IFN-γ	interferon gamma

IRF	interferon regulatory factor
ISG	interferon stimulated gene
ISGF3	interferon stimulated gene factor 3
ISRE	interferon stimulated response element
JAK	janus kinase
JAK1	janus kinase 1
kDa	kilo Dalton
MCA	methylcholanthren
Mda5	melanoma differentiation associated gene 5
MEF	mouse embryonic fibroblast
Mo-MuLV	Moloney murine leukemia virus
Nk cell	natural killer cell
n.s.	not significant
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PIAS	protein inhibitor of activated STAT
Ptn	pleiotropin
Rag2	recombinase activating gene 2
RIG-I	retinoic acid-inducible gene 1
rpm	rotation per minute
SDS	sodium dodecyl sulphat
SHP	SH-2 domain-containing protein tyrosine phosphatase

SOCS	suppressor of cytokine signaling
STAT1	signal transducer and activator of transcription 1
STAT2	signal transducer and activator of transcription 2
TLR	toll-like receptor
TYK2	tyrosine kinase 2
v-abl	viral Abelson oncogene
VEGF	vascular and endothelial growth factor

Curriculum Vitae

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Internships

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Conference contributions

September 2009	**7th International Symposium on the biology of endothelial cells**, Vienna, Austria Title of presented poster: "Role of type I interferon signaling in tumor development and angiogenesis"
February 2010	**FEBS-Special Meeting 2010:Jak-Stat Signalling: from Basics to Disease,** Vienna, Austria Title of presented poster: The role of type I Interferon in Abelson induced leukemia

Publications

Neugebauer N., **Moritz N**., Eckelhart E., Simma O., Müller M. , Sexl V. and Stoiber D. „ The role of type I interferon in Abelson induced leukemia“
Manuscript in preparation

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- English – spoken - excellent, written - excellent, comprehension - excellent
- Italian –spoken – basic, written – basic, comprehension – basic

Technical
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- Experienced with a variety of biological software (DIVA, tScratch, EP realplex)