**In vivo** tumor surveillance by NK cells requires TYK2 but not TYK2 kinase activity

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Abbreviations: CCL3, chemokine (C-C motif) ligand 3; CFSE, carboxyfluorescein diacetate succinimidyl ester; GzmB, granzyme B; IFN, interferon; IFNAR, interferon α and β receptor; IL, interleukin; IL-12Rβ1, interleukin-12 receptor subunit β-1; iNK, immature natural killer; JAK, xJanus kinase; MACS, magnetic-activated cell sorting; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; miR, microRNA; mNK, mature natural killer; NK, natural killer; NKP, natural killer precursor; Prf1, perforin; SEM, standard error of the mean; STAT, signal transducer and activator of transcription; T-ALL, T cell acute lymphoblastic leukemia; TYK2, tyrosine kinase 2; TYK2⁹⁹²³E, kinase-inactive tyrosine kinase 2; WT, wild-type

Tyrosine kinase 2 (TYK2) is a Janus kinase (JAK) that is crucially involved in inflammation, carcinogenesis and defense against infection. The cytotoxic activity of natural killer (NK) cells in TYK2-deficient (Tyk2⁻/⁻) mice is severely reduced, although the underlying mechanisms are largely unknown. Using Tyk2⁻/⁻ mice and mice expressing a kinase-inactive version of TYK2 (Tyk2⁹⁹²³E), we show that NK cell function is partly independent of the enzymatic activity of TYK2. Tyk2⁻/⁻ and Tyk2⁹⁹²³E NK cells develop normally in the bone marrow, but the maturation of splenic Tyk2⁻/⁻ NK cells (and to a lesser extent of Tyk2²⁹⁹²³E NK cells) is impaired. In contrast, the production of interferon γ (IFNγ) in response to interleukin 12 (IL-12) or to stimulation through NK cell-activating receptors strictly depends on the presence of enzymatically active TYK2. The cytotoxic activity of Tyk2²⁹⁹²³E NK cells against a range of target cells in vitro is higher than that of Tyk2⁻/⁻ NK cells. Consistently, Tyk2²⁹⁹²³E mice control the growth of NK cell-targeted tumors significantly better than TYK2-deficient mice, showing the physiological relevance of the finding. Inhibitors of TYK2’s kinase activity are being developed for the treatment of inflammatory diseases and cancers, but their effects on tumor immune surveillance have not been investigated. Our finding that TYK2 has kinase-independent functions in vivo suggests that such inhibitors will leave NK cell mediated tumor surveillance largely intact and that they will be suitable for use in cancer therapy.

Introduction

TYK2 is a member of JAK family of receptor-associated tyrosine kinases. Upon binding of cytokines or growth factors to their receptor complexes, JAKs undergo auto- and trans-phosphorylation and phosphorylate the signal transducing receptor chain(s), which then provide docking sites for signal transducers and activators of transcription (STATs). STATs translocate to the nucleus and initiate transcription to induce ligand specific cellular responses. Activation of TYK2 occurs upon binding of several different cytokines, such as type I and type III interferons (IFNs), interleukin (IL-) 10, IL-12, IL-22, IL-23 and cytokines utilizing the gp130 receptor chain.¹

The most prominent immunological alterations observed in gene-targeted TYK2-deficient mice²,³ and a naturally occurring TYK2 mutant mouse strain⁴ are high susceptibility to microbial infections, resistance to several inflammatory and autoimmune diseases and impaired tumor immune surveillance.¹,⁵ Breast cancer, thymoma and colon adenocarcinoma transplants grow more progressively in Tyk2⁻/⁻ mice.⁷⁻⁹ Similarly, TYK2 deficiency results in a higher incidence of Abelson-induced leukemia, which was attributed to an impaired NK cell cytotoxicity and NK cell-
mediated tumor immune surveillance.\textsuperscript{10,11} Impaired NK cell functions in the absence of TYK2 are not restricted to antitumor responses but were also observed in the context of \textit{Leishmania major} infections.\textsuperscript{12} To date, two TYK2-deficient patients have been reported who suffer from high susceptibility to infections among other immunological defects.\textsuperscript{13,14} The first TYK2 specific inhibitors have been recently developed and are considered as promising therapeutic agents for the treatment of inflammatory and autoimmune diseases.\textsuperscript{15,20}

Very recently, tumor cell-intrinsic TYK2 activity has been linked to the development of T cell acute lymphoblastic leukemia (T-ALL) and cutaneous T cell lymphoma development in humans.\textsuperscript{21,22} Therefore, specific inhibition of TYK2 activity might be considered as a new therapeutic opportunity for some hematologic malignancies. Furthermore, invasiveness of certain types of prostate and breast cancer could be blocked by TYK2 inhibition \textit{in vitro}.\textsuperscript{23,24}

It becomes increasingly evident that, besides their canonical function, JAKs also exert functions independent of their catalytic activity and/or cytokine receptor association. Kinase-independent functions of human TYK2 were reported for IFNAR, IL-6R and IL-10R signaling complexes.\textsuperscript{25-27} In murine cells, catalytically inactive TYK2 facilitates mitochondrial respiration and mediates crosstalk from the type I IFN receptor complex to the phosphoinositide 3-kinase (PI3K) pathway.\textsuperscript{28,29} Interestingly, nuclear translocation and chromatin association of TYK2 has been reported, however, the significance of this observation remains to be elucidated.\textsuperscript{30} Thus, before blocking TYK2 in therapy it is of great importance to assess its kinase-independent functions \textit{in vivo}.

Using gene-targeted mice that harbor a point mutation in the ATP-binding pocket of the TYK2 kinase domain (\textit{Tyk2}^{K923E}) we have recently reported that TYK2 kinase activity is essential for type I IFN signaling as well as antiviral responses \textit{in vivo}.\textsuperscript{31} In this study, we further investigated the role of TYK2 in NK cells and tumor immune surveillance and present the first evidence for kinase-independent functions of TYK2 \textit{in vivo}.

**Results**

NK cells in the bone marrow develop normally in the absence of TYK2 or in the presence of kinase-inactive TYK2

NK cells develop in the bone marrow before migrating to the periphery where they undergo full maturation.\textsuperscript{32} They develop from NK precursors (NKPs: Lin$^-$CD122$^+$NK1.1$^+$DX5$^+$) \textit{via} immature NK cells (iNK: Lin$^-$CD122$^+$NK1.1$^+$DX5$^+$) into mature NK cells (mNKs: Lin$^-$CD122$^+$NK1.1$^+$DX5$^+$). We found similar frequencies of total NK cells (Lin$^-$CD122$^+$) (Fig. 1A) and of all three developmental stages in the bone marrow of \textit{WT}, \textit{Tyk2}^{K923E} and \textit{Tyk2}^{K277E} mice (Fig. 1B).

**NK cell maturation depends on the presence of TYK2 and is partially restored by kinase-inactive TYK2**

Next, we analyzed the frequency and maturation of splenic NK cells. The percentage of CD3$^-$CD11b$^+$ NK cells was not differing from the \textit{WT} (Fig. 1C) but their maturation was severely impaired in TK2-deficient mice (Fig. 1D). Compared to \textit{WT}, NK cells from \textit{Tyk2}^{K277E} mice showed a lower percentage of the most mature (CD27$^-$CD11b$^+$) and a higher percentage of the least mature (CD27$^+$CD11b$^+$) NK cell population (Fig. 1D). Surprisingly, this defect was partially restored in NK cells expressing \textit{Tyk2}^{K923E} (Fig. 1D). In line with this, we found an intermediate abundance of NK cells positive for the late maturation marker KLRG1 in \textit{Tyk2}^{K923E} between \textit{WT} and \textit{Tyk2}^{K277E} NK cells (Fig. 1E). Although less pronounced, this also applied for the expression levels of KLRG1 (Fig. 1F). These results establish a novel function of TYK2 in driving NK cell maturation, which is partially independent of its kinase activity.

**Liver NK cell development depends on TYK2 kinase activity**

Liver-resident NK cells develop from a distinct lineage than conventional NK cells which are found in the spleen and circulate around the body.\textsuperscript{33} Liver-resident and conventional NK cells are distinguished by their exclusive expression of CD49a and CD49b (DX5).\textsuperscript{34} The percentage of NK cells among liver lymphocytes was not affected by the absence of TYK2 or its kinase activity (Fig. 1G). However, we observed a strong reduction in the abundance of liver-resident NK cells (CD3$^+$TCR$^-$NKp46$^+$CD49a$^+$) in \textit{Tyk2}^{K923E} and \textit{Tyk2}^{K277E} mice, whereas the number of conventional NK cells was similar in all three genotypes (CD3$^+$TCR$^-$NKp46$^+$CD49b$^+$) (Fig. 1H).

**NK cell receptor expression is differentially affected by the absence of TYK2 or the presence of kinase-inactive TYK2**

NK cell activity is controlled by the integration of signals derived from activating and inhibitory receptors.\textsuperscript{35} We did not detect any differences in the expression of the activating receptor Ly49H (Fig. 2A) and NKp46 (Fig. S1A) but we found fewer \textit{Tyk2}^{K277E} than \textit{WT} NK cells that express the inhibitory receptor Ly49G2 (Fig. 2B) and the activating receptor NKG2D (Fig. 2C). In contrast, \textit{Tyk2}^{K923E} NK cells showed similar frequencies of Ly49G2$^+$ and NKG2D$^+$ cells as \textit{WT} NK cells, although expression levels were slightly reduced (Fig. 2B and C). Surprisingly, the abundance of DNAM-1$^+$ NK cells was even higher in \textit{Tyk2}^{K923E} than in \textit{WT} mice, although the absence of TYK2 did not have any effect (Fig. 2D). Thus, expression of \textit{Tyk2}^{K923E} not only restores some of the defects of \textit{Tyk2}^{K277E} but also has consequences that differ from both, \textit{WT} and \textit{Tyk2}^{K277E} NK cells.

**Absence of TYK2 and presence of kinase-inactive TYK2 have distinct effects on the expression of miRNAs and mRNAs but not on the abundance of cytolytic proteins**

As it becomes increasingly evident that miRNAs regulate NK cell activity,\textsuperscript{36} we determined the expression levels of selected miRNAs in \textit{WT}, \textit{Tyk2}^{K923E} and \textit{Tyk2}^{K277E} cells. In general, the differences were rather subtle and the miRNA expression patterns differed between naive (Fig. 3A) and IL-2-activated NK cells (Fig. 3B). miR-155 expression was not significantly different in NK cells \textit{ex vivo} (Fig. 3A) but it was increased in IL-2-expanded
Tyk2<sup>K923E</sup> NK cells (Fig. 3B), miR-233 was increased in Tyk2<sup>K923E</sup> NK cells <i>ex vivo</i> (Fig. 3A) but decreased in Tyk2<sup>K923E</sup> and Tyk2<sup>−/−</sup> expanded NK cells (Fig. 3B). miR-27a* showed reduced expression only in the absence of TYK2 in <i>ex vivo</i> NK cells, whereas we did not detect differences in miR-30e expression (Fig. 3A and B).

We next analyzed the transcriptome of IL-2-expanded WT, Tyk2<sup>K923E</sup> and Tyk2<sup>−/−</sup> NK cells using microarray technology. We found very few differences between the genotypes: 27 transcripts were differentially expressed between Tyk2<sup>−/−</sup> and WT or Tyk2<sup>K923E</sup> and WT cells (≥2-fold change, <i>P</i> ≤ 0.05; Table S1). Among these transcripts, 11 differed at least 2-fold (<i>P</i> ≤ 0.05) between Tyk2<sup>−/−</sup> and Tyk2<sup>K923E</sup> NK cells (Table S1). Hierarchical cluster analysis of all genes (Fig. S2) confirmed that Tyk2<sup>K923E</sup> differ from both Tyk2<sup>−/−</sup> and WT NK cells.

IL-2 expansion of NK cells leads to a post-transcriptional upregulation of the cytolytic proteins granzyme B (GzmB) and perforin (Prf1).<sup>37</sup> Neither the absence of TYK2 nor the presence of TYK2K923E had an effect on the expression of GzmB and Prf1 (Fig. 3C).

**IFNγ production is dependent on TYK2 kinase activity**

IFNγ is one of the main NK cell effector molecules.<sup>38</sup> Consistent with previous reports,<sup>10,12</sup> we found strongly reduced STAT3 and STAT4 activation and impaired production of IFNγ in the absence of TYK2 in response to IL-12 (Fig. 4A). TYK2 is not acting as a receptor scaffolding protein as we found similar sur-

Figure 1. NK cell maturation is impaired in Tyk2<sup>−/−</sup> but partially restored in Tyk2<sup>K923E</sup> mice. (A) Frequency of all NK cells in bone marrow (Lin<sup>−</sup>CD122<sup>+</sup>) was assessed by flow cytometry. (B) Total NK cells were divided into subpopulations of three developmental stages: NK precursor (NKP), immature (INK) and mature (mNK) NK cells. Percentages of NKp46<sup>−</sup> cells among CD3<sup>e</sup> NK1.1<sup>−</sup>, iNKs (DX5<sup>e</sup> NK1.1<sup>−</sup>) and conventional NK cells (DX5<sup>e</sup> NK1.1<sup>−</sup>) among Lin<sup>−</sup>CD122<sup>+</sup> population in bone marrow obtained from WT, Tyk2<sup>K923E</sup> and Tyk2<sup>−/−</sup> mice are shown. (C) Percentages of NK cells in the spleen were assessed by flow cytometry. (D) Splenic CD3<sup>c</sup> NK1.1<sup>+</sup> cells were analyzed for the expression of maturation markers CD27 and CD11b. Percentage of NK cells in each of the four maturation stages: CD27<sup>−</sup> CD11b<sup>−</sup>, CD27<sup>−</sup> CD11b<sup>+</sup>, CD27<sup>+</sup> CD11b<sup>−</sup> and CD27<sup>+</sup> CD11b<sup>+</sup> was assessed in the spleen of WT, Tyk2<sup>K923E</sup> and Tyk2<sup>−/−</sup> mice. (E) The abundance of KLRG1<sup>+</sup> cells among CD3<sup>c</sup> NK1.1<sup>+</sup> population and the level of KLRG1 expression (MFI) were assessed in the spleen of WT, Tyk2<sup>K923E</sup> and Tyk2<sup>−/−</sup> mice. (F) Percentage of NK cells (CD3<sup>c</sup> TCR<sup>b</sup> NKp46<sup>−</sup>) among liver lymphocytes was assessed by flow cytometry. (H) Liver NK cells were divided into two subpopulations: liver resident NK cells (CD49b<sup>+</sup>) and conventional NK cells (CD49b<sup>−</sup>) and the total number of each population is presented. (A, C, E, F, G) Boxplots with whiskers from minimum to maximum show data derived from two (A, C, E, F, n = 6–8 per genotype) or three (C, G, n = 10 per genotype) independent experiments. (B, D, H) Mean ± SEM of two independent experiments is presented (n = 6–7 per genotype). (A–H) *<i>P</i> < 0.05, **<i>P</i> < 0.01, ***<i>P</i> < 0.001.
requirement for kinase-active TYK2 for IFN dynamism production to NK cell receptor activation and is not limited to its function in the IL-12 signaling cascade.

To assess if Tyk2K923E and Tyk2−/− NK cells show generally impaired responses to receptor stimulation we analyzed the production of CCL3. The percentage of CCL3+ cells after anti-NK1.1 antibody cross-linking was similarly reduced in Tyk2K923E and Tyk2−/− NK cells (Fig. 4C left panel). However, Tyk2K923E showed slightly higher expression levels than Tyk2−/− cells (Fig. 4C right panel), indicating that kinase-inactive TYK2 can partially mediate NK cell receptor responses. An intermediate CCL3 expression level in Tyk2K923E between WT and Tyk2−/− cells was also observed after cross-linking with anti-Ly49D antibody (Fig. S3B).

Kinase-inactive TYK2 partially restores cytotoxic capacity of NK cells

To further investigate how TYK2 and its kinase activity influence effector functions of NK cells, we assessed the ability of NK cells to form conjugates with target cells and to lyse them. We did not observe any differences in the formation of conjugates with YAC-1 target cells between WT, Tyk2K923E and Tyk2−/− NK cells (Fig. 5A). Similarly, expression of the degranulation marker CD107a following NK1.1 or Ly49D receptor stimulation was not influenced by the absence of TYK2 or the presence of kinase-inactive TYK2. (Fig. 5B and Fig. 5C). In line with previous studies, 11 cytotoxicity of NK cells against a range of target cells in vitro was severely impaired in the absence of TYK2 (Fig. 2E). However, Tyk2−/− NK cells were able to lyse RMA-S, RMA-Rae1 and YAC-1 cells more efficiently than Tyk2−/−, albeit not to the level reached by WT cells (Fig. 5C-E). To determine cytotoxic activity in an in vivo setting, we intravenously injected cells that are deficient for MHC class I molecules (β2m−/− splenocytes) into WT, Tyk2−/− and Tyk2K923E mice and determined their rejection. Consistent with our in vitro assays, Tyk2K923E mice killed β2m−/− cells significantly better than Tyk2−/− mice (Fig. 5F). However, the difference between Tyk2K923E and Tyk2−/− mice appeared less pronounced than in vitro, suggesting that the cytotoxic capacity of Tyk2K923E NK cells may depend on the type of target cell and/or environmental factors.

Kinase-inactive TYK2 mediates NK cell-dependent tumor surveillance

We next tested whether Tyk2K923E NK cells are able to mediate tumor surveillance in vivo using two different NK cell-targeted tumor transplant models. Tyk2K923E mice controlled the growth of RMA-S tumors similarly well as WT mice, whereas Tyk2−/− mice developed significantly bigger tumors (Fig. 6A). RMA-Rae1 tumors were again better controlled in Tyk2K923E than in Tyk2−/− mice, although not as efficiently as in WT mice (Fig. 6B). Differences in tumor surveillance may also be due to differences in migratory or proliferative capacity of NK cells, and thus we determined the number of infiltrating NK cells in RMA-Rae1 tumors. We found similar amounts of tumor-infiltrating NK cells in Tyk2−/− and Tyk2K923E mice, with respect to both the percentage (Fig. 6C, left panel) and the absolute numbers (Fig. 6C, right panel).

Taken together, our results clearly show that kinase-independent functions of TYK2 in NK cells are of physiological relevance.

Discussion

In this study we uncovered a key contribution of kinase-inactive TYK2 to NK cell-dependent tumor surveillance, thereby providing the first evidence for kinase-independent functions of TYK2 in vivo.

Previously we have shown that Tyk2−/− mice develop B lymphoid tumors with shorter latency and higher incidence than WT mice. This phenotype was attributable to decreased NK cell cytoxicity and tumor surveillance, although the underlying mechanisms remained unclear. Herein we show that NK cells develop normally in the bone marrow regardless of the absence of TYK2 or presence of a kinase-inactive TYK2. Splenic NK cell maturation, however, is impaired in Tyk2−/− but partially restored in Tyk2K923E mice. The decrease in the most mature NK cell population (CD27−CD11b+) in Tyk2−/− mice is similar to what has been described in Ifnar1−/− and Stat1−/− mice, 40–44 indicating that TYK2 exerts these functions through its role in IFNα/β signaling. IFNα/β responses are similarly reduced in Tyk2−/− and Tyk2K923E NK cells and mice show similar sensitivity against virus infections, 31 arguing against an impact of TYK2K923E on canonical IFNα/β signaling. However, it does not exclude a role of Tyk2K923E in mediating STAT-independent responses, such as crosstalk to other signaling cascades, not essential for antiviral activity.

We furthermore provide evidence for an involvement of TYK2 in the development of liver-resident NK cells. In contrast to the maturation of splenic NK cells, this fully depends on the presence of kinase-active TYK2. Liver-resident NK cells have just recently been discovered as an NK cell lineage distinct from conventional and other tissue resident NK cells. 33 It is unclear, how TYK2 impacts on their abundance in the liver. Type I IFN is likely not involved as normal numbers of liver-resident NK cells were reported in Ifnar1−/− mice. 42 In contrast to Tyk2K923E and Tyk2−/− mice, a dramatic decrease of total liver NK cells was found in Ifng−/− mice, but this study did not differentiate NK cell subpopulations. 45

With respect to NK cell function, we demonstrate that Tyk2K923E partially restores cytotoxic activity of conventional NK cells against a range of different target cells. It seems likely that the role of TYK2 is linked to its role in IFNα/β signaling, as NK cells from both Ifnar1−/− and Stat1−/− mice have impaired killing activity. 40–42,44 Effects on the expression of the lytic proteins GzmB and Prf1 and on degranulation could be excluded as the underlying mechanisms for IFNAR1 40 and TYK2. Furthermore, we show that TYK2 is not required for conjugate formation of NK cells with their target cells. In line with this, GzmB and Prf1 expression were not altered in the absence of STAT1. 41

Similar to our earlier findings for IFNα/β, we did not observe differences between Tyk2−/− and Tyk2K923E NK cells in...
miR-223 in naive and miR-155 in IL-2 expanded phenotype as for DNAM-1 was observed for the expression of increased expression of DNAM-1. A similar "gain-of-function" cascade is not due to an IL-12R

demonstrate that the requirement for TYK2 in the IL-12 signal-regulation of NK cell effector functions,36 miR-155 overexpression of the human NK cells, one may speculate that epigenetic remodeling of the Ifng locus might be dependent on kinase-active TYK2.49

Instead, TYK2 is needed for full activation of JAK2. This function may depend on TYK2 and regulate miR-155 and miR-233.52-54

Paracrine actions of cytokines during the IL-2 culture, which vivo and IL-2 cultivated NK cells may be explained by autocrine/paracrine actions of cytokines during the IL-2 culture, which may depend on TYK2 and regulate miR-155 and miR-233,52-54

While it has been shown that miR-233 has a redundant role in regulation of NK cell effector functions,36 miR-155 overexpression results in increased IFNγ production and cytotoxicity.47,52-53 Although we found IFNγ production unaffected, miR-155 upregulation might contribute to the improved killing activity of Tyk2K923E NK cells.

Partial restoration of NK cell cytotoxicity by kinase-inactive TYK2 also translates into NK cell-mediated tumor surveillance. Using two different NK cell-targeted tumor cell lines, we could show that tumor surveillance in Tyk2K923E is clearly better than in Tyk2−/− mice. NK cell infiltration was similar in WT, Tyk2K923E and Tyk2−/− mice, indicating that indeed functional differences account for the differences in tumor control.

Interestingly, surveillance of RMA-S tumors even reached WT levels in Tyk2K923E mice. Tumor cell-specificity may be explained by the differential expression of NK cell receptors. For example, DNAM-1 is particularly important for NK cell-mediated killing when NKG2D ligands are not present on tumor cells.55,56 Besides, tumor cell-specificity could also be due to differences in the tumor environment as other cell types, such as myeloid cells, dendritic cells or stromal fibroblasts, may crucially contribute to tumor surveillance. The slightly increased production of CCL3 by Tyk2K923E compared to Tyk2−/− NK cells may also contribute to an improved tumor surveillance, for instance by enhancing the recruitment and activation of dendritic cells.57

Figure 2. NK cells expressing NKG2D and DNAM-1 are more abundant in Tyk2K923E than in Tyk2−/− mice. Frequency of splenic NK cells expressing activating or inhibitory receptors and level of surface expression of these receptors (MFI) was assessed by flow cytometry. Boxplots with whiskers from minimum to maximum show percentage (left panel) and MFI (right panel) of CD3−CD11b− NK cells positive for receptors (A) Ly49H, (B) Ly49G2, (C) NKG2D and (D) DNAM-1. Data are derived from three (A, B, n = 10 per genotype) or two (C, D, n = 6–8 per genotype) independent experiments. (A-D) * P < 0.05, ** P < 0.01, *** P < 0.001.
In summary, we provide evidence that NK cell-dependent tumor surveillance does not strictly depend on the presence of kinase-active TYK2. This finding is of clinical relevance as there is growing interest in the use of TYK2 inhibitors for cancer therapy. Nevertheless, kinase-inactive TYK2 does not fully reconstitute the WT situation and it is thus necessary to carefully consider the beneficial effects of TYK2 inhibitors versus their potential harmful effects on antitumor immunity.

Materials and methods

Mice and cell lines

Wild-type (WT) C57BL/6N mice were purchased from Charles River Laboratories or Janvier Labs. Tyk2−/− (B6N.129P2-Tyk2tm1.Bia),2 Tyk2K923E (B6N.129P2-Tyk2tm3/x923E.Bia),3 and β2-microglobulin (B2m)−/− (B6.129-B2m−/−/N12),4,5 a kind gift from Prof. Wilfried Ellmeier) animals were on C57BL/6N background, gender- and age-matched (6–12 weeks) and maintained under specific pathogen-free conditions according to FELASA recommendations (2014). All animal experiments were approved by the Ethics and Animal Welfare Committee of the University of Veterinary Medicine Vienna and granted by the national authority (Austrian Federal Ministry of Science and Research) according to Section 8ff of Law for Animal Experiments under licenses GZ-66.099/0155-II/3b/2011, BMWF-68.205/0218-II/3b/2012 and were performed according to the guidelines of FELASA and ARRIVE.

Murine lymphoma cell lines YAC-1,59 RMA-S60 and RMA-Rae161 were cultured in RPMI1640 complete medium: RPMI1640 supplemented with L-glutamine (PAA), 10% heat-inactivated FCS (Invitrogen), 50 μM 2-mercaptoethanol (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma).

Tumor transplant experiments

Right and left flanks of WT, Tyk2K923E and Tyk2−/− animals were depilated 3 to 4 d prior to tumor cell injection. 10⁶ RMA-S or RMA-Rae1 cells were injected subcutaneously into each flank and tumor growth was monitored every other day. After 11 (RMA-Rae1) or 15 (RMA-S) d, the body and tumor weight were assessed. For flow cytometry analysis of tumor infiltrating cells, tumors were pressed twice through 100 μm cell striainers to obtain a single cell suspension.

NK cell isolation, expansion and stimulation

Spleens were isolated and single cell suspensions were prepared by pressing through 100 μm cell striainers. NK cells were isolated using DX5-labeled MACS® beads according to the manufacturer’s protocol (Miltenyi Biotec) and expanded in RPMI1640 complete medium supplemented with 5,000 U/mL recombinant human IL-2 (Proleukin®, Novartis) for 5–7 d. Purity of NK cells was assessed by flow cytometry and were routinely 85–95% CD3− NK1.1+ of total cells. Cultivated NK cells were further stimulated with 5 ng/mL recombinant mouse IL-12 (R&D Systems) for the times indicated and cells were used for Western blot analysis and supernatants for ELISA. For the analysis of the expression of GzmB, Prf1 and miRNAs, cells were deprived from IL-2 for 4 h prior to cell lysis.

Antibodies and flow cytometry

Single cell suspensions were prepared from spleen, bone marrow or liver. Liver lymphocytes were isolated by liver perfusion via the portal vein with 5–10 mL sterile PBS. Separation of lymphocytes and hepatocytes was performed using 37.5% percoll (GE Healthcare). CD16/CD32 (93), CD122 (TM-4), CD49b (DX5), NK1.1 (PK136), Nkp46 (29A1.4), CD3e (145-2C11), KLRG1 (2F1), CD27 (LG.7F9), CD11b (M1/70), Ly49H (3D10), Ly49G2 (4D11), NKG2D (CX5), DNAM-1 (10E5), IFNγ (XMG1.2), CD107a (1D4B), Nkp46 (29A1.4) and CCL3 (DNT3CC) were purchased from ebioscience. Mouse lineage antibodies (CD3: 145-2C11, CD19: 1D3, Ly6-C and...
Ly6G: RB6-8C5, Ter-119: TER119), Ly49D (4E5), CD49a (Ha31/8), TCR-β (H57-597) and IL-12Rβ1 (114) were purchased from BD Biosciences. Analyses were performed on a BD FACSCanto II (BD Bioscience, Heidelberg, Germany) and analyzed using BD FACSDiva software V8.0.

**Western blot**

Cell lysis, SDS-PAGE and Western blots were performed as described previously. For pJAK2 analysis cells were lysed directly in 1 x Laemmli sample buffer. Antibodies used were: pSTAT3 (Tyr705, CS#9131), STAT3 (CS#9132S), STAT4 (clone C46B10, CS#2653), pJAK2 (Tyr1008, D4A8, CS#8082), JAK2 (D2E12, CS#3230), GzmB (CS#4275) and Prf1 (CS#3693) all from Cell Signaling Technology; panERK (clone 16/ERK) and pSTAT4 (Tyr693, clone 38/pSTAT4) from BD Transduction Laboratories. Quantification of Western blots was done with ImageJ software.

**miRNA analysis**

RNA was isolated from expanded NK cells or ex vivo DX5 MACS-purified NK cells using miRNeasy Mini Kit (Qiagen). Reverse transcription was performed using miScript II RT Kit (Qiagen) according to manufacturer’s instructions. Quantitative PCR was performed on Stratagene MX3000 (Agilent Technology, Boebelingen, Germany) using miScript SYBR Green PCR Kit (Qiagen). Following miScript Primer Assays (Qiagen) were used: Hs_SNORD72_1 as an internal control, Mm_miR-27a*-1, Mm_miR-155_1, Mm_miR-30e-2, Mm_miR-223_2. The qPCR was analyzed using the standard curve method as described previously. Expression levels are shown relative to expression in WT NK cells.

**Transcriptional profile analysis**

RNA was isolated from expanded NK cells using peqGOLD TriFast (PEQLAB) and further purified using RNeasy Mini Kit (Qiagen) according to the manufacturers’ instructions. RNA quality was determined by 2100 Bioanalyzer Instrument (Agilent Technologies, Boebelingen, Germany) and only samples with RNA integrity number (RIN) over 8.7 were used for further analysis. The transcriptional profile of WT, Tyk2<sup>+/+</sup> and Tyk2<sup>−/−</sup> NK cells in biological triplicates was determined using GeneChip microarray assay (Affymetrix Mouse Gene 2.0 ST arrays). Sample preparation for microarray hybridization was carried out as described in the Affymetrix GeneChip WT PLUS Reagent Kit User Manual (Affymetrix). Sample processing was performed at an Affymetrix Service Provider and Core Facility, “KFB – Center of Excellence for Fluorescent Bioanalytics” (Regensburg, Germany). Data analysis was performed.

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**Figure 4.** IFN<sub>γ</sub> production by NK cells is dependent on TYK2 kinase activity. (A) DX5<sup>+</sup> cells were MACS-purified from spleens of WT, Tyk2<sup>K923E</sup> and Tyk2<sup>−/−</sup> mice, expanded for 7 d with IL-2, stimulated with IL-12 for indicated time points or left untreated (w/o) and further analyzed for activation of STATs and JAK2 and for IFN<sub>γ</sub> production. Levels of pSTAT3, pSTAT4 and pJAK2 were assessed by Western blot and a representative blot out of two independent experiments is shown (left panel). The production of IFN<sub>γ</sub> by NK cells was assessed by ELISA (right panel) and mean ± SEM is presented from three independent experiments (n = 3 per genotype per treatment). (B) WT, Tyk2<sup>K923E</sup> and Tyk2<sup>−/−</sup> splenocytes were stimulated with anti-NK1.1 antibody for 5 h. Intracellular IFN<sub>γ</sub> levels were assessed by flow cytometry and the percentage of IFN<sub>γ</sub><sup>+</sup> cells within the CD3<sup>−</sup>DX5<sup>+</sup> population (left panel) or level of IFN<sub>γ</sub> expression (MFI, right panel) is shown as boxplots with whiskers from minimum to maximum from three independent experiments (n = 3 per genotype). (C) WT, Tyk2<sup>K923E</sup> and Tyk2<sup>−/−</sup> splenocytes were stimulated with anti-NK1.1 antibody for 8 h. Intracellular CCL3 levels were assessed by flow cytometry and the percentage of CCL3<sup>+</sup> cells within the CD3<sup>−</sup>DX5<sup>+</sup> population (left panel) or level of CCL3 expression (MFI, right panel) is shown as boxplots with whiskers from minimum to maximum from two independent experiments (n = 4 per genotype). (A, B, C) *P < 0.05, **P < 0.01, ***P < 0.001.
(robust multi-chip average) normalization including background correction, quantile normalization across all arrays and median polished summarization based on log2 transformed expression values. For detection of differentially expressed genes, two-way ANOVA was performed. Genes with a $P$ value ≤ 0.05 and a fold change of at least two were considered to be deregulated. For visualization samples were assessed for batch effects and further normalized by the Partek’s batch removal tool using experiment as factor. Hierarchical clustering was done using standardized normalization. Pearson’s correlation as similarity measure and Ward’s method as linkage criteria to identify most global differences between the three genotypes. The microarray data have been deposited in the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE68294.

Conjugate formation

NK cells expanded for 5–7 d were stained with CD3ε and NK1.1 antibodies and YAC-1 cells were stained with Cell Proliferation Dye eFluor® 670 (eBioscience) according to the manufacturer’s protocol. 2 × 10^5 NK cells were mixed with 2 × 10^5 YAC-1 cells in 200 μL of cold RPMI1640 complete medium and centrifuged 600 rpm for 1 min (Heraeus Multifuge 1S, Thermo Scientific). Conjugate formation was induced by incubation at 37°C for 10 min and then stopped by adding ice cold PBS or prevented by keeping cells on ice. The percentage of duplets double positive for NK1.1 and eFluor® 670 among the CD3ε population was analyzed by flow cytometry.

NK cell IFNγ and CCL3 production

For in vitro expanded NK cells, IFNγ production was assessed in the cell culture supernatant using Mouse IFN-gamma Quantikine ELISA (R&D Systems). For α vivo NK cells, IFNγ and CCL3 production was analyzed by flow cytometry as follows: one day prior to the experiment, tubes were...
coated with 10 µg/mL of anti-NK1.1 antibody (PK136) or anti-Ly49D (4E5) and incubated overnight at 4°C. Splenocytes were seeded at 5 x 10^6 cells/tube in 400 µL RPMI1640 complete medium. After 1 h of incubation at 37°C, brefeldin A (eBioscience) was added and cells were incubated for another 4 h for IFNγ or 7 h for CCL3. Splenocytes were stained for CD3ε and DX5 and intracellular IFNγ or CCL3 using Foxp3/Transcription Factor Staining Buffer Set according to manufacturer’s instructions (eBioscience). The percentage of IFNγ^+ or CCL3^+ NK cells was assessed by flow cytometry.

Degranulation assay

Tubes were coated with anti-NK1.1 or anti-Ly49D antibody and splenocytes were seeded as described above. Cells were incubated for 4 h at 37°C in the presence of anti-CD107a antibody. Splenocytes were then stained for CD3ε and DX5 and the expression of CD107a on NK cells was analyzed by flow cytometry. The data are presented as a difference between CD107a mean fluorescence intensities (MFI) of anti-NK1.1/anti-Ly49D antibody stimulated and unstimulated sample.

NK cell cytotoxicity assays

In vitro NK cell cytotoxicity assays were performed as previously described using YAC-1, RMA-S and RMA-Rae1 as target cells. In vivo cytotoxicity assays were performed as follows: splenocytes from β2m−/− and WT mice were isolated and stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) according to the manufacturer’s instructions (Molecular Probes, CellTrace™ CFSE Cell Proliferation Kit) at 5 µM and 0.5 µM, respectively. β2m−/− and WT splenocytes were mixed at a 1:1 ratio and injected intravenously into recipient mice. The input sample was stored at 4°C. After 16 h, spleens of recipient mice were isolated and the ratio of CFSE^low//CFSE^high cells was assessed by flow cytometry. The rejection of β2m−/− cells was calculated as follows:

\[
\text{% rejection} = \left\{1 - \left(\frac{\text{CFSE}^\text{low}}{\text{CFSE}^\text{high}}\right)_{\text{output}}\right\} \times 100%.
\]

Statistical analysis

Unpaired t-tests or one-way ANOVA with Bonferroni post tests were performed using GraphPad Prism version 5.00 or 6.00 for Mac (GraphPad Software). The level of significance is indicated for each experiment (*P < 0.05; **P < 0.01; ***P < 0.001).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Supplemental Material
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