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# DIPLOMARBEIT

Titel der Diplomarbeit

„Dissection of kinase-dependent and -independent functions of TYK2 in NK cells“

verfasst von

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## 1. ABSTRACT

The tyrosine kinase 2 (TYK2) is one of four Janus kinases (JAKs) that together with the signal transducers and activators of transcription (STATs) performs cytokine and growth factor signal transduction. TYK2 is critical in innate and adaptive immunity. It mediates resistance to viral and bacterial infections and has crucial roles in tumour surveillance. Natural killer (NK) cells are part of the cellular innate immune defence brigade in host immunity as they rapidly recognise and clear pathogen-infected and malignant cells. Previous research has shown that TYK2 in NK cells contributes to effective killing of malignant cells. In the last years JAKs, including TYK2, were shown to possess biological functions independent from their kinase activity. To determine kinase-independent functions of TYK2 *in vivo*, our lab has generated kinase-inactive *Tyk2* knockin mice by replacing the lysine at position 923 in the ATP-binding site with glutamic aicd (*Tyk2*$^{K923E}$). The presented work is based on the finding that *Tyk2*$^{K923E}$ NK cells show an intermediate killing phenotype between wild-type and *Tyk2*$^{-/-}$ NK cells in cytotoxicity assays with various tumour cells lines. The main aim of this study was to dissect kinase-dependent and -independent functions of TYK2 in the regulation of selected maturation factors and cytotoxic proteins in cultivated and *ex vivo* NK cells. In addition, activation of signalling pathways during cytotoxicity assays should be analysed.

As a main finding, we show reduced STAT5 protein expression in *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ compared to the wild-type NK cells. *Tyk2*$^{K923E}$ NK cells had slightly higher levels of STAT5 than *Tyk2*$^{-/-}$ NK cells, indicating a contribution of kinase-inactive TYK2 to the regulation of STAT5. Interestingly, this expression pattern was dependent on the presence of interleukin-2 (IL-2) as it was neither observed in cells cultivated in the presence of IL-15 nor in NK cells collected *ex vivo*. In addition, we found activation of STAT5 in NK cells incubated with a murine T cell lymphoma cell line (YAC-1) only in wild-type and not in *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ NK cells. We furthermore identified genes regulated in a TYK2 kinase-dependent manner in NK cells grown in the presence of IL-2: Vascular endothelial growth factor (*Vegf)* was upregulated in *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ compared to wild-type NK cells, whereas the activating receptor *Nkg2d* and the death receptor Fas ligand (*FasL)* showed lower expression. Finally, we could show that the levels of the lytic proteins perforin (PRF1) and granzyme B (GZMB) are dramatically higher in *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ than in wild-type NK cells when limiting concentrations of IL-2

(1000U/ml) are used for NK cell growth but not under our standard culturing conditions (5000U/ml).

## 2. ZUSAMMENFASSUNG

Die Tyrosin-Kinase 2 (TYK2) ist eine der vier Janus Kinasen (JAKs), die gemeinsam mit den Signaltransduktoren und Transkriptionsaktivatoren (STATs), die Signaltransduktion von Zyktokinen und Wachstumsfaktoren vermittelt. TYK2 ist ein zentraler Faktor in der angeborenen und adaptiven Immunität, vermittelt Resistenzen gegen virale und bakterielle Infektionen und besitzt eine essentielle Rolle in der Tumor Überwachung. Natürliche Killer (NK) Zellen sind Teil der zellulären Immunabwehr-Brigade, die in der Lage sind pathogen-infizierte wie auch maligne Zellen rasch zu erkennen und zu beseitigen. Vorangehende Studien belegten, dass TYK2 in NK Zellen einen wichtigen Beitrag zur effektiven Bekämpfung maligner Zellen leistet. In den vergangenen Jahren wurde gezeigt, dass JAKs und im Speziellen auch TYK2 biologische Funktionen unabhängig von ihrer Kinase-Aktivität besitzen. Zur *in vivo* Analyse dieser TYK2 Kinase-unabhängigen Funktionen wurden in unserem Labor Kinase-inaktive *Tyk2* „knockin" Mäuse hergestellt, indem Lysin an der Position 923 in der ATP-Bindungsstelle durch Glutaminsäure ersetzt wurde (*Tyk2*$^{K923E}$). Die hier vorliegende Arbeit basiert auf der Erkenntnis, dass *Tyk2*$^{K923E}$ NK Zellen einen intermediären „Killing"-Phänotyp im Zytotoxizitäts-Assay mit diversen Tumorzelllinien zwischen wildtyp und *Tyk2*$^{-/-}$ NK Zellen aufweisen. Vorrangiges Ziel dieser Studie war es Kinase-abhängige und -unabhängige Funktionen von TYK2 in der Regulation von ausgewählten NK Zell-Reifungsfaktoren und zytotoxischen Proteinen in kultivierten und in *ex vivo* NK Zellen zu analysieren. Zusätzlich sollte auch die Aktvierung der Signalwege während des Zytotoxizitäts-Assays dokumentiert werden.

Im Rahmen dieses Projekts gelang es uns reduzierte Expression von STAT5 in *Tyk2*$^{-/-}$ und *Tyk2*$^{K923E}$ im Vergleich zu wildtyp NK Zellen nachzuweisen. *Tyk2*$^{K923E}$ zeigten zudem geringfügig höhere STAT5 Expression als *Tyk2*$^{-/-}$ NK Zellen, was auf eine Beteiligung von Kinase-inaktivem TYK2 an der Regulierung von STAT5 schließen lässt. Interessanterweise war dieses Expressionsmuster von der Anwesenheit von Interleukin-2 (IL-2) im Kulturmedium abhängig, da es weder in IL-15 kultivierten noch in NK Zellen *ex vivo* beobachtet wurde. Überdies konnten wir STAT5 Aktivierung in wildtyp NK Zellen nach Inkubation mit einer murinen T Zell-Lymphoma Zelllinie (YAC-1) nachweisen, nicht aber in *Tyk2*$^{-/-}$ und *Tyk2*$^{K923E}$ NK Zellen. Unsere Studie identifizierte zudem auch Gene, die in IL-2 kultivierten NK Zellen TYK2 Kinase-abhängig reguliert sind: der vaskuläre Endothelwachstumsfaktor *Vegf* war in *Tyk2*$^{-/-}$

und *Tyk2*$^{K923E}$ NK Zellen hochreguliert, wogegen der aktivierende Rezeptor *Nkg2d* sowie der „Todesrezeptor“ *FasL* eine niedrigere Expression als in wildtyp Zellen aufwies. Darüber hinaus fanden wir eine starke Zunahme der lytischen Proteine Perforin (PRF1) und Granzym B (GZMB) in *Tyk2*$^{-/-}$ und *Tyk2*$^{K923E}$ NK Zellen nach Kultivierung mit einer limitierenden IL-2 Konzentration (1000U/ml), nicht aber in Zellen die unter Standardbedingungen (5000U/ml) expandiert wurden.

## 3. INTRODUCTION

### 3.1. JAK-STAT signalling

The very first detection of interferons (IFNs) by Isaacs and Lindemann in the late 1950s[1] fired the starting pistole for great interest in cytokine signalling, leading to the discovery of Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) around three decades later[2]. The pathway is utilized by many cytokines as well as by growth factors and enables rapid and adequate responses to extracellular stimuli. JAK-STAT signalling regulates very diverse processes, such as cell growth and differentiation, and is required for embryonic development, differentiation, metabolism, hematopoiesis and host immunity[3 4].

STATs are transcription factors that transduce signals to alter gene expression patterns. Cell surface receptors bind to extracellular signalling proteins and activate the receptor-associated JAKs. The JAKs then phosphorylate STAT dimers, which subsequently accumulate in the nucleus to drive appropriate gene transcription[5 6 7] (*figure 1A*). Phosphorylation triggers conformational changes within the STAT dimers and induces migration to the nucleus, however it has been shown that unphosphorylated STATs (U-STATs) can induce the expression of specific genes[8].

There are four known JAK family members, JAK1, JAK2, JAK3 and TYK2, sized from 120 to 140kDa, that are expressed in most tissues, except for the leukocyte-specific Jak3. JAKs share seven homologous regions (JH1-JH7)[6 9] (*figure 1B*). The two C-terminal regions JH1 and JH2 correspond to the kinase and pseudokinase domains, respectively, whereas JH3 and part of JH4 form the src-homology (SH2) domain and JH4-7 build the 4.1 protein- ezrin- radixin-moesin- domain (FERM domain). The N-terminal FERM domain fosters binding to cytokine receptor chains, can have scaffolding function for receptor surface expression and regulates kinase activity[10]. Data derived from JAK1 and JAK3 research indicate that also the kinase domain mediates receptor binding[11]. The function of the SH2 domain of JAK proteins is still unclear. Tasks of the pseudokinase domain include scaffolding of domains and regulation of the kinase activity of the kinase domains. However, similar to other pseudokinases[12], recent data suggest that the JAK2 pseudokinase is catalytically active, although with different specificity as the kinase domain and not cytokine inducible[13].

Seven STATs (STATs1-4, STAT5a, STAT5b and STAT6) exist in mammalian cells. Several conserved structures are shared by STATs: the amino terminal domain, a coiled-coil domain, a DNA-binding domain, a linker domain, an SH2 domain and a transactivation domain. The C-terminal transcriptional activation domain is not conserved in structure but in function. Two conserved amino acids (tyrosine and serine) at the C-terminal end are inducibly modified by phosphorylation (*figure 1C*)[5 6].

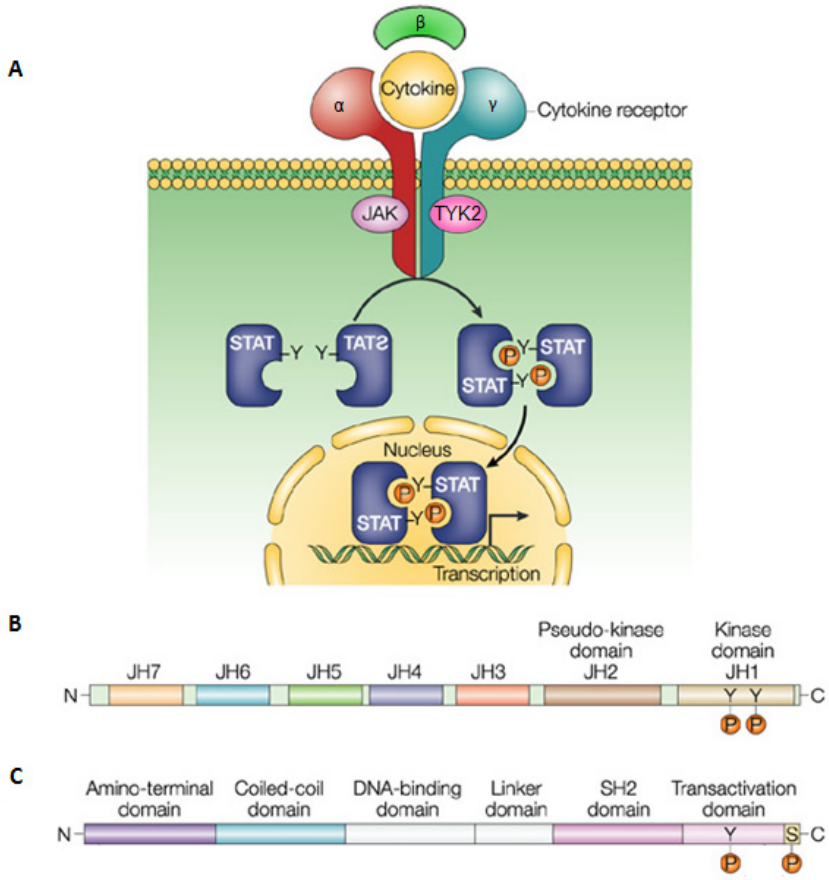


**Figure 1: JAK-STAT Pathway.**
(A) phosphorylation cascade (B) JAK-structure (C) STAT-structure (modified from Nature Review Immunology: Regulation of JAK-STAT signalling in the immune system. Liu B. et al. 2003).

The JAK-STAT pathway consists of a limited number of main participants but is multifarious in its effects and feature to cross-talk with other signalling cascades. Diverse cytokines and growth factors bind to specific receptors on the cell surface and activate specific combinations of JAKs and STATs to transduce ligand-specific signals to the nucleus. Signalling via the JAK-STAT pathway fulfils various functions, whereby some JAKs and STATs contribute exclusively to the immune response. Research using gene-targeted mice enabled the dissection of the specific functions of each JAK and STAT protein. Severe defects in

embryonic development for instance, as well as immunodeficiencies and enhanced tumour development were accredited to single JAK or STAT knockout or mutant mice[6].

### 3.1.2. Tyrosine kinase 2

Tyrosine kinase 2 (TYK2) was the first of the four JAKs identified and its biological significance has been originally discovered by the complementation of a mutant human fibrosarcoma cell line defective in IFNα and β signalling[14]. Further research using gene-targeted *Tyk2* knockout mice[15 16 17] and naturally occurring *Tyk2* mutant mice (B10.D1-H2$^{q}$/SgJ), enabled insight into TYK2 functions *in vivo*[18].

The *Tyk2* gene is located on chromosome 19 in humans and 9 in mice and encodes the multi-domain protein with a molecular weight of around 130 kDa. *Tyk2* is ubiquitously expressed but several TYK2 functions are cell type-specific due to restricted expression of cytokine receptor chains. TYK2 is associated with IFNAR1, IL-12Rβ1, IL-10Rβ, gp130-containg receptor complexes and IL-13Rα1 (*figure 2*). Cell surface receptors chains associated with TYK2 always act in concert with receptor chains associated with JAK1 and/or JAK2 but never JAK3[19].

Loss of TYK2 in murine cells only leads to partial loss of cytokine responses, indicating that TYK2 is never the initiating but rather the signal amplifying kinase. The most prominent defect in the absence of *Tyk2* lies in a strong reduction of IL-12 induced signalling and a consequent defect in IFNγ production. *Tyk2*-deficient mice are highly susceptible to viral, bacterial and parasitic infections but, on the other hand, more resistant to several inflammatory and autoimmune diseases[19]. IL-12 is important for NK cell function and impaired NK cell cytotoxicity has for example been shown in *Tyk2*-deficient mice after infection with the protozoan pathogen *Leishmania major*[20]. *Tyk2* deficiency in human cell lines results in a complete loss of responsiveness to IFNα due to reduced surface expression of IFNAR1[21 22]. This receptor stabilizing property is independent of TYK2 kinase activity and, interestingly, not observed in murine cells[23].

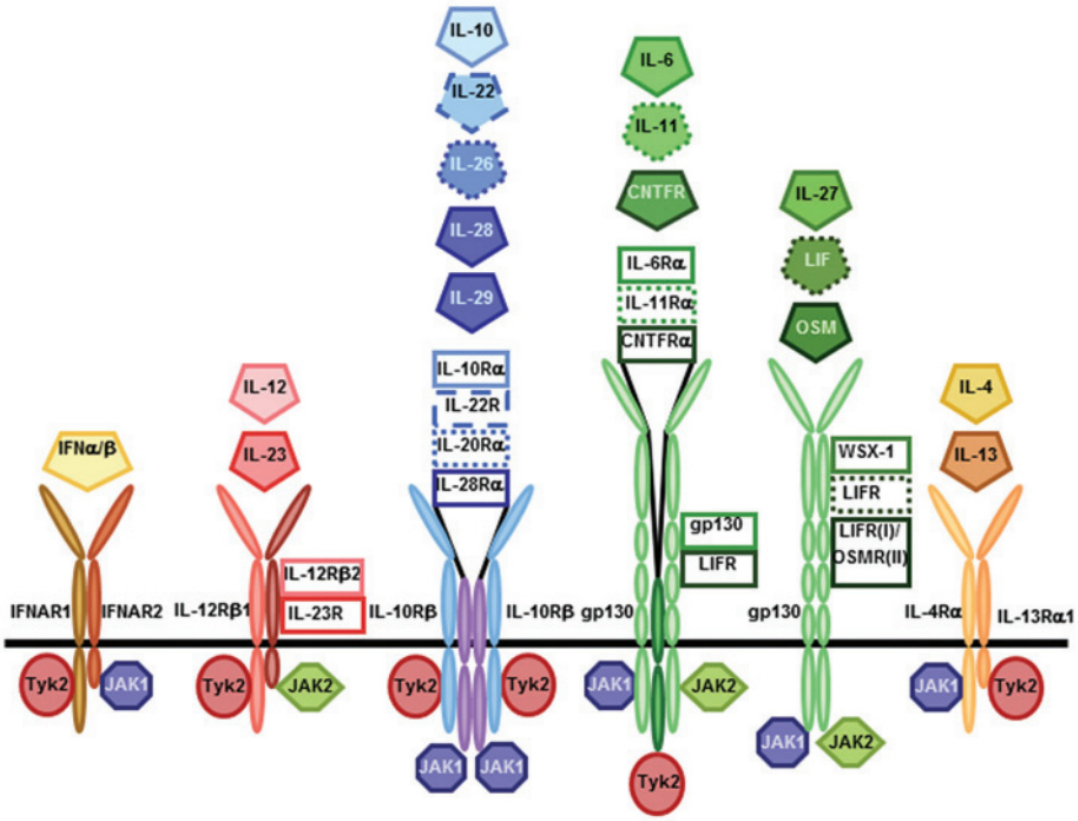


**Figure 2: Overview of cytokine receptors and associated Janus kinases (JAKs).** TYK2 is associates with IFNAR1, IL12Rβ1, IL-10Rβ, IL-6Rα, IL-11Rα1, CNTFRα and IL-13Rα1. Signalling of various cytokines is mediated via TYK2 associated receptors. (picture taken from: Establishing the role of tyrosine kinase 2 in cancer. Übel C, Mousset S, Trufa D, Sirbu H, Finotto S. OncoImmunology 2013).

Two cases of *Tyk2* deficiency in human patients have been described by now and differ slightly in clinical symptoms. One patient displayed hyper-IgE syndrome (HIES), atopic dermatitis and recurrent infectious diseases, such as herpes simplex virus (HSV) infection, oral candidiasis, salmonella gastroenteritis and Bacille Calmette-Guerin (BCG) infection[24]. The second patient also showed severe infections after vaccination with BCG along with neurobrucellosis and cutaneous herpes zoster infection, but totally lacked high IgE levels[25].

TYK2 gene locus polymorphisms have been linked with several autoimmune and inflammatory diseases[19 26 27]. More recently, a TYK2 polymorphism was reported to be protective against infertility and/or endometriosis[28]. A role of TYK2 in cancer was first described in mice, which developed Abelson-induced B lymphoid leukemia/ lymphoma as well as TEL-JAK2-induced T lymphoid leukemia with higher incidence and shorter latency in the absence of *Tyk2*. This was ascribed to an impaired tumour surveillance caused by a decreased IL-12/IL-18-dependent cytotoxic activity of *Tyk2*$^{-/-}$ NK/NKT cells[29]. TYK2 also plays an important role in T cell-mediated tumour surveillance, as *Tyk2*-deficient mice showed higher susceptibility to tumours that are controlled by cytotoxic T cells compared to wild

type mice[30]. Moreover, *Tyk2* deficiency was shown to enhance breast cancer cell growth and metastasis due to increased myeloid suppressor cell activity[31]. More recently, a cell-intrinsic tumour-promoting function of human TYK2 has been reported in T cell acute lymphoblastic leukemia (T-ALL)[32]. Furthermore TYK2 overexpression was found in several cancerous tissues, such as breast and prostate cancer, as well as in squamous cervical carcinoma[33].

### 3.2. NK cells

Natural killer (NK) cells were first discovered in 1975[34] and were defined as large granular lymphocytes (LGL) with a selective cytolytic activity and the ability to lyse murine leukaemia cells. NK cells are crucial in host immunity, in particular for the clearance of pathogen infected and malignant cells[35 36]. During the last years a great deal of research was done on NK cells and their potential use for cancer immunotherapy[37 38 39].

NK cells belong to the innate, evolutionary more ancient branch of the immune defence but recently it was shown that they also act in adaptive immunity as they can have immunological memory[40 41]. Unlike antigen-specific immunity of T or B cells, NK cells do not somatically rearrange T cell receptors or immunoglobulin genes from their germline configuration[42]. In contrast to cytotoxic T cells, NK cells can directly induce apoptosis of target cells without the need for specific immunization. NK cells are thought to be transient as they have a short life expectancy with a half-life of around 7-10 days[43]. Importantly, NK cells differ significantly from natural killer T cells (NKT), as the latter are basically T cells that express the common αβ T cell receptor (TCR) and, additionally, several NK cell surface molecules[44 45].

#### 3.2.1. Definition and identification of NK cells by their surface markers

Several molecules are considered as NK cell surface markers, namely NK1.1 (NKR-P1C, a C-type lectin) in C57BL/6 mice, CD56 in humans, integrin $\alpha_2$ (CD49b/DX5) and NKp46 (Ncr1)[46] across all mammalian species[47]. However, these markers are not unique for NK cells, as they are also expressed on T cells, granulocytes and mast cells. Murine NK cells can be distinctly identified as $CD3^-$ $NK1.1^+$ $CD122^+$ or $CD3^-$ $DX5^+$ $CD122^+$ cells. The lack of CD3 enables the discrimination of NK cells from T cells that may also express NK1.1, DX5 and CD122. In addition, murine NK cells express the dendritic cell (DC) marker CD11c, the B cell marker CD45R (B220) and the B and T cell marker CD2. Many of these molecules are regulated

during NK cell development and maturation (see below) and different surface marker combinations can define distinct NK cell stages[48].

### 3.2.2. Development and maturation of NK cells

NK cell development takes place in the bone marrow and requires a specific microenvironment providing growth factors and cytokines such as IL-15, IL-7 and IL-21[49]. This is followed by phenotypic and functional maturation in the spleen, liver, thymus and lymph nodes. Surface markers used for the determination of NK cell developmental and maturation stages by flow cytometry analysis[50] are indicated in *figure 3*. Some markers are only present in early stages and disappear later on, whereby others start expression with proceeding maturation and remain on the surface later on.

Haematopoietic stem cells (HSCs) are located in the medulla of the bone marrow and are able to give rise to all different blood cell types. The first step is the commitment of HSC to the lymphoid cell lineage, whereby differentiation results in the generation of common lymphoid progenitor cells (CLPs). From the very beginning CLPs express the surface marker CD244, which is continually expressed until the most mature stage of NK cells[51]. Further on, cells develop into pre-natural killer precursor cells (pre-NKPs) by loosing differentiation ability to T cells, B cells or DCs. However, the potential of pre-NKPs to act as a precursor for other innate lymphoid cells still needs to be verified. A further differentiation step leads to the refined NK cell precursor (rNKP)[51], representing the earliest precursor that is committed to the NK cell lineage. The surface marker CD127 (IL-7rα) is present in CLPs and NKPs, but gets down-regulated in rNKPs, whereby cytokine receptor IL-2Rβ (CD122) and activating receptor NKG2D get upregulated during the rNKP cell stage. In further development immature NK (iNK) cells show upregulation of NK1.1[52] and tumour necrosis factor ligand (TRAIL) expression. iNK cells rest in the bone marrow until they get stimulated mainly by IL-15 to promote expression of further maturation markers. These act as cytokine and chemokine receptors to enable the iNK cells to enter the periphery. In the late iNK stage, cells also start expression of natural cytotoxicity triggering receptor 1 (Ncr1/ NKp46) and C-lectin proteins such as Ly49-family members[53]. Further maturation is hallmarked by the expression of a number of surface markers such as CD49b (DX5), CD11b and CD16. The maturation process is ongoing until the cells exhibit a set of surface markers that characterizes a fully mature NK cell (mNK), furthermore also the loss of CD27 hallmarks this

cell stage. They additionally express the markers CD43 as well as the killer cell lectin-like receptor subfamily G member 1 (KLRG1). CD86 and CD69 are only expressed in activated peripheral NK cells[48]. mNK cells are found in bone marrow, spleen, liver, lungs, omentum, blood, intestine and placenta, however mechanisms that direct NK cells to these tissues under normal, non-infectious situations, are still unclear[54]. Degranulation and expression of granzymes (GZMs) and perforin (PRF1) take place in mNK cells that are called effector NK cells in the activated state. Interestingly, PRF1 is also present in memory cells, however GZM expression is found in iNK and mNK cells[41 55]. Memory NK cells show all markers that can be detected on the surface of mNK cells, except that CD69, CD89, CD122 and GZMs are downregulated.

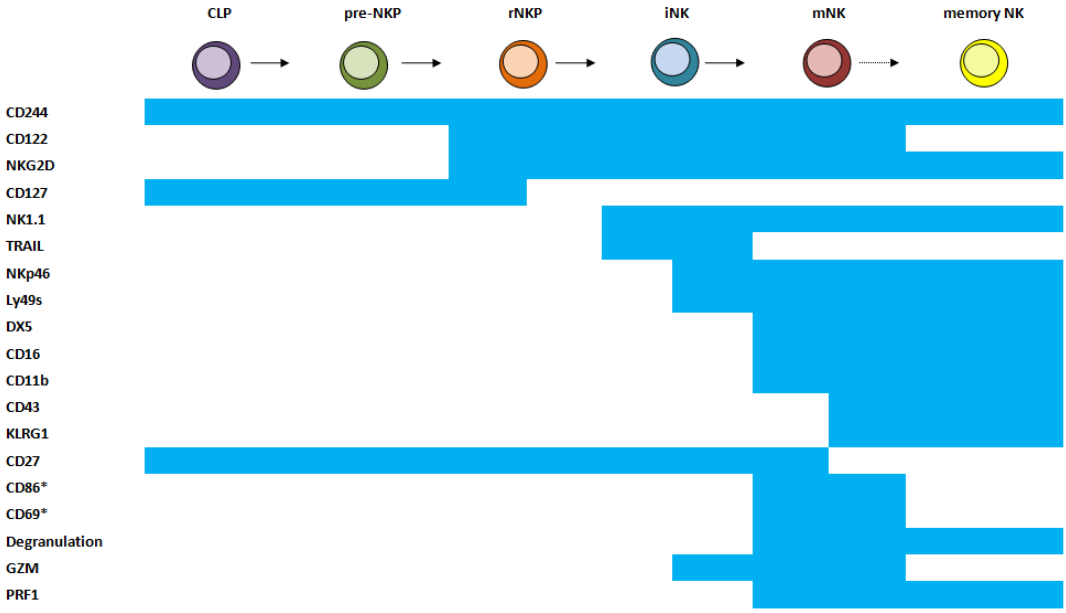


**Figure 3: Factors characterizing stages of NK cell development and maturation**
*cell surface markers CD86 and CD69 are only expressed in activated NK cells that have already entered the periphery. Different stages af NK cells development are defined as follows: common lymphoid progenitor cells (CLPs), pre-natural killer precursor cells (pre-NKPs), refined NK cell precursor (rNKP), immature NK (iNK),mature NK cell (mNK) and memory NK cells. The figure is modified from: Developmental programming of natural killer and innate lymphoid cells. Di Santo J.P. et al., 2013, Current Opinion in Immunology and Epigenetic regulation of NK cell differentiation and effector functions. Bryceson Y.T. et al., 2013, Frontiers in Immunology.

Several transcription factors involved in NK cell development and maturation have been identified. Members of the ETS family transcription factors (i.e. Ets-1 and PU.1), inhibitor of DNA-binding 2 (Id2), and Ikaros family zink finger protein 1 (IKZF1) are required for the generation of NKP cells. Subsequently, GATA binding protein 3 (Gata-3), IFN regulatory factor 2 (IRF2), and T-box 21 protein (Tbet) mediate the generation of iNK cells and CCAAT

enhanced binding protein gamma (CEBPγ) and microphthalmia-associated transcription factor (MITF) are essential for the maturation to mNK cells[56]. Transcription factors Tbet and eomesodermin (EOMES) were found to be of main importance for NK cell maturation as they mediate control of distinct maturation checkpoints[51].

### 3.2.3. NK cell recognition of target cells

NK cells are able to distinguish between normal and infected or malignant cells in their surrounding by scanning with a broad spectrum of cell surface receptors[54]. Activating receptors (ARs) activate NK cells *via* binding to a target cell, and inhibitory receptors (IRs) transmit inhibitory signals if they come across MHC class I molecules or MHC class I-related molecules on target cells. This strategy is called the "missing self" recognition, was put forward by Ljunggren and Karre in 1990[57 58] and implies that the loss or down-regulation of self MHC class I already induces NK cell sensitivity and activation. Interaction of IRs with MHC class I recruits the src homology region 2 domain-containing protein tyrosine phosphatase-1 (SHP-1) and SHP-2, which subsequently inhibit NK cell activating signals[59 60]. NK cell activation arises from a sensitive balance of activating and inhibitory signals[61 62]. In human NK cells, for example, ARs such as the natural cytotoxicity receptors (NCRs, e.g. NKp44 and NKp46) bind to target cells and mediate their rapid cell death when binding of IRs, such as killing inhibitory receptors (KIRs), is missing. NKG2D is a well-known and important AR, which triggers cytotoxicity as well as cytokine/chemokine production (e.g. TNFα, IFNγ, MIP-1β)[63]. Interestingly, ligands of NKG2D, such as Rae-1 and MHC class I polypeptide-related sequence A (MICA) in humans, are structurally similar to MHC class I molecules and can be found on the surface of stressed, virally infected and transformed cells[64 65 66]. It has been shown that there are two splice variants of NKG2D in mice, the long variant NKG2D-L is expressed on all NK cells and associates only the adaptor molecule DAP10, whereas the short variant NKG2D-S was found on the surface of activated NK cells in association with either DAP10 or DAP12[67]. The murine AR Ly49H also triggers NK cell cytotoxicity by association with DAP12 and stimulates NK cell cytokine and chemokine production. In addition to ARs and IRs, cytokines (see below) and costimulatory molecules (e.g. CD27, CD28, inducible co-stimulator molecule (ICOS) and CD40L) can impact on NK cell activation and cytotoxicity[68 69 70].

Another way of target cell recognition is provided by CD16 (FcγIII) binding to the Fc-part of

antibodies, such as IgG, bound to the surface of opsonized cells. In the cytoplasmic domain, the NK cell CD16 contains an activating motif, the immunotyrosine-activating motif (ITAM) that is able to induce further signal transduction resulting in target cell killing *via* granule exocytosis (see below). This process is generally referred to as antibody-dependent cell-mediated cytotoxicity (ADCC) and is of major importance during infectious diseases. Although tumour cells are normally not opsonized, this pathway might be an important tool in monoclonal antibody therapy against cancer[71].

### 3.2.4. Cytokines in NK cell development and function

The cytokines IL-2 and IL-15 are known to be important for development, maturation and activation of NK cells *in vivo*[72]. IL-2 is critical for NK cell homeostasis, but is not absolutely needed for differentiation. IL-15 is considered as the most critical cytokine in NK cell development and homeostasis *in vivo*, as mice deficient for IL-15[73] exhibit more severe defects than mice with IL-2 signalling defects[74]. IL-15 mainly acts as factor for NK cell survival, generation and maintenance and it supports proliferation and development. Moreover, IL-15 is essential for NK cell differentiation from progenitor cells[75] and affects proliferation as well as NK cell survival more than IL-2[49]. Importantly, IL-2 and IL-15 have similar functions, as both enable NK cell proliferation and increase NK cell cytotoxicity[54]. IL-2 and IL-15 bind the same NK cell surface signalling receptor IL-2Rβ-γc[54], however each cytokine also binds its own alpha chain, IL-2Rα and IL-15Rα, respectively. IL-2 signalling is also fully functional in absence of IL-2rα[76]. IL-15 shows no sequence homology with IL-2[77 78] and uses a mechanism named trans-presentation. Thereby the IL-15 trans-presenting cell binds the cytokine to the IL-15Rα on its cell surface and stimulates neighbouring cells through the IL-15/IL-15Rα complex. This complex is, for example, efficiently formed by dendritic cells after Toll-like receptor (TLR) stimulation. Interestingly, only membrane-bound complexes can stimulate NK cells, as secreted complexes do not show the same effect[79]. IL-2 and IL-15 activate JAK1, JAK3 and STAT5, but only IL-2 is able to also activate STAT1 and STAT3[80 81]. Furthermore, IL-2 induces STAT4 activation in primary NK cells and NK cell lines, but not in T cells, whereas IL-15 does not activate STAT4. IL-2 and IL-15 trigger overlapping but also distinct NK cell responses[82], however molecular reasons for these differences remain unknown. IL-2 *in vivo* is mainly produced by T cells and DCs, whereas IL-15 is produced by DCs, monocytes and epithelial cells, but not by T cells[83].

Apart from IL-2 and IL-15 a number of other cytokines affect NK cell development, maturation and function[49]. NK cell activation and IFNγ production is triggered by pro-inflammatory cytokines, such as IL-12, IL-18, IL-21 and IFNα/β[84]. Type I IFNs are extremely important for NK cell cytotoxicity[85] as they stimulate upregulation of FasL and PRF1[86] as well as of NKG2D[87]. IL-12 and IL-18 in concert strongly activate NK cell cytotoxicity. IL-18 alone mainly induces FasL expression, but does not activate IFNγ production, whereas IL-12 strongly induces IFNγ production but only triggers cytotoxicity in synergy with other cytokines[88].

Several cytokines that share the common cytokine receptor IL-2Rγc act on NK cells. IL-4, IL-7, IL-9 and IL-21 all utilize a second and specific receptor chain, namely IL-4R, IL-7Rα, IL-9R and IL-21R and signal through JAK1/JAK3 and STAT3, 5 or 6 (*figure 4)*. The effects of IL-4 on NK cells are not well known. Human NK cells cultured with IL-4 are not able to secrete IFNγ and are not cytolytic after stimulation. IL-4 causes downregulation of NKG2D in murine NK cells and thereby impairs killing of target cells *via* this AR, suggesting a negative influence of IL-4 on NK cell cytotoxicity. IL-7 does not play a role in NK cell killing, but is involved in the regulation of NK cell development. IL-9 appears to only have narrow effects and acts synergistically with IL-2 to reinforce IL-2 activity. Finally IL-21 was shown to costimulate proliferation of NK cells as well as to promote cytotoxicity[49].

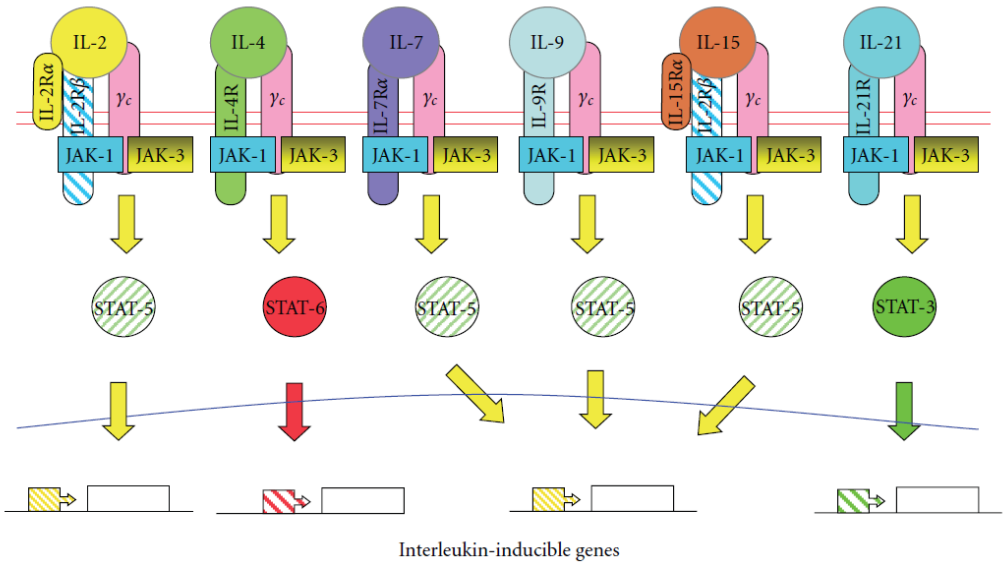


**Figure 4: Cytokines that share the common cytokine-receptor IL-2Rγc for signalling in NK cells.**
Figure taken from: Role of Common Gamma-Chain Cytokines in NK Cell Development and Function: Perspectives for Immunotherapy. Ferrini S., et al., 2011, Journal of Biomedicine and Biotechnology.

### 3.2.5. NK cell cytotoxicity

Once an NK cell is activated, NK cell killing is possible by two different direct mechanisms, using either cytolytic killing through granzymes and perforin or the death-receptor pathway (*figure 5*). Additionally, NK cells are also capable to kill in an indirect manner through secretion of tumour necrosis factor (TNF) superfamily-members and IFNγ[89].

#### *3.2.5.1. Granule exocytosis*

NK cells and cytotoxic T cells share the killing mechanism of granule exocytosis that is provided by the main players perforin (PRF1), a membrane-disrupting protein, and granzymes (GZMs), a family of structurally related serine proteases with various substrate specificities. Granule exocytosis is extremely efficient as killing of infected or malignant cells proceeds within minutes[90]. NK cells release cytotoxic granule contents into the intercellular space between NK and target cell. Formerly PRF1 was assumed to form pores into cell membranes of target cells to enable channelling of GZMs. Today the process of cytotoxic protein uptake is thought to be transmitted by receptor-mediated endocytosis[90], in particular the mannose-6-phosphat receptor (MPR) can act as a receptor for GZMB[91]. It is suggested that PRF1 uses the same mechanism to enter the target cell, although it has not been shown yet. GZMs and PRF1 may bind to the target cell surface as parts of a single macromolecular complex associated with serglycin[92]. After endocytosis, PRF1 is suggested to disrupt the endosome membrane and to trigger the release of GZMs into the cytosol, as it was shown for GZMB[93]. GZMB then triggers apoptotic cell death of target cells directly by mitochondrial BID/tBID-regulated cytochrome c release followed by cellular caspase activation (*figure 5*). However, caspase-blocking does not prevent target cell killing, indicating that an unknown caspase-independent pathway is required[94].

Eleven granzymes have been described (GZM A-H, K, M and N)[95] that mainly differ in preferential cleavage sites. GZMA and K are trypsins, cleaving after basic residues arginin and lysine, whereas GZMB is an aspartic protease, cleaving after aspartic acid residues, mediating oligonucleosomal fragmentation of DNA[90]. GZMA has been shown to induce target cell death indirectly by activating factors that generate single-strand DNA nicks[96]. In concert with PRF1, GZMB and GZMA are able to induce nuclear damage. Although PRF1 is not inducing cell death by itself it is vital for NK cell cytotoxicity, as target cell killing is dramatically impaired in *Prf1*-deficient mice[97]. In tumour target cells GZMB definitely

requires PRF1 for fully cytotoxic function[98]. Mice lacking one or several GZMs have been reported to exhibit more serious immune deficits than *Prf1*-deficient mice on the same genetic background[99]. However, there is evidence for a central role for PRF1 in situations of immune system disturbance due to pathogen infection, autoimmunity or the loss of other cell death pathways[99].

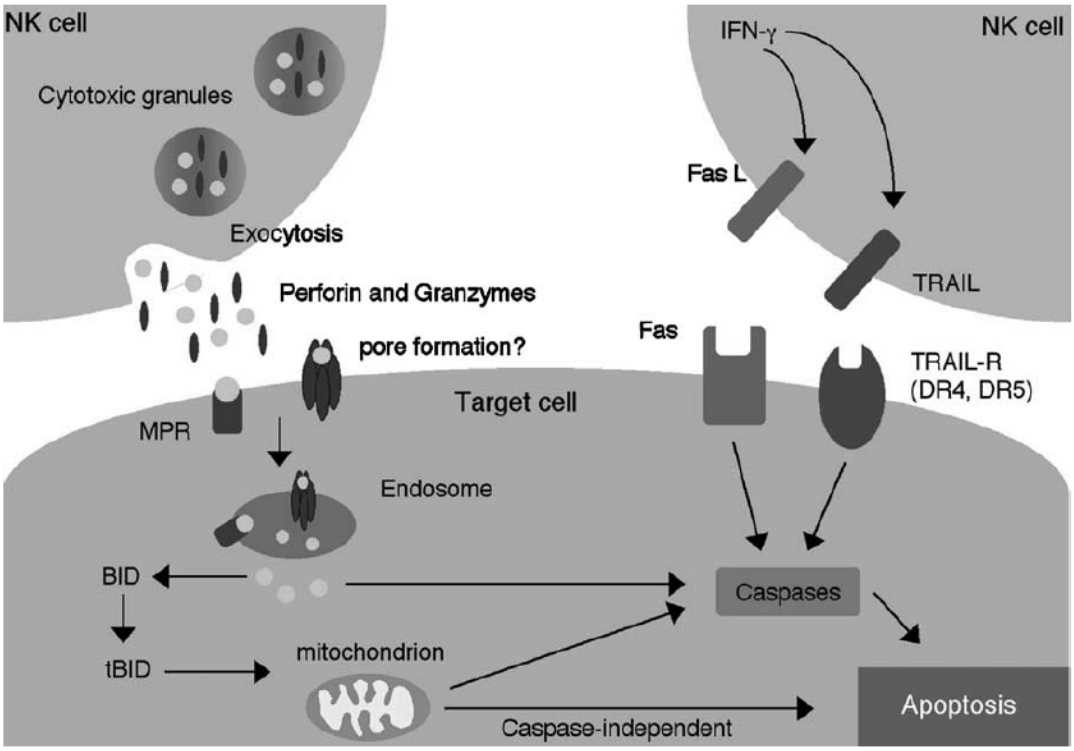


**Figure 5: NK cells exert two main direct killing mechanisms.**
The left side of the picture shows granule exocytosis that is mediated by Gzms and Prf1 which enter the target cell *via* the mannose 6-phosphat receptor (MPR) or pore formation, respectively. Gzms are able to follow caspase-dependet or -independet pathways to induce apoptosis, whereby one includes a family of pro-apoptotic regulators BCL-2 interacting domain (BID) and the truncated BID (tBID). On the right side the classical death receptor pathway is depicted, which uses TRAIL and/or FasL to induce target cell death. Figure taken from: Activation of NK cell cytotoxicity. Hayakawa Y., Smyth M.J., et al., 2004, Molecular Immunology.

### *3.2.5.2. Death receptor pathway*

The classical death receptor pathway in NK cells is mediated by Fas ligand (FasL) and/or TNF-related apoptosis inducing ligand (TRAIL/ Apo2L) binding to corresponding receptors and inducing apoptosis in a caspase-dependent manner[90] (*figure 5*). TRAIL binds to DR4, DR5 and osteoprotegerin (OPG) in humans[100] and to DR5 in mice[101], whereas FasL binds to Fas receptor (FasR/ CD95) in both species. TRAIL expression is highly upregulated on the NK cell surface after stimulation with IL-2 and/or IL-15 and was shown to be involved in NK cell killing of TRAIL-sensitive tumour cells[102]. TRAIL-mediated killing strongly depends on IFNγ,

which induces expression of TRAIL on NK cells and predisposes tumour cells to TRAIL-mediated cytotoxicity[103]. Control of encephalomyocarditis virus (EMCV) is also highly dependent on NK cell activation including upregulation of TRAIL[104]. FasL-mediated killing is facilitated by the induction of FAS receptor expression on tumour cells through NK cell-derived IFNγ. FasL-mediated killing is known to play a crucial role in tumour clearance[90].

#### *3.1.5.3. Cytokine secretion*

Various cytokines secreted by NK cells are essential for the activation of other immune cells[105], but some also act in an autocrine/paracrine manner. NK cells and T cells are the main producers of IFNγ. IFNγ production of activated NK cells is of particular importance as it induces appropriate adaptive immune responses and restricts tumour angiogenesis[106] [107]. Furthermore, IFNγ is a main activator of macrophages and is capable to inhibit viral and bacterial replication directly[108]. Activated NK cells also secrete TNFα, which exhibits important regulatory functions in immunity. For example, it activates macrophages (e.g. stimulates their competence for phagocytosis) and it promotes migration of neutrophil granulocytes to the site of infection[109]. Activated NK cells secrete a broad repertoire of additional cytokines and growth factors, such as IL-10, IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF). They also secrete various chemokines, such as MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), XCL1 and IL-8 (CXCL8)[110] [111].

## 4. AIMS OF THE STUDY

### 4.1. Background of the project

JAKs can have biological functions independent from their kinase activity. Non-catalytical functions of TYK2 are essential for stable surface expression of the human IFNAR1[22]. In line with this, two recently characterized rare disease-associated human TYK2 variants that lack catalytical activity mediate fully functional IFNα signalling[27]. TYK2 kinase-dependent and -independent functions are involved in the regulation of murine mitochondrial respiratory processes[112] and activation of the phosphoinositol3-kinase-pathway (PI3-K) in response to IFNβ requires TYK2 but not its kinase-activity[113].

To determine kinase-independent functions of TYK2 *in vivo*, our lab has generated kinase-inactive *Tyk2* knockin mice. The tyrosine kinase was inactivated by replacing the lysine at position 923 in the ATP-binding site with glutamic aicd (K923E). Analysis of primary cells derived from *Tyk2*$^{K923E}$ mice revealed an essential requirement for the TYK2 kinase activity for efficient signalling by IL-12 and IFNβ. In line with this, *Tyk2*$^{K923E}$ mice displayed similar susceptibility to viral infections as *Tyk2* knockout mice[114]. Interestingly, TYK2 kinase-independent functions were found in tumour surveillance. *Tyk2*$^{K923E}$ and wild-type mice showed higher resistance to tumour growth upon subcutaneous transplantation of several distinct tumour cell lines (i.e. MC38 adenocarcinoma, EG7 thymoma and RMA-S lymphoma) than *Tyk2*$^{-/-}$ mice. This correlated with an increased cytotoxic activity of *Tyk2*$^{K923E}$ compared *to Tyk2*$^{-/-}$ NK cells, whereas cytotoxic T cell (CTL) activity was similarly impaired in *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ mice (Prchal-Murphy et al., manuscript in preparation).

### 4.2. Aims

The aims of this study are based on the finding that NK cells expressing a kinase-inactive *Tyk2* (*Tyk2*$^{K923E}$) show an intermediate cytotoxic activity against several different tumour cell lines and an intermediate maturation phenotype between wild-type and *Tyk2*$^{-/-}$ NK cells (Prchal-Murphy et al., manuscript in preparation). The mechanisms are still unclear and the goal of the study was to determine kinase-dependent and -independent functions of TYK2 in the regulation of genes known to impact on NK cell maturation and cytotoxicity under different culturing conditions and *ex vivo*.

*Tyk2* kinase-dependent and -independent functions in candidate gene regulation in NK cells.

The first aim was to compare the expression of candidate genes in NK cells derived from wild-type, *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ mice. NK cells that were *in vitro* expanded in the presence of IL-2, similar to those used in cytotoxicity assays, and genes known from literature to be important for cytotoxicity and/or maturation of NK cells were selected for the analysis. Dependent on the results, gene expression should also be determined in non-activated NK cells (*ex vivo*).

Activation of JAK-STAT signalling in NK cells during cytotoxicity assay.

The second aim was to determine whether the JAK-STAT pathway is activated in the course of a cytotoxic assay of NK cells with YAC-1 tumour cells. In particular, expression and phosphorylation of STAT1, STAT3, STAT4 and STAT5 and a potential influence of TYK2 and TYK2$^{K923E}$ should be investigated. Furthermore, expression of the lytic proteins GZMB and PERF1 should be determined and compared between NK cells incubated with or without YAC-1 cells.

Elucidating the impact of culturing conditions on NK cells and the role of TYK2.

NK cells are commonly cultured with IL-2 and/or IL-15, , whereby different concentrations are applied in literature. Thus, we wanted to investigate if the culture conditions used impact on the phenotype of *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ NK cells. To this end, we firstly wanted to determine limiting IL-2 and IL-15 concentrations for wild-type NK cell growth. Secondly, wild-type, *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ NK cells should be grown under selected conditions and compared with respect to NK cell yield, levels of lytic proteins and, dependent on the results from above, expression of further candidate genes.

Determination of *Tyk2*-deletion efficiency in *Tyk2*$^{\Delta NK}$ cells.

To study NK cell-intrinsic functions of TYK2 our lab has generated mice with *Tyk2* specifically deleted in NK cells. These mice (*Tyk2*$^{\Delta NK}$*)* have been generated by crossing *Tyk2*$^{fl/fl}$ mice to *Ncr1-Cre* mice, which contain a transgene with the *Cre* recombinase under the control of the Ncr1 (NKp46) promoter[115]. NK cells derived from these mice should be tested for the *Tyk2* deletion efficiency on the mRNA expression level.

# 5. MATERIALS AND METHODS

## 5.1. Buffers and reagents

| | |
|---|---|
| 1x SDS-PAGE running buffer: | 192mM glycine<br>25mM Tris-HCl pH 7.4<br>0.1% SDS |
| 1x Western blot transfer buffer: | 25mM Tris.base<br>150mM glycine<br>20% methanol |
| Stacking gel: | 5% acrylamid/ bisacrylamid (37.5:1)<br>126mM Tris-HCl pH 6.8<br>0.1% SDS<br>0.06% APS<br>0.07% TEMED |
| Running gel: | 8% or 10% acrylamid/ bisacrylamid (37.5:1)<br>375mM Tris-HCl pH 8.8<br>0.1% SDS<br>0.075% APS<br>0.09% TEMED |
| 2x Lämmli sample buffer (LSB): | 126mM Tris-HCl pH 6.8<br>20% glycerin<br>4% SDS<br>200mM DTT<br>0.02% bromophenol blue |
| 1x PY-TBST: | 10mM Tris-HCl pH 7.4<br>75mM NaCl |

| | |
|---|---|
| | 1mM EDTA pH 8<br>0.1% Tween-20 |
| Strip buffer: | 2M glycine-HCl pH 2.5<br>0.25% SDS |
| Blocking solution: | 5% milk powder in 1x PY-TBST or<br>5% BSA in 1x PY-TBST |
| 1x Schindler lysis buffer: | 50mM Tris-HCl pH 8<br>150mM NaCl<br>0.5% NP40 (IPEGAL)<br>10% glycerol<br>1mM DTT<br>0.1mM EDTA<br>2mM sodium-vanadate<br>25mM sodium-fluoride<br>1mM PMSF<br>1ng/µl pepstatin<br>10ng/µl leupeptin<br>0.1U/µl aprotinin |
| DEPC-$H_2O$: | 0.01% DEPC in sterile MQ-$H_2O$, incubated o/n and autoclaved |
| TE buffer: | 10mM Tris-HCl pH 8<br>1mM EDTA pH 8 |

| Materials | Company | Code/ Cat. No. |
|---|---|---|
| 2-mercaptoethanol | Invitrogen, Lofer, Austria | 1242924 |
| Bio-Rad Protein Assay | Bio-Rad Laboratories Inc., Hercules, USA | 500-0006 |
| Bovine serum albumin (BSA) | Roth Lactan, Graz, Austria | T844.3 |
| Dithiothreitol (DTT) | Invitrogen/ Gibco, Lofer, Austria | D-1532 |
| ™Prestained Protein Ladder | Thermo Scientific | 26620 |
| Western Blotting Detection Reagents (ECL™ Amersham™) | GE Healthcare, Munich, Germany | RPN2106 |
| Fetal calf serum (FCS), heat-inactivated | Invitrogen/Gibco, Lofer, Austria | 10270-106 |
| Nitrocellulose-Hybond™- ECL membrane | GE Healthcare, Munich, Germany | RPN303D |
| Mouse IL-2 recombinant protein | eBioscience, San Diego, USA | REF14-8021 |
| Mouse IL-15 recombinant protein | eBioscience, San Diego, USA | REF14-8153 |
| CD49b (DX5) MicroBeads, mouse | Miltenyi Biotec GmbH, Bergisch Gladbach, Germany | 130-052-501 |
| autoMACS separation buffer | Miltenyi Biotec GmbH; Bergisch Gladbach, Germany | 130-091-221 |
| Red bood cell lysing buffer | Sigma-Aldrich Chemie GmbH, Vienna, Austria | R7757 |
| Trypan blue solution 0,4% | Sigma-Aldrich Chemie GmbH, Vienna, Austria | T8154 |
| peqGOLD TriFast | Preqlab Biotechnologie GmbH, Erlangen, Germany | 30-2020 |
| Dulbecco´s phosphate buffered saline | PAA Laboratories, Pasching, Austria | H15-002 |
| Penicillin/streptomycin (100x) | PAA Laboratories, Pasching, Austria | P11-010 |
| RPMI + L-glutamin | PAA Laboratories, Pasching, Austria | E15-840 |

**Table 1: Buffers and reagents**

## 5.2. Mice

Wild-type (WT, C57BL/6N) mice were purchased from Charles River Laboratories. *Tyk2*$^{-/-}$ (B6N.129P2-*Tyk2*$^{tm1Biat}$)[15], *Tyk2*$^{K923E}$ (B6.129P2-*Tyk2*$^{tm3(K923E)Biat}$)[114] and *Tyk2*$^{\Delta NK}$ (B6.129P2-*Tyk2*$^{tm1(fl)Biat}$-*Tg(Ncr1-Cre)*$^{Biat265}$) mice were on C57BL/6N background. *Tyk2*$^{\Delta NK}$ mice have been generated by crossing *Tyk2*$^{fl/fl}$ (B6.129P2-*Tyk2*$^{tm1(fl)Biat}$) mice (Vielnascher et al., manuscript in preparation) with transgenic mice expressing the *Cre* recombinase under the control of the Ncr1(NKp46) promoter[115]. Mice were kept under specific pathogen-free conditions according to FELASA guidelines.

### 5.3. Cell culture

For all centrifugation steps a „Hereaus Multifuge 1S" (Thermo Fisher Scientific, Waltham, USA) was used with standard settings of 1000rpm at room temperature (RT) for 5 minutes. All cells were grown at 37°C and 5% $CO_2$.

#### 5.3.1. Isolation of murine NK cells

Male mice aged 6-7 weeks were sacrificed using $CO_2$ for euthanasia. Spleens were taken, homogenized through cell strainers 100µm nylon (BD Bioscience Europe, Erembodegem, Belgium) and splenocytes from 4 spleens per genotype were pooled and washed with PBS. Red blood cell lysis was done with red blood cell lysing buffer (Sigma-Aldrich Chemie GmbH, Vienna, Austria) using 1ml per spleen. Splenocytes were incubated with 50µl of MACS microbeads (CD49b/DX-5) and 200µl of MACS buffer per spleen for 20min at 4°C in the darkness. NK cell purification was done with MACS LS columns, washing and eluting the columns was done with MACS buffer according to the manufacturer's instructions. Eluted cells were either used directly for the analysis (*ex vivo* NK cells) or expanded *in vitro*.
The purity of NK cells directly harvested after MACS isolation was usually around 60%, obtaining around $3x10^6$ cells. Purity could be increased by an additional MACS separation, however this would lead to a considerable loss of cells (Agnieszka Witalisz, personal communication). Typical yield of protein and total RNA after one MACS separation was 60µg and 6µg, respectively, from 2 spleens.

#### 5.3.2. *In vitro* expansion of NK cells

Eluted cells from 4 spleens were washed in RPMI, resuspended in 1ml of NK cell medium and cultured on 24-well plate for 3-4 days. Cells were splitted 1:3 as soon as 95% confluency was reached (usually on day 3 or 4) and pooled into a 6-well plate on day 6 with the addition of 2ml NK cell medium. In case NK cells were not sufficiently confluent, 300µl of medium was exchanged with fresh medium every second day. Cells were counted right after isolation, before each splitting step and before harvesting. A Neubauer counting chamber (depth = 0.100mm; $0.0025mm^2$) was used applying 10µl of a 1:1 mix of resuspended cell suspension and trypan blue solution (0.4%) to one chamber. Before RNA and protein isolation, NK cells were counted and seeded at $1x10^6$ cells/well in 24-well plate with 500µl of cytokine-free

RPMI medium for 4 hours at 37°C. This incubation step without cytokine addition enables generation of NK cells with an equal status for further analysis.

With this protocol a maximum of up to $1x10^7$ cells and approximately 750µg protein or 120µg of total RNA were obtained per 4 spleens.

NK cell medium: RPMI with L-glutamin, 10% FCS (heat-inactivated), penicillin/streptomycin (100U/ml and 100µg/ml), 50µM 2-mercaptoethanol and mouse IL-2 recombinant protein (5000U/ml).

### 5.3.3. Isolation of thymocytes and bone marrow cells

Male mice aged 6-7 weeks were sacrificed using $CO_2$ for euthanasia. Thymus was dissected and homogenized through cell strainers 100µm nylon, cells were pooled from three mice per genotype and counted directly without any further treatment. For the isolation of bone marrow cells, femur and tibia were cut under sterile conditions on both ends and flushed with PBS. Cells were collected in tubes and counted. Cell counting was performed as described above. RNA was isolated from $1x10^7$ cells in 4 technical replicates for each single cell suspension.

### 5.4. Protein isolation/ SDS-PAGE and Western blot

NK cells were harvested ($1x10^6$ cells per sample), washed with PBS (centrifugation with Hereaus Multifuge 1S 1000rpm, RT, 5min) and lysed in 60µl Schindler lysis buffer for 20 minutes on ice. Cell lysates were centrifuged (13000rpm, 4°C, 5 min) and supernatant was collected. The protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., Hercules, USA) diluted 1:5 with MQ-$H_2O$. Concentrations were adjusted to 1µg/µl with Schindler lysis buffer and mixed 1:1 with 2x Lämmli sample buffer (LSB).

SDS-PAGE was performed to separate proteins (100min, 120V, 8 or 10% final acrylamide/bisacrylamide concentration dependent on the size of proteins of interest). ™Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, USA; diluted 1:5 in 2x LSB) was used as a molecular weight marker. Semidry Western blot was performed with a Bio-Rad Trans-Blot SD, Semidry Transfer-Cell (Bio-Rad Laboratories Inc., Hercules, USA) using nitrocellulose membranes (GE Healthcare, Munich, Germany) and Whatman™ (GE Healthcare, Munich, Germany) filter papers equilibrated in Western blot transfer buffer. Blotting was performed with $1mA/cm^2$ for 2 hours. Blocking of membranes was done in 1x PY-TBST + 5% milk powder at RT for 1 hour, except for the detection of GZMB and PRF1,

where blocking was performed in 1x PY-TBST + 5% BSA. Membranes were probed with primary and secondary antibodies listed below (*table 3* and *4*).

Primary antibodies were diluted 1:2000 in 1x PY-TBST buffer (+1% BSA, 0.01% sodium azide), except for GZMB and PRF1. Due to strong differences in expression levels, detection conditions for GZMB and PRF1 had to be optimized in the distinct NK cell cultures and optimized antibody dilutions are listed in *table 2*. HRP-linked secondary antibodies were diluted 1:2000 in 1x PY-TBST (+1% milk powder). Blot incubation was performed for 4 hours or overnight at 4°C for primary antibodies, and for 1 hour at RT for secondary antibodies. Chemiluminescent detection was done using Western Blotting Detection Reagents (ECL™ Amersham™, GE Healthcare, Munich, Germany), exposing the blot to an X-ray film and processing with Optimax X-ray film processor (PROTEC Processor Technology GmbH & Co KG, Oberstenfeld, Germany).

| | protein | cell source | blocking | conc. of primary antibody | conc. of secondary antibody | % of gel |
|---|---|---|---|---|---|---|
| *ex vivo* | **GZMB** | rabbit | 5% BSA | 1:10000 in 1% BSA | 1:2000 in 1% BSA | 10% |
| | **PRF1** | rabbit | 5% BSA | 1:5000 in 1% BSA | 1:2000 in 1% BSA | 10% |
| expanded *in vitro (IL-2)* | **GZMB** | rabbit | 5% BSA | 1:2000 in 1% BSA | 1:2000 in 1% BSA | 10% |
| | **PRF1** | rabbit | 5% BSA | 1:2000 in 1% BSA | 1:2000 in 1% BSA | 10% |

**Table 2: GZMB and PRF1 Western blot conditions**

| Primary antibody | Source | Company | Cat. no. | Size |
|---|---|---|---|---|
| anti-GZMB | rabbit | Cell Signaling Technology, Danvers, USA | 4275 | 30-37 kDa |
| anti-PRF1 | rabbit | Cell Signaling Technology, Danvers, USA | 3693 | 70-75 kDa |
| anti-STAT1 | rabbit | Cell Signaling Technology, Danvers, USA | 9172 | 84 + 91 kDa |
| anti-phospho-STAT1 (Tyr 701) | rabbit | Cell Signaling Technology, Danvers, USA | 9171 | 84 + 91 kDa |
| anti-STAT3 | rabbit | Cell Signaling Technology, Danvers, USA | 4904 | 79 + 86 kDa |
| anti-phospho-STAT3 (Tyr 705) | rabbit | Cell Signaling Technology, Danvers, USA | 9137 | 79 + 86 kDa |
| anti-STAT4 | rabbit | Cell Signaling, Technology, Danvers, USA | 2653 | 81 kDa |
| anti-phospho-STAT4 (Tyr 693) | mouse | BD Biosciences, New Jersey, USA | 612738 | 81 kDa |

| | | | | |
|---|---|---|---|---|
| anti-STAT5 | rabbit | Santa Cruz Biotechnology, CA, USA | sc-835 | 90 kDa |
| anti-phospho-STAT5 (Tyr 695) | mouse | Cell Signaling Technology, Danvers, MA | 9356 | 90 kDa |
| panERK | mouse | BD Biosciences, New Jersey, USA | 610124 | 85, 56, 44 and 42 kDa |
| anti-actin | rabbit | Santa Cruz Biotechnology, CA, USA | sc-7210 | 44 kDa |

**Table 3: Western blot primary antibodies**

| Secondary antibody* | Source | Company | Cat. no. |
|---|---|---|---|
| *anti-mouse IgG HRP-linked F(ab')$_2$* | sheep | GE Healthcare, Munich, Germany | NA9310 |
| *anti-mouse IgG HRP-linked* | horse | Cell Signaling, Danvers, USA | 7076 |
| *anti-rabbit IgG HRP-linked* | goat | Cell Signaling, Danvers, USA | 7074 |
| *anti-rabbit IgG HRP-linked F(ab')$_2$* | donkey | GE Healthcare, Munich, Germany | NA9340 |

**Table 4: Western blot secondary antibodies**
* Anti-mouse and anti-rabbit IgG secondary antibodies were used from two different companies with no obvious difference in results.

### 5.5. RNA isolation/ cDNA synthesis/ qPCR

RNA was isolated from NK cells with peqGOLD TriFast according to the manufacturer's instructions. Briefly, samples in 850µl TriFast were mixed with 180µl of chloroform, inverted and centrifuged (Hereaus Multifuge 1S, 15min, 13500rpm, 4°C). Upper aqueous phase was aliquoted and mixed with 450µl of isopropanol, incubated for 10min and centrifuged (Hereaus Multifuge 1S, 10min, 13500rpm, 4°C). Supernatant was removed and pellet was washed with ethanol (75%) and centrifuged (Hereaus Multifuge 1S, 5 min, 8500rpm, 4°C). Ethanol was removed and pellet was dried (60°C, thermo block). The pellet was resolved in 20-50µl DEPC water, incubated on ice for 15 min followed by incubation at 60°C for 15 min. Aliquots of RNA samples were diluted 1:25 in TE buffer and RNA concentration was determined by measurement of the optical density at 260nm and 280nm using a spectrophotometer U-3000 (HITACHI) and quartz cuvettes. RNA samples were adjusted to equal concentrations and DNAse treatment was performed with iSCRIPT DNase treatment kit, applying 1µg of RQ1 DNase per µg of RNA. Subsequently, RNA was transcribed into cDNA

using iSCRIPT cDNA synthesis kit (1µg RNA/20µl reaction volume). qPCR was performed in 96 well PCR plates, using Stratagene Mx3000P (Agilent Technologies, Santa Clara, USA) and Mx Pro program for Windows. Depending on the particular target gene, assays with either a FAM-labeled probe (e.g. *Ube2d2*) or EvaGreen® (e.g. *GZMB, PRF1*) were used (*table 5*). The fluorescent dye EvaGreen® intercalates into double stranded nucleic acid and therefore specificity of PCR products was verified with melting curve analysis.

qPCR reaction for assays using a FAM-labeled probe: $MgCl_2$ (4mM), 1x Hotfire polymerase buffer, dNTP mix (200µM), primers (each 300nM) and Hotfire polymerase (1U/rxn), FAM-probe (100nM) + 1µl cDNA for a prerun and 2µl cDNA for a main run were applied from each sample.

qPCR reaction for EvaGreen®-based assays: $MgCl_2$ (4mM), 1x Hotfire polymerase buffer, dNTP mix (200µM), primers (each 300nM) and Hotfire polymerase (1U/rxn), EvaGreen® (100nM) + cDNA as described above.

qPCR reaction for QuantiTect® (Quiagen, Venlo, Netherlands) assays with EvaGreen® dye and ready-to-use mixtures of forward and reverse primers: $MgCl_2$ (2,5mM), 1x Hotfire polymerase buffer, dNTP mix (200µM), Quiagen assay primers (100nM) and Hotfire polymerase (1U/rxn), EvaGreen® (100nM) + cDNA as described above. Quiagen primers are listed in *table 6*.

Cycling conditions for all assays: 95°C for 15 min, 95°C for 20 sec and 60°C for 1 min (40 cycles). To test the quality of the cDNA, preruns of all samples including $RT^-$ controls (no reverse transcriptase added) were performed. In main runs duplicate measurements were performed and appropriate standard curves were included. Relative expression levels of target genes, normalized to the housekeeping gene *Ube2d2*, were calculated with the standard curve method using Microsoft Excel.

| Primers | Sequence | Reference |
|---|---|---|
| *Ube2d2* | fw.: 5´-AGGTCCTGTTGGAGATGATATGT-3´<br>rev.: 5´-TTGGGAAATGAATTGTCAAGAA-3´<br>FAM: 5´(6FAM)AGCCCACGTCGTAGCAAACCACC (BHQ1)-3´ | RTPrimerDB<br>ID 3377* |
| *Gzma* | fw.: 5´-GGAGCAGAGAGGGAGATAATCGGA-3´<br>rev.: 5´-ATTGCGCCTGGGCAGGTTGAG-3´ | designed by Putz E.M. |
| *Gzmb* | fw.: 5´-CCAATCAGATATGTGCGGG-3´<br>rev.: 5´-GGAAACTATGCCTGCAGGG-3´ | designed by Putz E.M. |

| | | |
|---|---|---|
| *Gmzc* | fw.: 5´-CCAATCAGATATGTGCGGG-3´<br>rev.: 5´-GGAAACTATGCCTGCAGGG-3´ | designed by Putz E.M. |
| *Prf1* | fw.: 5´-GATGTGAACCCTAGGCCAGA-3´<br>rev.: 5´-GGTTTTTGTACCAGGCGAAA-3´ | designed by Putz E.M. |
| *mVegf* | fw.: 5´-ATGCGGATCAAACCTCACCAA-3´<br>rev.: 5´-TTAACTCAAGCTGCCTCGCCT -3´ | designed in Department of Medical Biotechnology (Prof. Dr. Jozef Dulak), Jagiellonian University, Krakow, Poland |
| *mTnfa* | fw.: 5´-CAAAATTCGAGTGACAAGCCTG-3´<br>rev.: 5´-GAGATCCATGCCGTTGGC -3´ | RTPrimerDB<br>ID 3747* |
| *Tgfb1* | M00441724_m1 | Applied Bioscience, Foster City,USA[116] |

**Table 5: qPCR primers with EvaGreen®**
all acquired from Sigma, St. Louis, USA, except for *mTnfa* from Invitrogen, Lofer, Austria, and *Tgfb1* from Applied Biosystems, Foster City, USA**.** *details available through the RT primer and probe database (RTPrimerDB): http://medgen.ugent.be/rtprimerdb

| Primers | Sequence |
|---|---|
| *Il2ra* | QuantiTect® Primer Assay: Mm_Il2ra_1_SG<br>Cat.no.: QT00101108 |
| *Il15* | QuantiTect® Primer Assay: Mm_Il15_1-SG<br>Cat. no.: QT00107653 |
| *Tbet* | QuantiTect® Primer Assay: Mm_ Tbx21_1-SG<br>Cat. no.: QT00129822 |
| *Nkg2d* | QuantiTect® Primer Assay: Mm_Klrk1_1-SG<br>Cat. no.: QT00104363 |
| *Eomes* | QuantiTect® Primer Assay: Mm_Eomes_1-SG<br>Cat. no.: QT01074332 |
| *Fasl* | QuantiTect® Primer Assay: Mm_Tnfsf6_1-SG<br>Cat. no.: QT00102970 |
| *Trail* | QuantiTect® Primer Assay: Mm_Tnfrsf8_1-SG<br>Cat. no.: QT00116599 |

**Table 6: qPCR assays purchased from Quiagen (Venlo, Netherlands).**

### 5.6. Enzymed-linked immunosorbent assay (ELISA)

ELISA was performed using the Mouse IFN-gamma Platinum ELISA Kit (eBiosciences, San Diego, USA; Cat. No.: BMS 606TWO), according to the standard protocol supplied by the company.

# 6. RESULTS

## 6.1. Candidate gene expression in wild-type, *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ NK cells (*in vitro* expanded)

To identify mechanisms underlying the differences in maturation and cytotoxicity of wild-type, *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ NK cells, we first analysed mRNA expression of candidate genes in NK cells, which were expanded for 7 days in the presence of IL-2. We selected genes that are known to be involved in target cell killing (*figure 6A*), genes encoding relevant cytokines/cytokine receptors (*figure 6B*) and genes involved in NK cell maturation and/or activation (*figure 6C*). As shown in *figure 6A*, we found lower expression of *FasL* in *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ compared to wild-type and slightly increased levels of *Gzmc* in *Tyk2*$^{-/-}$ compared to *Tyk2*$^{K923E}$ and wild-type NK cells, whereas no differences were observed for *Gzma*, *Gzmb* and *Prf1*. Interestingly, vascular endothelial growth factor (*Vegf),* which has an important role in angiogenesis, was higher expressed in *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ than in wild-type cells, whereas *Ifng*, *Tgfb* and *Il2ra* mRNAs were similarly expressed in all cells (*figure 6B*). *Tbet* and *Eomes,* which are crucial transcription factors in NK cell maturation, did not show any differences in expression levels between the genotypes (*figure 6C*). Similar to *FasL*, the prominent activatory receptor *Nkg2d* was lower expressed in *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ compared to wild-type NK cells, although statistical significance was only reached in *Tyk2*$^{K923E}$ cells. In summary, data suggest that TYK2 positively regulates the expression of some candidate genes involved in NK cell function. Reduced *Nkg2d* and *FasL* expression in the absence of TYK2 may reduce NK cell killing capacity dependent on the target cells and their expression of inhibitory receptors. The role of NK cell-derived VEGF is unclear, but increased expression may lead to increased vascularization of tumours in *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ mice. Importantly, regulation of these genes was similar in the absence of TYK2 and the presence of a kinase-inactive TYK2 and hence represents a kinase-dependent function of TYK2.

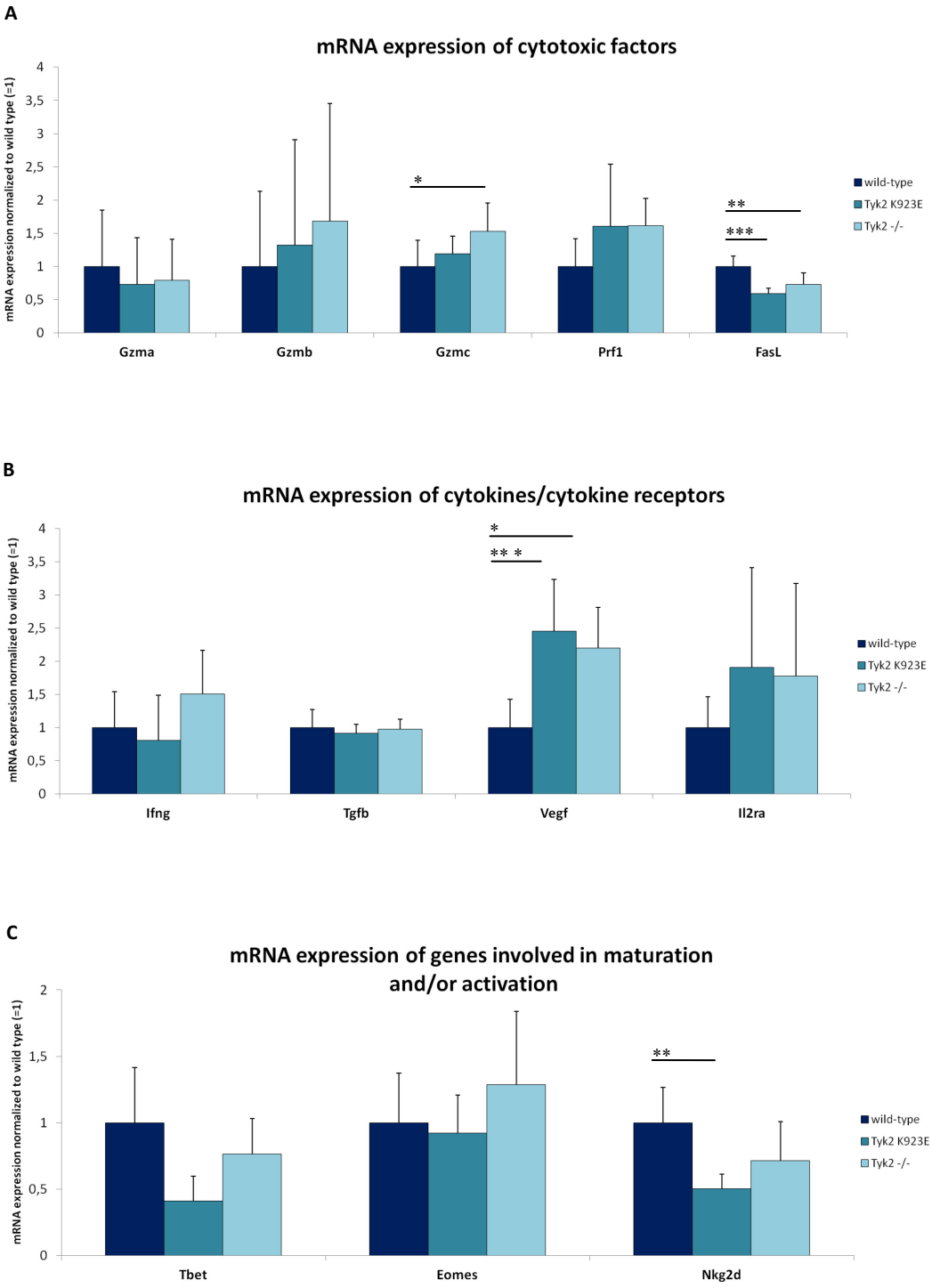


**Figure 6: Candidate gene expression in NK cells isolated from *Tyk2*$^{K923E}$, *Tyk2*$^{-/-}$ and wild-type mice and *in vitro* expanded in the presence of IL-2 of (A) cytotoxic factors (B) cytokines/cytokine receptors and (C) genes involved in maturation and/or activation.**

NK cells were expanded in the presence of IL-2 (5000U/ml) for 7 days, seeded in duplicates or triplicates ($1x10^6$ cells/sample) and harvested after 4h of incubation w/o IL-2. 3 biological replicates per genotype (each 4 spleens pooled) were used for the first experiment, whereas 1 biological replicate per genotype (each 4 spleens pooled) was used for the second experiment. mRNA levels were determined by RT-qPCR. Data were normalized to the housekeeping gene *Ube2d2* and are given relative to wild-type cells. The figures show mean of 6-10 samples per genotype from 2 independent experiments +SD, *p<0.05, **p<0.01 and ***p<0.001.

### 6.2. Candidate gene expression in wild-type, *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ NK cells (*ex vivo*)

In addition to the *in vitro* expanded and IL-2 activated NK cells, we analysed candidate gene expression in splenic, non-activated NK cells. As the cell number is limiting, we only selected genes that are involved in NK cell maturation and genes that appeared interesting from the results obtained from the *in vitro* cultivated NK cells. No gross differences in expression levels of *Tbet, Eomes*, *Nkg2d, Il2ra* and *Vegf* were found between wild-type, *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ cells (*figure 7)*. *Gzmb*, *Prf1* and *Ifng* mRNA expression was below detection limit. Although data are from one experiment only and thus need confirmation, these results indicate that expression of these genes is regulated *Tyk2*-independently in *ex vivo* collected NK cells.

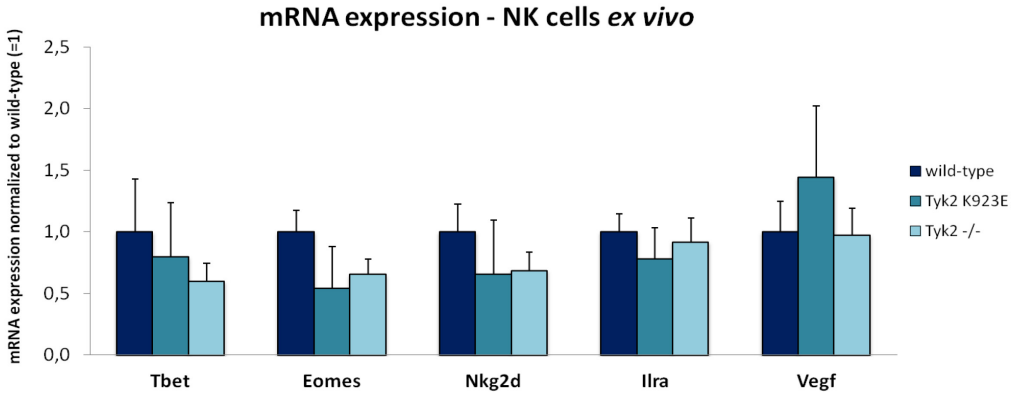


**Figure 7: Candidate gene expression in NK cells from *Tyk2*$^{K923E}$, *Tyk2*$^{-/-}$ and wild-type mice *ex vivo*.**
NK cells from 2 independent experiments were analysed. 4 biological replicates per genotype (each 1 spleen) were used for the first, and also 4 biological replicates per genotype (each 2 spleens pooled) were used for the second experiment. mRNA levels were determined by RT-qPCR. qPCR data were normalized to *Ube2d2* (housekeeping gene) and are shown relative to wild-type cells. Mean of 4 samples per genotype from one experiment +SD are given.

### 6.3. Expression of lytic proteins and activation of STATs during cytotoxicity assay

As GZMB and PRF1 are mainly regulated post-transcriptionally[99 117], we also checked their expression at the protein level. In addition, we wanted to determine if incubation of NK cells with target cells leads to changes in lytic protein levels. IL-2 cultured NK cells were incubated with YAC-1 cells as it is done in cytotoxicity assays and protein expression was determined by Western blot analysis. IL-2 cultured NK cells without YAC-1 cell-stimulation, YAC-1 cells and unstimulated T cells from mice of the three different genotypes were used as controls. As shown in *figure 8*, PRF1 and GZMB were similarly expressed across the genotypes and the treatments. The slightly lower expression of GZMB in YAC-1 stimulated *Tyk2*$^{K923E}$ compared to *Tyk2*$^{-/-}$ and wild-type NK cells was not consistently observed. As expected neither YAC-1 cells nor unstimulated T cells showed detectable levels of lytic proteins.

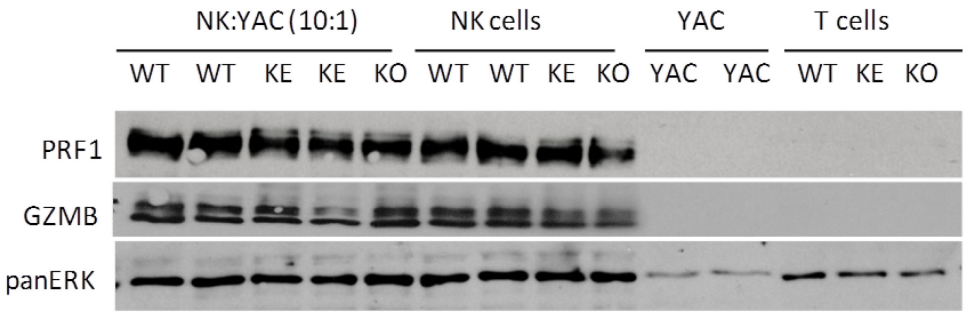


**Figure 8: Expression of GZMB and PRF1 in wild-type, *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ NK cells with or without incubation with YAC-1 target cells (10:1 effector:target ratio).**
Splenic NK cells from 4 mice per genotype of *Tyk2*$^{K923E}$ (KE), *Tyk2*$^{-/-}$ (KO) and wild-type (WT) mice were isolated, pooled and expanded *in vitro* with IL-2 (5000U/ml) for 7 days. NK cells were counted and 1x10$^6$ cells in 0,5ml were incubated for 4 hours (37°C, 5%$CO_2$) in the presence or absence of YAC-1 cells in duplicates. Cells were lysed and protein expression analysed using 10% SDS-PAGE and Western Blot (10µg protein/ slot). As negative controls YAC-1 cell and T cell lysates (WT, KE and KO) were used. Membranes were probed with antibodies against GZMB, PRF1 and panERK as a loading control (42 kDa). Depicted is one representative from two independent experiments.

We next analysed if STATs get activated during NK cell cytotoxicity assays (e.g. through autocrine/paracrine cytokine actions) and whether this depends on the presence of TYK2 and/or its kinase activity. STAT1 tyrosine 701 phosphorylation (*figure 9A*) was not detectable, whereas STAT3 tyrosine 705 (*figure 9B*) and STAT4 tyrosine 693 (*figure 9C*) showed a weak constitutive phosphorylation. In contrast, STAT5 showed a weak constitutive and a clearly YAC-1 cell-inducible tyrosine 694 phosphorylation in wild-type NK cells (*figure 9D*). Interestingly, STAT5 phosphorylation was neither induced in *Tyk2*$^{K923E}$ nor in *Tyk2*$^{-/-}$ NK cells incubated with target cells. Constitutive STAT5 phosphorylation was also lower in both *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ compared to wild-type cells. Moreover, *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ NK cells expressed lower total STAT5 protein than wild-type cells, irrespective of the presence of target cells (*figure 9D*). STAT5 protein levels appeared more reduced in *Tyk2*$^{-/-}$ than in *Tyk2*$^{K923E}$ cells, indicating that kinase-inactive TYK2 can to some degree compensate for the loss of Tyk2. In contrast, total STAT1 (*figure 9A*), STAT3 (*figure 9B*) and STAT4 (*figure 9C*) protein did not show differences in expression levels between the genotypes. Taken together these results show, that STAT5 but not STAT1, STAT3 or STAT4, is activated upon incubation of NK cells with their target cells. Data indicate that constitutive and induced STAT5 activation depends on the presence of a kinase-active TYK2. In contrast, both TYK2 kinase-dependent and -independent mechanisms seem to upregulate or maintain STAT5 protein expression in IL-2 cultivated NK cells, irrespective of the presence of YAC-1 target cells.

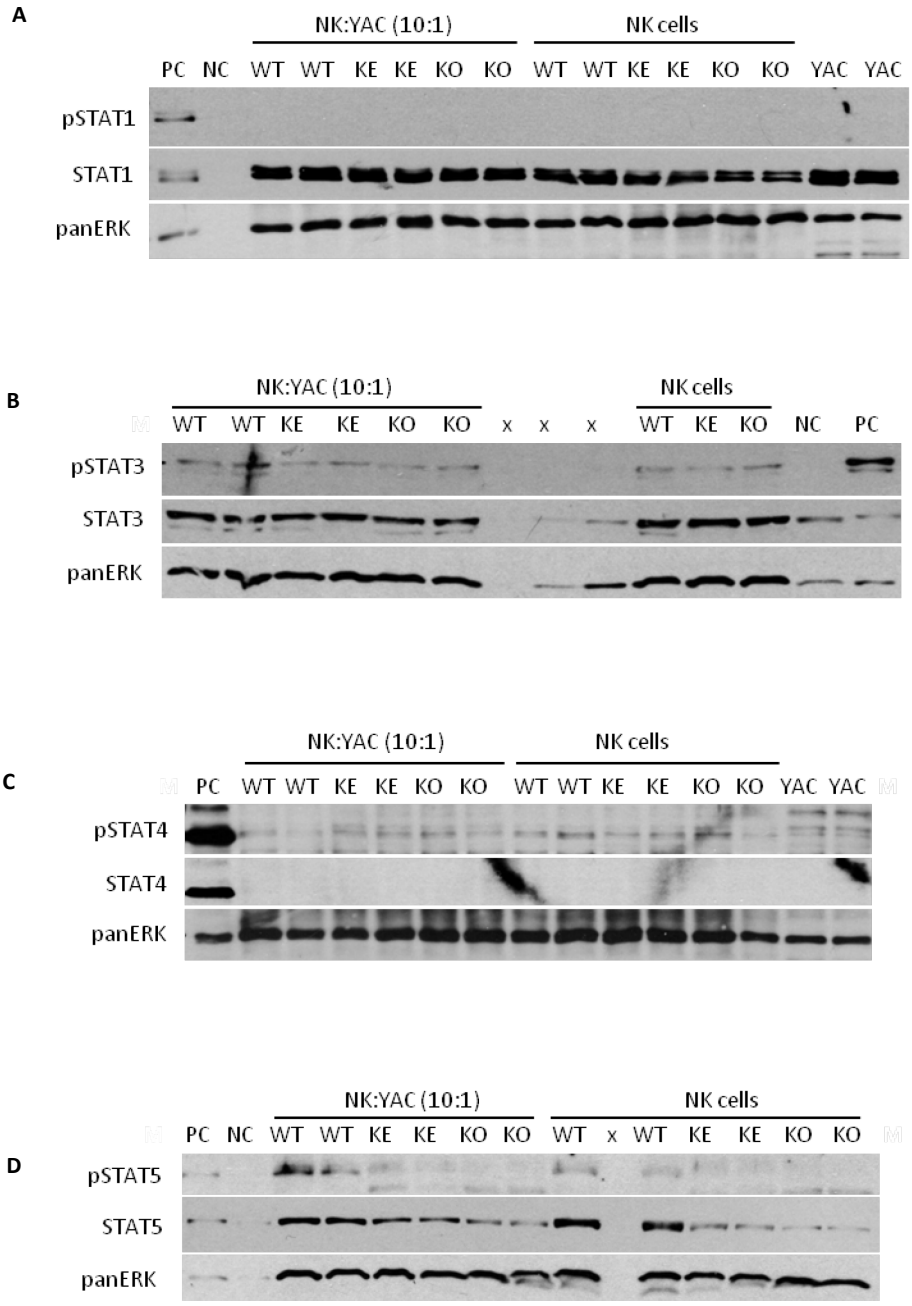


**Figure 9: Activation of (A) STAT1, (B) STAT3, (C) STAT4 and (D) STAT5 in wild-type (WT), *Tyk2*$^{K923E}$ (KE) and *Tyk2*$^{-/-}$ (KO) NK cells during cytotoxicity assay with YAC-1 target cells (10:1 effector: target ratio).**
Lysates from the same experiments as in *figure 3* were used and analysis was done as indicated in the respective legend. Membranes were probed for (A) STAT1 tyrosine 701 phosphorylation (pSTAT1) and total STAT1 protein (STAT1), (B) STAT3 tyrosine 705 phosphorylation (pSTAT3) and total STAT3 protein (STAT3), (C) STAT4 tyrosine 693 phosphorylation (pSTAT4) and total STAT4 protein (STAT4) and (D) STAT5 tyrosine 694 phosphorylation (pSTAT5) and total STAT5 protein (STAT5). As positive controls (PC) following samples were used: (A) for pSTAT1, macrophages stimulated with IFNγ for 15 minutes, (B) for pSTAT3, protein lysate from whole spleen of MCMV infected mice 72h post infection (C) for pSTAT4, T cells stimulated with IFNβ for 15 minutes and (D) for pSTAT5, macrophages stimulated with GM-CSF for 15 minutes. For STAT5 as a negative control (NC) protein lysate of unstimulated T cells was used. (A-D) panERK was used as a loading control (42 kDa). One representative of two independent experiments is depicted.

To confirm these findings and to exclude that signals for GZMB and PRF1 were saturated, we down-titrated samples from 10µg to 1µg of protein per slot and repeated the analysis. As shown in *figure 10*, pSTAT5 was below the detection limit, whereas STAT5 protein showed reduced expression in *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ compared to wild-type cells. However, *Tyk2*$^{-/-}$ samples were either loaded inaccurately or the transfer of proteins was inefficient at this part of the membrane. Importantly, GZMB and PRF1 expression seemed similar in *Tyk2*$^{K923E}$ and wild-type NK cells also under clearly non-saturating Western blot conditions.

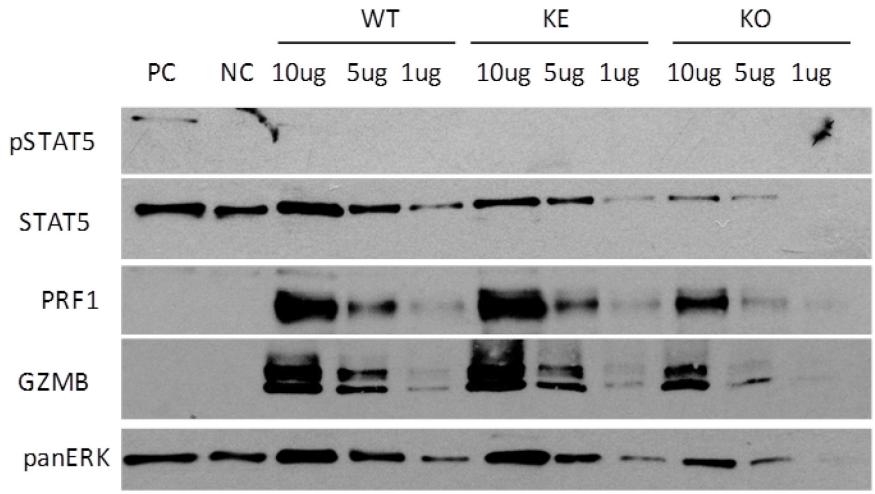


**Figure 10: STAT5 phosphorylation and expression of STAT5, PRF1 and GZMB: down-titration of protein lysates.**
NK cells *in vitro* expanded with IL-2 (5000U/µl) for 7 days were treated as described in *figure 3*. Analysis was done by 10% SDS-PAGE and Western Blot. Three different amounts of protein lysates (10µg/slot, 5µg/slot and 1µg/slot) per genotype were applied on the gel and membranes were probed for STAT5 tyrosine 694 phosphorylation (pSTAT5), total STAT5 protein (STAT5), PRF1 and GZMB. panERK was used as a loading control (42 kDa). As a positive control (PC) for pSTAT5, macrophages stimulated with GM-CSF for 15 minutes were used, as a negative control (NC) protein lysate of unstimulated macrophages was used.

### 6.4. STAT5 expression in IL-2 cultivated and in *ex vivo* wild-type, *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ NK cells

We next tested whether TYK2 also affects STAT5 phosphorylation and expression in untreated splenic NK cells (i.e. *ex vivo*). Similar to *figure 9A*, low level of pSTAT5 was detectable in IL-2 cultured wild-type, but not *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ NK cells (*figure 11*, *top panel*). As expected, pSTAT5 was not detectable in *ex vivo* NK cells and only background bands were visible. In contrast to the IL-2 cultivated NK cells, STAT5 expression in *ex vivo* NK cells did not grossly differ between the genotypes and seemed even a bit higher in *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ than in wild-type cells. This suggests that TYK2 is required to upregulate or maintain STAT5 protein levels in IL-2 activated, but not in naive NK cells.

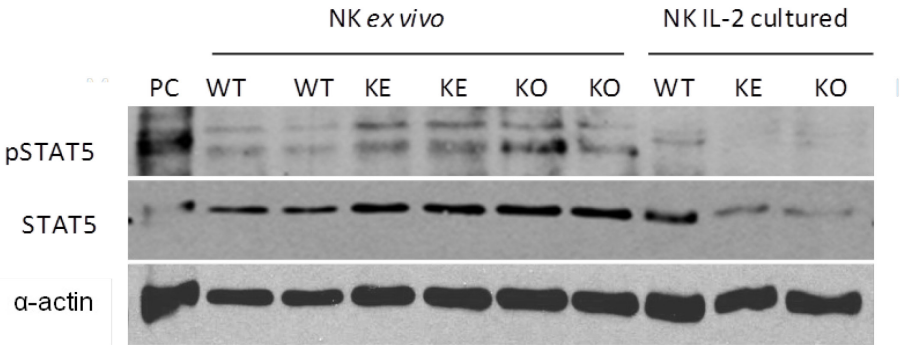


**Figure 11: Comparison of STAT5 protein levels in wild-type, *Tyk2*$^{K923E}$ (KE), and *Tyk2*$^{-/-}$ (KO) NK cells *ex vivo* and expanded with IL-2.**
NK cells *ex vivo* from 2 independent experiments were analysed. 2 biological replicates per genotype (each 3 spleens pooled) were used for each experiment. NK cells *in vitro* expanded in the presence of IL-2 were used from experiment 6.3. Analysis was done by 10% SDS-PAGE and Western Blot (10µg protein/slot). Membranes were probed for STAT5 tyrosine 694 phosphorylation (pSTAT5) and total STAT5 protein (STAT5). As a positive control (PC) for pSTAT5 macrophages stimulated with GM-CSF for 15 minutes were used. α-actin was used as a loading control.

### 6.5. Kinetics of IL-2 responses in *ex vivo* NK cells: STAT5 phosphorylation and upregulation of PRF1 and GZMB

As according to our standard protocol NK cells are cultivated for 7 days with IL-2 before they are used for cytotoxicity assays, we next wanted to define the kinetics of STAT5 phosphorylation and the upregulation of GZMB and PRF1. Splenic NK cells were stimulated with IL-2 (5000U/ml) for 20 minutes, 24 hours, 48 hours or 90 hours and protein expression was analysed by Western Blot. Surprisingly, we could not detect STAT5 tyrosine 694 phosphorylation in this experiment, whereas STAT5 total protein expression increased after IL-2 stimulation with a maximum at 90 hours (*figure 12A, top and second panel*). PRF1 was highly induced after 24 hours of IL-2 treatment and further increased with time (*figure 12A*, *third panel*). GZMB was upregulated with similar kinetics as PRF1 (*figure 12B*, *third panel*). For GZMB, weak bands could be detected in untreated cells and early after stimulation, but these appeared at slightly higher molecular weight and likely are unspecific bands. STAT3 phosphorylation was not detected and STAT3 protein was modestly upregulated after 90 hours stimulation (*figure 12B*, *top and second panel*). STAT3 and STAT5 were comparatively modestly upregulated and also showed highest expression at the latest time point analysed.

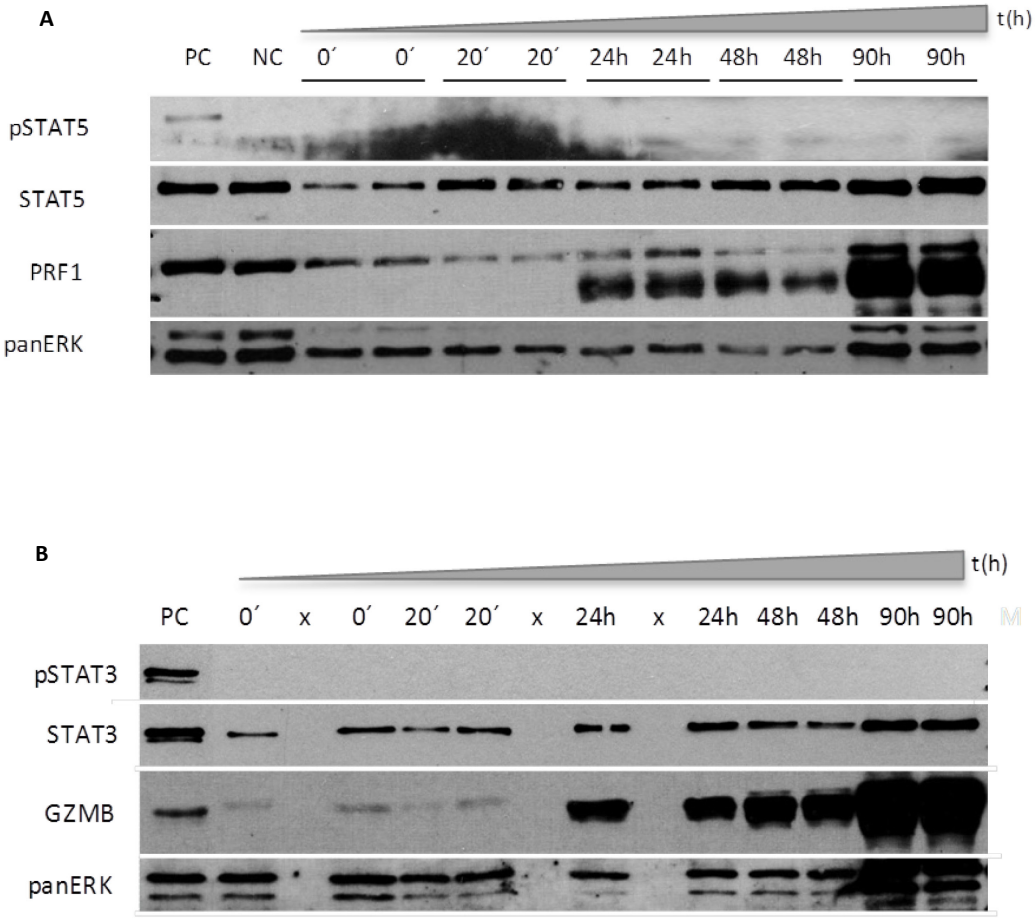


**Figure 12: Kinetics of IL-2 responses in NK cells: (A) pSTAT5, STAT5 and PRF1 and (B) pSTAT3, STAT3 and GZMB.**

NK cells isolated from 2 spleens/sample were pooled and treated with IL-2 (5000U/ml) for the times indicated or left untreated (0'). Each treatment was performed in duplicate. Analysis was done by 10% SDS-PAGE and Western Blot. Membranes were probed for (A) STAT5 tyrosine 694 phosphorylation (pSTAT5), total STAT5 protein (STAT5) and PRF1, (B) STAT3 tyrosine 705 phosphorylation (pSTAT3) total STAT3 protein (STAT3) and GZMB. As a positive control (PC) following samples were used: (A) macrophages stimulated with GM-CSF for 15 minutes and (B) protein lysates from whole spleen of mouse cytomegalovirus (MCMV) infected mice at 72h post infection. panERK was used as a loading control.

### 6.6. *In vitro* expansion of wild-type NK cells with different doses of IL-2 and IL-15

Based on our finding that TYK2 affects STAT5 protein expression in IL-2 cultured NK cells, we next wanted to determine whether this also holds true for IL-15 cultivated cells. Furthermore, we wanted to test whether NK cell growth is affected by the absence of TYK2 if limiting cytokine concentrations are used. To define limiting concentrations, IL-2 and IL-15 were used at three different concentrations and cell expansion, IFNγ secretion, GZMB and PRF1 protein expression and expression of selected genes (i.e. *Tbet*, *Gzmb* and *Il2ra*) was monitored in wild-type NK cells.

In the presence of 5000U/ml or 1000U/ml IL-2 (*figure 13A*) and 1000ng/ml or 100ng/ml IL-15 (*figure 13B*) NK cell numbers increased over time, whereas the lowest concentrations of either cytokine (200U/ml and 10ng/ml, respectively) did not support NK cell growth. Similar cell numbers of around $1,3x10^7$ to $1,2x10^7$ were obtained after 7 days cultivation with either 5000U/ml IL-2 or 100ng/ml IL-15. The middle concentration in both cases stimulates NK cell proliferation less than the highest, indicating that these are already limiting conditions with respect to NK cell proliferation. As indicated in *figure 13C* we observed a similar pattern of IFNγ secretion by NK cells cultured with either IL-2 or IL-15. With the lowest cytokine concentration used, IFNγ was barely detectable in the supernatants, which is in line with the continuous dying of cells shown in *figure 13A*. Surprisingly, we found higher IFNγ levels in supernatants of cells grown with the middle concentration than in those from cells cultivated with the highest cytokine concentration, indicating that proliferation does not strictly correlate with the production of IFNγ. In contrast, GZMB and PRF1 expression increased with the IL-2 and IL-15 concentration (*figure 14A*). NK cells cultured with IL-15 showed higher expression of both GZMB and PRF1 than those cultured with IL-2 at the concentrations used. Interestingly at the resepective middle concentrations, *Tbet* mRNA expression was higher in IL-15 than in IL-2 cultivated cells, whereas *Il2ra* mRNA appeared distinctly higher in IL-2 than in IL-15 cultivated cells (*figure 14B*). *Gzmb* mRNA levels (*figure 14B*) were similar in all tested conditions. However, RT-qPCR analysis was only done once and data have to be interpreted cautiously.

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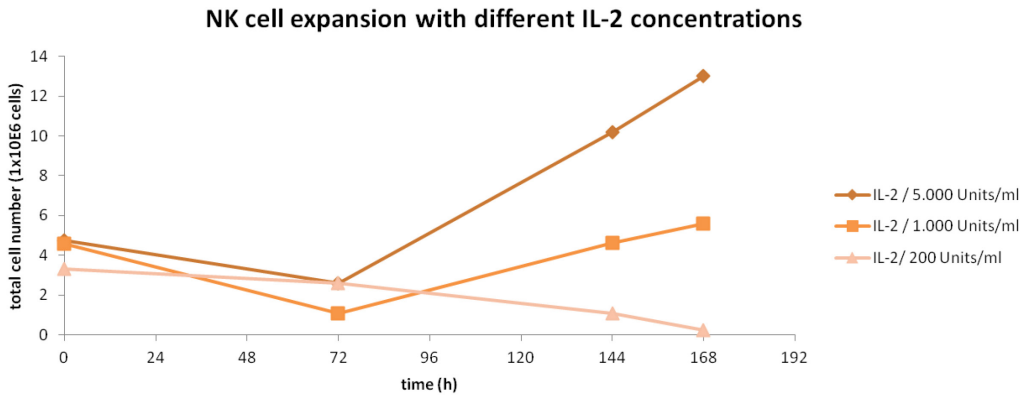


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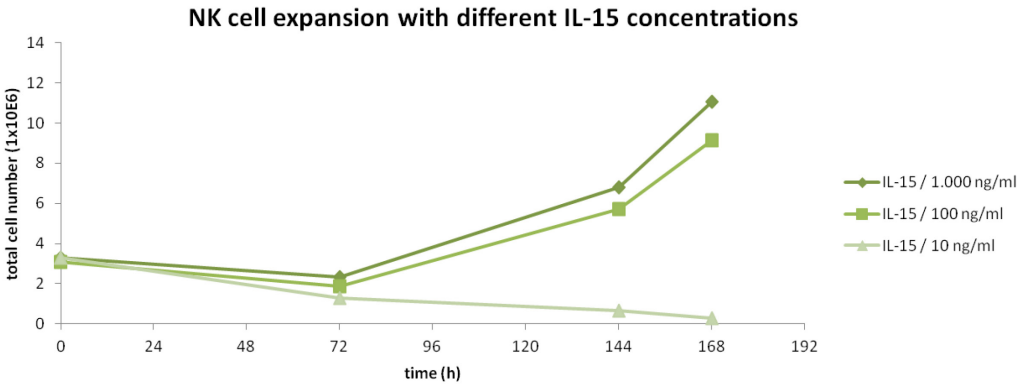


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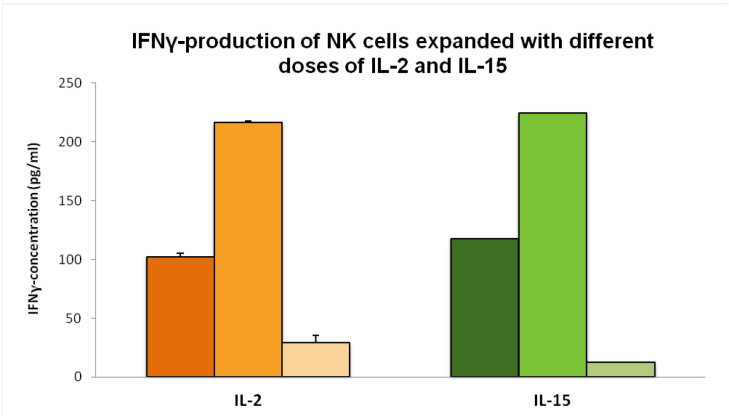
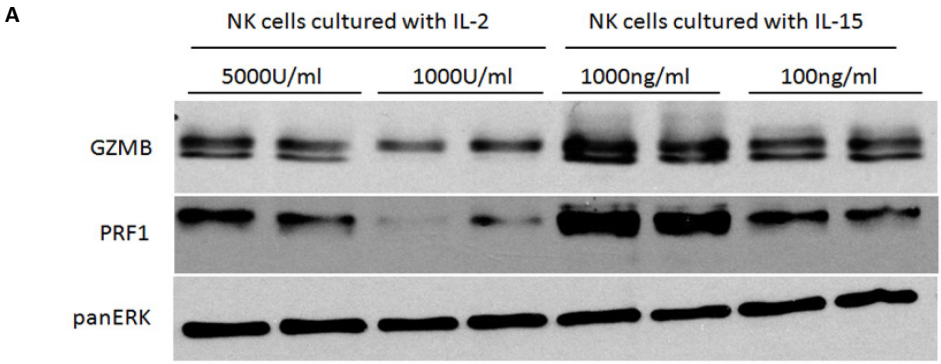
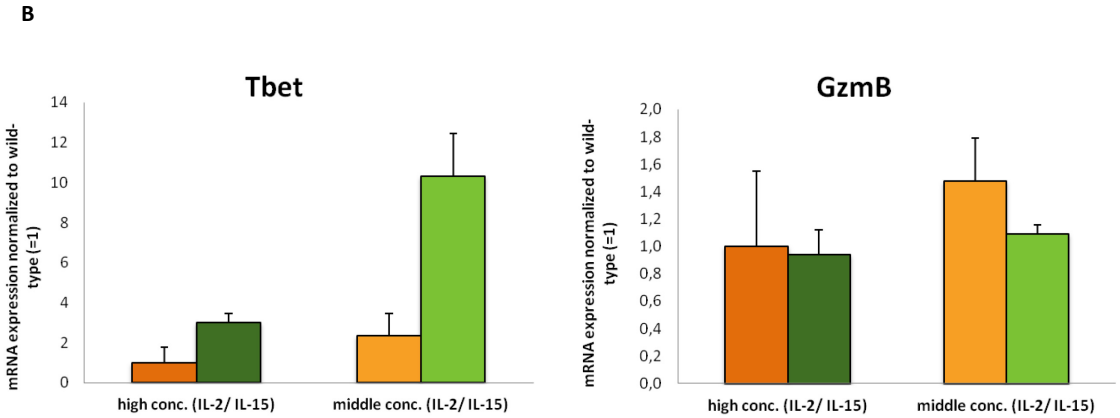



**Figure 13: (A, B) *In vitro* expansion of NK cells from wild-type mice with 3 different concentrations of (A) IL-2 and (B) IL-15 and (C) IFNγ production.**
NK cells from 4 spleens were pooled for each genotype and expanded in the presence of (A) IL-2 at 5000U/ml, 1000U/ml and 200U/ml and (B) IL-15 at 1000ng/ml, 100ng/ml and 10ng/ml. (A, B) NK cells were counted directly after isolation (0) and prior to splitting (72h, 144h) and harvesting (168h). The total amount of cells at the indicated time point is given. (C) IFNγ production was measured from cell supernatants after 168 hours of cultivation with ELISA. Colour code is same as in A and B. Mean values +SD of duplicate measurements are given.



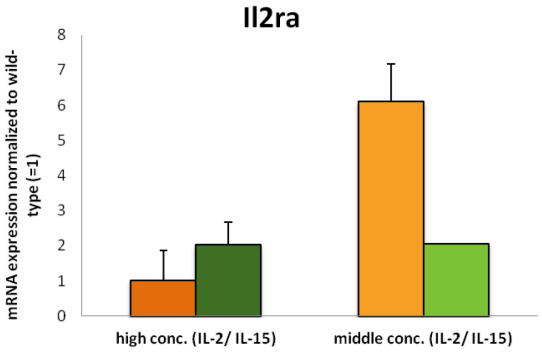




**Figure 14: (A, B) *In vitro* expansion of NK cells from wild-type mice at different concentrations of IL-2 or IL-15 and analysis of (A) GZMB and PRF1 protein expression and (B) *Tbet*, *Gzmb* and *Il2ra* mRNA expression.**

NK cells from the experiment shown in *figure 8A and 8B* were harvested and 1x10$^6$ cells seeded in duplicate or triplicate per treatment for (A) protein and (B) RNA isolation. (A) Analysis of protein expression was done from duplicates per treatment with 10% SDS-PAGE and Western Blot (10µg protein/slot). Membranes were probed for GZMB, PRF1 and panERK as a loading control. (B) mRNA expression was analysed withRTqPCR, expression was normalized to the house-keeping gene *Ube2d2* and mean values of 2-3 samples per treatment from one experiment +SDs are given .

### 6.7. Impact of TYK2 on NK cell growth, IFNγ production and the expression of PRF1 and GZMB in the presence of limiting concentrations of IL-2 and IL-15

As we have defined 1000U/ml IL-2 and 100ng/ml IL-15 to be the limiting concentrations, we next analysed *Tyk2* dependency of NK cell growth, IFNγ production and the expression of lytic proteins under these conditions. The experiments were done analogously to the previous one (*figure 13A-C*) with wild-type, *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ NK cells.

As shown in *figure 15A* and *B, Tyk2* did not grossly influence NK cell expansion in the presence of IL-2 (*figure 15A*) or IL-15 (*figure 15B*). However, NK cell growth exhibited rather high variability within the two independent experiments in particular when grown with IL-15 (*figure 15B*). Consistent with the experiment shown in *figure 13B*, total NK cell yields were considerably lower with 1000U/ml IL-2 than with 100ng/ml IL-15. Furthermore, NK cells cultured in IL-2 proliferated slower and cells were harvested one day later (i.e. day 8) than those cultivated with 100ng/ml IL-15.

IFNγ secretion was slightly higher in *Tyk2*$^{K923E}$ than in *Tyk2*$^{-/-}$ and wild-type NK cells both after IL-2 and IL-15 cultivation, however statistical significance was obtained only for *Tyk2*$^{K923E}$ *versus* wild-type cells cultured with IL-2 (*figure 15C)*. Strikingly, *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ NK cells expressed much higher levels of GZMB and PRF1 than wild-type cells (*figure 16*). Furthermore, *Tyk2*$^{-/-}$ cells expressed more PRF1 than NK cells of *Tyk2*$^{K923E}$ mice. This indicates a suppressive function of TYK2 on the expression of these proteins which can be partially exerted by a kinase-inactive TYK2. Importantly, this difference was only observed for IL-2 but not for IL-15 cultured NK cells (*figure 16*). GZMB and PRF1 showed similar or slightly lower expression after IL-15 than after IL-2 cultivation in wild-type cells. STAT5 protein showed reduced expression in *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ compared to wild-type cells (*figure 16*), which is, although less pronounced, consistent with NK cells grown in the presence of 5000U/ml IL-2 (compare *figures 9, 10 and 11*). In contrast, this was not observed in NK cells expanded in the presence of IL-15 (100ng/ml), suggesting that the TYK2 effects are specific for IL-2.

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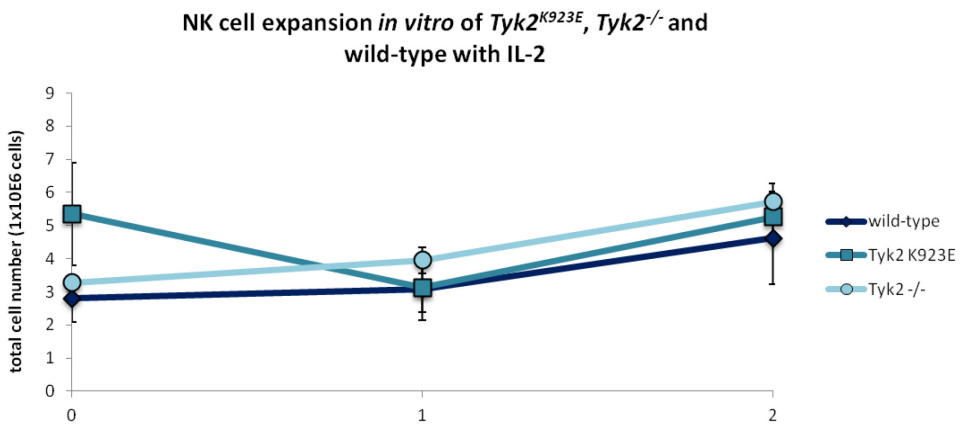


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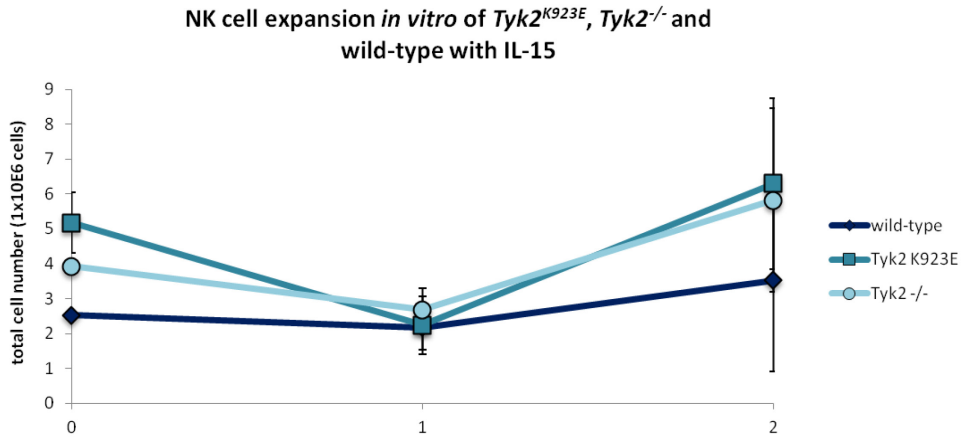


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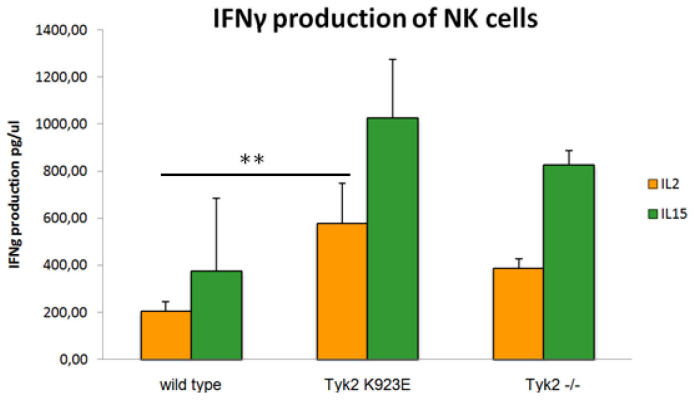


**Figure 15: (A, B) *In vitro* expansion of NK cells from WT, *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ mice in chosen concentrations of (A) IL-2 1000U/ml and (B) IL-15 100ng/ml cytokine and (C) IFNγ production.**
(A, B) NK cells from 4 spleens per genotype were pooled and expanded in the presence of (A) IL-2 1000U/ml and (B) IL-15 100ng/ml. NK cells were counted directly after isolation (0), prior to splitting (72h or 96h) and before harvesting (168 or 192h) - indicated on x-axis as points 0, 1, 2. The figures show mean values from 2 independent experiments ± SD. (C) IFNγ production was measured from cell supernatants after 168/192 hours of cultivation with ELISA. Mean values from 2 independent experiments + SD are depicted; ** p<0.01

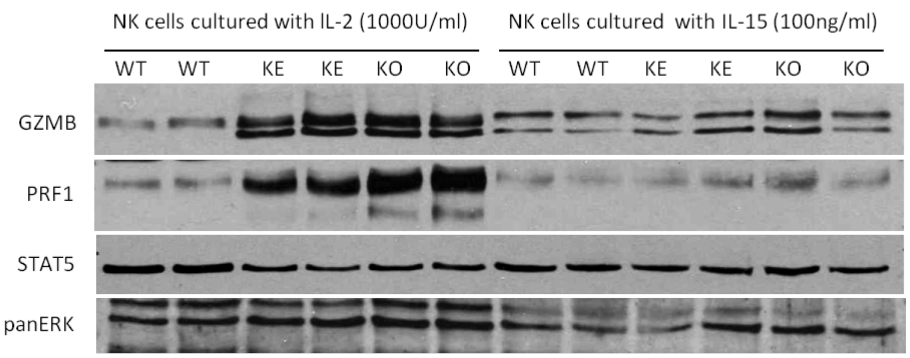


**Figure 16: GZMB, PRF1 and STAT5 expression in wild-type (WT) *Tyk2*$^{K923E}$ (KE), *Tyk2*$^{-/-}$ *(KO)* NK cells grown in the presence of IL-2 (1000U/ml) or IL-15 (100ng/ml).**
NK cells derived from the experiments depicted in *figure 10A* and *10B* were harvested and seeded 1x10$^6$ cells per sample for protein isolation. Analysis was done with 10% SDS-PAGE and Western Blot (10µg protein/slot), whereby 2 technical replicates per genotype and treatment were applied on the gel. Membranes were probed for GZMB, PRF1 and STAT5 protein. One representative of two independent experiments is depicted. panERK (42 kDa) was used as a loading control.

## 6.8. Determination of *Tyk2* deletion efficiency in *Tyk2*$^{\Delta NK}$ mice.

*Tyk2*$^{\Delta NK}$ mice have been previously generated by crossing *Tyk2*$^{fl/fl}$ mice to *Ncr1-iCre* mice, a mouse line containing a transgene with the Cre recombinase under the control of the *Ncr1* (NKp46) promoter. This causes the deletion of *Tyk2* specifically in Ncr1$^+$ NK cells. To determine the deletion efficiency mRNA expression was analysed in NK cells from *Tyk2*$^{\Delta NK}$ compared to *Tyk2*$^{fl/fl}$ mice. *In vitro* expanded NK cells were used to ensure high purity of the cell population. To check whether deletion of *Tyk2* affects NK cell proliferation, we additionally monitored the growth of cells. Single cell suspensions from thymus, bone marrow and DX5$^-$ cells (i.e. flow through from the NK cell purification by MACS for DX5) were taken as controls. As shown in *figure 17A Tyk2* is efficiently deleted in NK cells isolated from *Tyk2*$^{\Delta NK}$ mice. A deletion efficiency of approximately 85% at the mRNA level could be obtained. Importantly, DX5$^-$ cells from *Tyk2*$^{\Delta NK}$ mice exhibit similar *Tyk2* expression as DX5$^+$ cells from *Tyk2*$^{fl/fl}$ mice. Thymocytes and bone marrow cells from *Tyk2*$^{\Delta NK}$ showed an approximately 40% lower *Tyk2* mRNA expression than NK cells from *Tyk2*$^{fl/fl}$ mice. As thymus and bone marrow contain only very few NK cells (less than 1% of total cells[118]) and the deletion by the *Ncr1-Cre* is highly specific[115], these results indicate organ-specific differences in *Tyk2* mRNA expression. However, analysis of deletion efficiency at the genome level and a direct comparison of *Tyk2*$^{fl/fl}$ and *Tyk2*$^{\Delta NK}$ thymocytes and bone marrow cells would be

required to confirm this observation. As shown in *figure 17B,* NK cells isolated from *Tyk2*$^{fl/fl}$ and *Tyk2*$^{\Delta NK}$ mice exhibited similar growth over 7 days in the presence of IL-2, suggesting that proliferation under these conditions is not *Tyk2*-dependent.

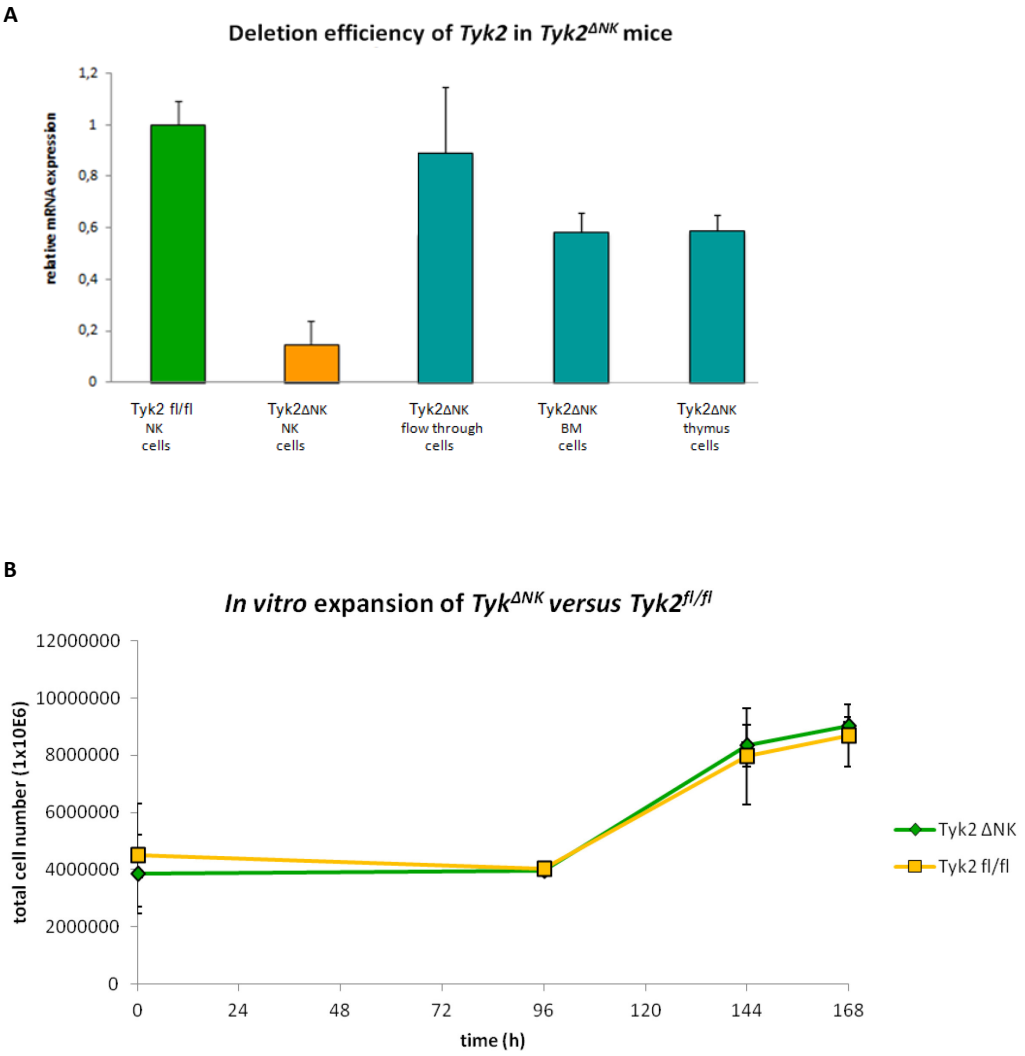


**Figure 17: (A, B) Deletion efficiency of *Tyk2* in *Tyk2*$^{\Delta NK}$ mice.**
NK cells from *Tyk2*$^{\Delta NK}$ and *Tyk2*$^{fl/fl}$ were isolated (MACS for DX5) from 3 spleens per genotype, pooled and expanded in NK cell medium with IL-2 (5000U/µl) for 7 days in triplicates. In addition, thymus and bone marrow cells were isolated from Tyk2$^{\Delta NK}$ mice as controls. The flow through from NK cell isolation was used as a negative control and cells were directly harvested at 1x10$^6$ cells/sample without culturing. (A) 1x10$^6$ cells were used for RNA isolation and *Tyk2* mRNA expression was determined by RT-qPCR. Data were normalized to *Ube2d2* (housekeeping gene) and calculated relative to *Tyk2*$^{fl/fl}$ NK cells. Mean values of 6 samples from 2 independent experiments + SD are given. (B) Growth curve of NK cells from *Tyk2*$^{\Delta NK}$ and *Tyk2*$^{fl/fl}$ mice. Mean values of 6 samples from 2 independent experiments ± SD are given.

## 7. DISCUSSION

Based on the previous finding that TYK2 is required for NK cell cytotoxicity against tumour cells (Stoiber et al., 2004) and that this does only partially depend on its kinase activity (Prchal-Murphy et al.; manuscript in preparation), we investigated *Tyk2* kinase-dependent and -independent functions in NK cells in more detail. In particular, expression of candidate genes, known to be involved in cytotoxicity and maturation was compared between NK cells derived from wild-type, *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ mice (*in vitro* cultivated and *ex vivo* cells). Furthermore, the activation of the JAK-STAT pathway during cytotoxicity assays was analysed.

One main finding of this study was that *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ NK cells show reduced STAT5 protein expression compared to wild-type cells. STAT5 expression was slightly higher in *Tyk2*$^{K923E}$ cells than in *Tyk2*$^{-/-}$ cells, indicating that kinase-dependent and -independent TYK2 functions are involved in STAT5 regulation. Interestingly, differences in STAT5 expression were observed in NK cells cultivated in the presence of IL-2 but neither in IL-15 cultivated nor in NK cells isolated *ex vivo*, suggesting that TYK2 specifically affects IL-2 responses. As TYK2 is not directly involved in IL-2 signalling the underlying mechanism is currently unclear. One possible explanation could be that TYK2 affects the expression of the IL-2 specific IL-2R alpha chain (IL-2Rα), as the main other signalling components, including the receptor chains IL-2Rα and γc, JAK1/JAK3 and STAT5, which are shared by IL-2 and IL-15. *IL-2ra* is not expressed in naive NK cells but is induced by IL-2[119] and IL-12[120]. As we did not observe differences in IL-2Rα mRNA expression between wild-type, *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ IL-2 cultivated or freshly isolated NK cells, FACS analysis will be required to determine whether there are differences in IL-2Rα surface expression. Another possibility is that autocrine/paracrine TYK2-activating cytokines cross-talk with the IL-2 signalling cascade.

We could furthermore show a clear YAC-1 target cell-induced activation of STAT5 in wild-type NK cells. This was totally missing in *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ NK cells and may reflect lower STAT5 protein levels in these cells. Notably, we found low levels of constitutive STAT5 phosphorylation in IL-2 cultivated wild-type but not *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ NK cells. In contrast to STAT5, STAT1, STAT3 and STAT4 were not activated upon incubation of NK cells with YAC-1 tumour cells and did not show any differences in expression levels between the genotypes. STAT5 has multiple pivotal roles in in NK cell development and function, including

regulation of cytotoxicity[115 121]. Phosphorylated STAT5 can activate PI3K *via* binding to the adaptor protein GAB-2[115 122], thus PI3K pathway can increase cytotoxicity by facilitating granule assembly and accumulation towards target cells[123]. Based on this it would be interesting to determine intracellular distribution of granules in *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ NK cells upon target cell contact. Furthermore, IL-2 activated STAT5 induces PRF1 gene expression, which together with GZMB constitutes the weapons of granule exocytosis[124 125]. Despite the differences in STAT5 phosphorylation, we did not observe differences in PRF1 mRNA or protein expression under our standard NK cell growth conditions, i.e. 7 days cultivation in the presence of 5000U/ml IL-2. However, PRF1 is induced by STAT5B[121] and we did not differentiate between STAT5A and STAT5B in our analysis. Gene expression levels of other STAT5A/B induced genes (e.g. Cis, c-myc, Bcl-2, Bcl-$X_L$)[126 127] should be investigated to determine potential down-stream effects of the reduced STAT5 activation in the absence of TYK2 and the presence of TYK2$^{K923E}$.

Surprisingly, we found strongly increased levels of PRF1 protein in *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ compared to wild-type NK cells when we cultivated cells with a 5-fold lower dose of IL-2. This is opposite to the expectations as STAT5 protein expression was again lower in *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ than in wild-type cells under these conditions. However, PRF1 is strongly regulated on the translational level by unknown mechanisms[117]. PRF1 seemed modestly higher in *Tyk2*$^{-/-}$ than in *Tyk2*$^{K923E}$ NK cells arguing for a contribution of kinase-inactive TYK2 to the suppression of PRF1 protein expression. Similar to PRF1, GZMB protein was much higher in *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ than in wild-type cells. The reason for this is unclear, but it may be due to the lack of negative feedback inhibition. This possibility could easily be assessed by kinetic analyses of IL-2 responses. Furthermore, it would be interesting to determine, whether under these conditions (i.e. 1000U/ml IL-2 for 7 days) *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ NK cells still show reduced cytotoxicity despite an increased expression of lytic proteins. Another explanation for increased PRF1 and GZMB in *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ cells could be that a substantial fraction of these proteins is already exocytosed without target cell contact in wild-type but not *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ cells. Although spontaneous degranulation has not yet been reported in NK cells, it will be interesting to test the culture media for the presence of PRF1 and GZMB. Interestingly, PRF1 and GZMB were similarly expressed in all genotypes if cells were grown in the presence of IL-15 at different doses, again indicating that differences are IL-2-specific.

In addition, we monitored growth of wild-type, *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ NK cells expanded in presence of IL-2 (1000U/ml) or IL-15 (100ng/ml), which were defined as limiting concentration using wild-type cells. By means of cell counting we did not observe any differences between the genotypes, but the variation between experiments was rather high. More precise methods, such as BrdU cell proliferation assays, could reveal whether there are modest differences in NK cell growth.

We furthermore found some genes that were regulated in a *Tyk2*-dependent manner in IL-2 expanded NK cells. As they did not show differences between *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ NK cells, regulation is TYK2 kinase dependent. Vascular endothelial growth factor (*Vegf*) was higher expressed in *Tyk2*$^{K923E}$ and *Tyk2*$^{-/-}$ than wild-type NK cells. This was not seen in NK cells *ex vivo*, indicating a role of TYK2 in *Vegf* regulation by IL-2. *Ex vivo* NK cells are only around 60-70% pure as they were enriched with a single round of MACS and thus this needs to be repeated with FACS sorted NK cells. Nevertheless, IL-2 can induce *Vegf* expression in T cells[128]. The ability of NK cells to produce *Vegf* has just very recently been described for human cells[129 130], but its role in cancer remains to be determined. *Vegf* is best known for its function in angiogenesis[131 132] but it can also promote NK cell adhesion, preferentially of IL-2-activated NK cells, to the tumour vasculature[133 134]. Our finding may suggest that TYK2 represses tumour vascularization, which is in line with the observation from our laboratory that several transplanted tumours display higher vascularization in *Tyk2*$^{-/-}$ than in wild-type mice (Prchal-Murphy M. and Witalisz A., personal communication). In contrast to *Vegf,* we found decreased expression *Nkg2d* and *FasL* in *Tyk2*$^{-/-}$ and *Tyk2*$^{K923E}$ NK cells, suggesting that recognition of cells expressing *Nkg2d* ligands (e.g. Mult1, Rae-1, Ulbp)[135] and Fas/FasL-mediated killing may be reduced in the absence of TYK2.

To enable studies defining NK cell intrinsic and extrinsic functions of TYK2, our laboratory has generated mice with a floxed *Tyk2* allele (*Tyk2*$^{fl/fl}$, Vielnascher R. et al., manuscript in preparation) and crossed them to mice expressing the Cre recombinase under an NK cell-specific promoter (Tg-Ncr1-Cre[115]). We could show that in IL-2 expanded NK cells derived from these mice (*Tyk2*$^{\Delta NK}$) *Tyk2* is very efficiently deleted. Deletion efficiency was around 85% on the mRNA level, which is in the same range as reported previously for the deletion of *Stat5* in NK cells from *Stat5*$^{fl/fl}$*-Tg-Ncr1-Cre* mice[115]. In addition,this result goes in line with 81.0% ± 2.42% GFP-positive cells in spleens reported in *Ncr-1-iCreTg*-transgenic mice that express enhanced GFP upon *Cre*-mediated excision of a *loxP*-flanked stop cassette[115].

Moreover, we could show that loss of *Tyk2* in NK cells does not influence *in vitro* proliferation in the presence of IL-2 compared to $Tyk2^{fl/fl}$ control.

Taken together, we could show that TYK2 represses *Vegf* and promotes *Nkg2d* and *FasL* mRNA expression in IL-2 expanded NK cells in a kinase-dependent manner. In contrast, we found that TYK2 upregulates STAT5 protein expression and that this regulation occurs partly kinase-independent. As STAT5 can regulate NK cell cytotoxicity, these results possibly provide a mechanistic basis for the intermediate tumour cell killing phenotype of $Tyk2^{K923E}$ between wild-type and $Tyk2^{-/-}$ NK cells. New insights into *Tyk2*-dependent regulation of NK cell cytotoxicity *in vitro* might constitute crucial principles for future research on cytolytic NK cell functions and tumour surveillance *in vivo*.

## 8. ABBREVIATIONS

| | |
|---|---|
| APS | Ammonium persulfate |
| DEPC | Diethylpyrocarbonate |
| DTT | Dithiothreitol |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FELASA | Federation of European Laboratory Animal Science Associations |
| GZMB | Granzyme B |
| IL-2 | Interleukin-2 |
| IL-15 | Interleukin-5 |
| JAK | Janus kinase |
| KE | *Tyk2*$^{k923E}$ |
| KO | *Tyk2*$^{-/-}$ |
| MACS | Magnetic cell separation (Miltenyi Biotec GmbH) |
| MQ-$H_2O$ | Milli-Q-$H_2O$ (EMD Millipore Corporation, Billerica, MA) |
| o/n | Overnight |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PMSF | Phenylmethylsulfonyl fluoride |
| PRF1 | Perforin 1 |
| rpm | rotations per minute |
| RPMI | Roswell Park Memorial Institute medium |
| RT | Room temperature |
| rxn | reaction |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| STAT | Signal transducers and activators of transcription |
| TE | Tris/EDTA buffer |
| TEMED | Tetramethylethylenediamine |
| TYK2 | Tyrosine kinase 2 |
| WT | Wild-type |

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## 10. CURRICULUM VITAE

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ABSTRACT/POSTER

***Characterisation of kinase-dependent and -independent role of tyrosine kinase 2 in NK cell biology***
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