NK Cells Require Cell-Extrinsic and -Intrinsic TYK2 for Full Functionality in Tumor Surveillance and Antibacterial Immunity

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Tyrosine kinase 2 (TYK2) is a widely expressed receptor-associated kinase that is involved in signaling by a variety of cytokines with important immune regulatory activities. Absence of TYK2 in mice results in impaired NK cell maturation and antitumor activity, although underlying mechanisms are largely unknown. Using conditional ablation of TYK2 in NK cells we show that TYK2 is required for IFN-γ production by NK cells in response to IL-12 and for an efficient immune defense against *Listeria monocytogenes*. Deletion of TYK2 in NK cells did not impact NK cell maturation and IFN-γ production upon NK cell activating receptor (actR) stimulation. Similarly, NK cell–mediated tumor surveillance was unimpaired upon deletion of TYK2 in NK cells only. In line with the previously reported maturation-associated *Ifng* promoter demethylation, the less mature phenotype of *Tyk2*$^{-/-}$ NK cells correlated with an increased CpG methylation at the *Ifng* locus. Treatment with the DNA hypomethylating agent 5-aza-2-deoxycytidine restored the ability of *Tyk2*$^{-/-}$ NK cells to produce IFN-γ upon actR but not upon IL-12 stimulation. NK cell maturation was dependent on the presence of TYK2 in dendritic cells and could be rescued in Tyk2-deficient mice by treatment with exogenous IL-15/IL-15Rα complexes. IL-15 treatment also rescued the in vitro cytotoxicity defect and the impaired actR-induced IFN-γ production of *Tyk2*$^{-/-}$ NK cells. Collectively, our findings provide the first evidence, to our knowledge, for a key role of TYK2 in the host environment in promoting NK cell maturation and antitumor activity. ***The Journal of Immunology*****, 2019, 202: 1724–1734.**

Natural killer cells are effector lymphocytes of the innate immune system and are characterized by their strong cytotoxic activity against infected and transformed cells. NK cell effector functions are tightly regulated by several mechanisms, including activating and inhibitory NK cell receptor and cytokine signaling (1). Most of the cytokines that act on NK cells signal through the JAK/STAT pathway (2). All STAT family members positively or negatively regulate NK cell activities, although underlying mechanisms are just beginning to emerge (3). Little is known about the impact of the individual JAK family members (JAK1-3 and tyrosine kinase 2 [TYK2]). *Jak1*$^{-/-}$ and *Jak2*$^{-/-}$ mice die soon after birth and during embryonic development, respectively (4–6). Conditional deletion of JAK2 in adult mice revealed a critical role of JAK2 in the maintenance of peripheral NK cell numbers and their maturation state (7). Treatment of mice with the JAK2-specific inhibitor BSK805 or the JAK1/JAK2 inhibitor ruxolitinib mimics NK cell defects upon conditional deletion of JAK2 and results in accelerated metastasis of transplanted breast cancer cells (7). Ruxolitinib treatment of patients suffering from myeloproliferative neoplasms impairs NK cell proliferation, maturation, and cytolytic capacity (8). *Jak3*$^{-/-}$ mice and mice with a *Jak3* loss-of-function mutation fail to develop NK cells (9–11), a phenotype that is recapitulated in patients bearing *Jak3* mutations (12, 13). NK cells from *Tyk2*$^{-/-}$ mice fail to produce IFN-γ in response to IL-12 and/or IL-18 and have an impaired early control of *Leishmania major* infections (14, 15). Defective IFN-γ production by NK cells in response to IL-12/IL-18 cotreatment has been described in *TYK2*-deficient patients (16), albeit with one exception (17). TYK2 is also involved in signaling by type I IFNs, IL-23, and IL-10, which directly act on NK cells but also on other cell types that might affect NK cell development and activity, such as dendritic cells (DCs), macrophages, or stromal cells (18). The first evidence for a role of TYK2 in NK cell–mediated tumor surveillance was provided by studies using the Abelson murine leukemia virus–induced B cell tumor model (19), which was later confirmed with tumor transplants (20). NK cells from *Tyk2*$^{-/-}$ mice show reduced maturation

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Abbreviations used in this article: actR, activating receptor; 5-Aza, 5-aza-2-deoxycytidine; BM, bone marrow; DC, dendritic cell; *Ly5.1*, *B6.SJL-Ptprc*^{a}; β2*m*$^{-/-}$, β2-*microglobulin*$^{-/-}$; MHC I, MHC class I; p.i., postinfection; rm, recombinant murine; S, sequencing primer; TSS, transcriptional start site; TYK2, tyrosine kinase 2; *WT*, wild-type.

and cytotoxicity and produce considerably less IFN-γ upon NK cell activating receptor (actR) stimulation than wild-type (*WT*) NK cells, although the underlying mechanisms remain unclear (19, 20). JAK/STAT signaling components are frequently altered in cancers, and considerable effort is directed toward the development of specific inhibitors for tumor therapy (21–24). TYK2 inhibitors are considered for the treatment of inflammatory diseases and tumor therapy (25–32). However, to realize their full potential and to identify harmful side effects, a better understanding of how TYK2 regulates NK cell function and antitumor activity is required.

In this study, we made use of mice that lack TYK2 selectively in NK cells to discriminate between direct and indirect effects of TYK2 on NK cell functionality. We demonstrate that TYK2 in $CD11c^+$ and other non–NK cells regulates NK cell maturation, target cell cytotoxicity, and tumor surveillance, whereas TYK2 in NK cells is required for IL-12 signaling and an efficient immune defense against the intracellular bacterium *Listeria monocytogenes*. We furthermore provide evidence that NK cell–extrinsic, TYK2-dependent signals increase the competence of NK cells to produce high amounts of IFN-γ in response to actR stimulation and provide, to our knowledge, the first indication for an involvement of TYK2 in *Ifng* promoter demethylation.

Materials and Methods

Ethics statement

All animal experiments were approved by the Ethics and Animal Welfare Committee of the University of Veterinary Medicine Vienna and the national authority (Austrian Federal Ministry of Science and Research) according to §§ 26ff. of Animal Experiments Act, Tierversuchsgesetz 2012: TVG 2012 (BMWF-68.205/0218-II/3b/2012, BMWFW-68.205/0032-WF/II/3b/2014, BMWFW-68.205/0103-WF/V/3b/2015, BMWFW-68.205/0212-WF/V/3b/2016).

Mice and cell lines

WT, C57BL/6N mice were purchased from Janvier Labs. $Tyk2^{-/-}$ (*B6.129P2-Tyk2*^{tm1Biat} and *B6.129P2-Tyk2*$^{tm1.1Biat}$), $Tyk2^{fl/fl}$ (*B6.129S2-Tyk2*$^{tm1(fl/fl)Biat}$), $Tyk2^{\Delta DC}$ (*B6.129P2-Tyk2*$^{tm1[fl/fl]\ Biat}$*-Tg[Itgax-cre]*$^{1.1Reiz}$), and $Tyk2^{\Delta M}$ (*B6.129P2-Tyk2*$^{tm[fl/fl]Biat}$*-Lyz2*$^{tm[cre]Ifo}$*/J*) mice were described previously (33, 34). To generate mice that lack TYK2 in NK cells ($Tyk2^{\Delta NK}$), $Tyk2^{fl/fl}$ mice were crossed to *Ncr1-iCre–Tg (7BL/6N-Tg[Ncr1-icre]*^{265Sxl}) mice (35). *B6.SJL-Ptprc*^{a} (*Ly5.1*) mice were purchased from Charles River Laboratories, *β2-microglobulin*$^{-/-}$ ([$\beta 2m^{-/-}$]; *B6.129-B2m*^{tm1Jae}) mice (36) were kindly provided by Wilfried Ellmeier (Medical University of Vienna, Austria). All mice were on C57BL/6 background and bred at University of Veterinary Medicine Vienna under specific pathogen-free conditions. Age- and sex-matched (6–12 wk) mice were used for all experiments. For in vivo experiments, male mice (age 8–12 wk) were used. Murine lymphoma cell lines RMA-S (37) and RMA-Rae1 (38) were cultured in RPMI 1640 complete medium (R8758; Sigma-Aldrich) supplemented with 10% heat-inactivated FCS (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin (both from Sigma-Aldrich), and 50 μM 2-ME (Life Technologies).

In vivo tumor challenge

A total of 1×10^6 RMA-Rae1 tumor cells were s.c. injected into both flanks of mice depilated 3–4 d prior to tumor cell injection. Tumor growth was monitored every other day. After 10–13 d, the body and tumor weight were assessed.

In vivo L. monocytogenes *infection*

Mice were infected i.p. with 5×10^5 CFU *L. monocytogenes* strain EGD in 200 μl of PBS or were mock infected with PBS. Survival of mice was monitored for 2 wk. To determine bacterial burden, spleens and livers were harvested on day 5 postinfection (p.i.) and homogenized in PBS. Serial dilutions of homogenates were plated on Oxford agar plates (Biolife), and colonies were counted after 48 h growth at 37°C.

In vivo IL-15/IL-15Rα treatment

WT and $Tyk2^{-/-}$ mice were injected i.p. with recombinant murine (rm) IL-15 and IL-15Rα–Fc (both R&D Systems), which were preincubated for complex formation, as previously described (39), or PBS as a control. Injections were given every 2–3 d for 2 wk (four doses). Two days after the last injection, splenic NK cells were analyzed for the expression of maturation markers, or isolated splenocytes were analyzed for IFN-γ in response to anti-NK1.1 Ab stimulation as described below.

Abs and flow cytometry

NK cells from in vitro cultures and splenic single-cell suspensions were stained with the following Abs (all from eBioscience) against: CD16/CD32 (clone 93), CD49b (DX5), NK1.1 (PK136), NKp46 (29A1.4) CD3ε (145-2C11), CD3 (17A2), TCRβ (H57-597), CD8a (53-6.7), CD11c (N418), KLRG1 (2F1), CD27 (LG.7F9), CD11b (M1/70), MHC class II (M5/114.15.2), Ly6G (1A8), Ly6C (HK1.4), F4/80 (BM8), IFN-γ (XMG1.2), Ly5.2 (clone 104), and T-bet (eBio4B10). Biotinylated Ab to IL-15Rα and the isotype control were purchased from R&D systems. Intracellular T-bet and IFN-γ levels were analyzed using Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer's instructions. Analyses were performed on a FACSCanto II (BD Biosciences) and analyzed using BD FACSDiva software version 8.0 or on a CytoFLEX (Beckman Coulter) and analyzed using CytExpert version 2.2.0.97.

Mixed bone marrow chimeric mice

Ly5.1 recipient mice were lethally irradiated (9 Gy) 24 h prior to transplantation. Bone marrow (BM) of donor *WT*, $Tyk2^{-/-}$, and *Ly5.1* mice was isolated and depleted of mature T and NK cells using CD5- and DX5-labeled MACS beads (Miltenyi Biotec) according to the manufacturer's instructions. A total of 4×10^6 of *WT* or $Tyk2^{-/-}$ BM cells were mixed at a 1:1 ratio with *Ly5.1* BM cells and i.v. injected into *Ly5.1* recipient mice. Spleens of recipient *Ly5.1* animals were isolated 6 wk after receiving the donor BM and NK cell maturation, and T-bet levels were analyzed by flow cytometry.

NK cell isolation, expansion, and stimulation

NK cells were isolated from splenic single-cell suspensions from $Tyk2^{fl/fl}$, $Tyk2^{\Delta NK}$, $Tyk2^{\Delta M}$, $Tyk2^{\Delta DC}$, and $Tyk2^{-/-}$ mice using DX5-labeled MACS beads according to the manufacturer's instructions (Miltenyi Biotec). NK cells were expanded in RPMI 1640 complete medium supplemented with 5000 U/ml IL-2 (Proleukin; Novartis) or 100 ng/ml IL-15 (eBioscience) for 3–7 d. Purity of NK cells was assessed by flow cytometry and was typically 70–85% $CD3\varepsilon^-NK1.1^+$ cells of all living cells. Expanded NK cells were stimulated with 5 ng/ml recombinant mouse IL-12 (R&D Systems) for 10 min and used for Western blot analysis. For the analysis of actR-induced IFN-γ production, test tubes were precoated with 10 μg/ml of anti-NK1.1 Ab (PK136) (BioLegend or purified from the HB-191 hybridoma cell line) 1 d prior to the experiment. Splenocytes or cultured NK cells were seeded at 5×10^6 or 5×10^5 cells per tube, respectively, in RPMI 1640 complete medium. For the analysis of IL-12–induced IFN-γ production, 5×10^6 splenocytes or 5×10^5 cultured NK cells were seeded on uncoated tubes in RPMI 1640 complete medium with 5 ng/ml IL-12 (R&D Systems) and 5000 U/ml IL-2. Brefeldin A (eBioscience) was added after 1 h of incubation at 37°C. After additional 4 h of incubation, cells were stained for CD3ε, DX5, NKp46, and IFN-γ, and the frequency of $IFN\text{-}\gamma^+$ NK cells ($CD3\varepsilon^-DX5^+$ or $CD3\varepsilon^-NKp46^+$) was assessed by flow cytometry. For the analysis of IL-15Rα surface-levels, spleens were digested by collagenase D (1 mg/ml; Roche Applied Sciences) and DNase I treatment (20 μg/ml; Roche Applied Sciences).

NK cell cytotoxicity assays

In vitro cytotoxicity assays were performed as previously described (40) using RMA-S or RMA-Rae1 as target cells. For in vivo cytotoxicity assays, *WT* and $\beta 2m^{-/-}$ splenocytes were isolated, stained with different concentrations of CFSE, and mixed at a 1:1 ratio (input sample). Recipient mice were i.v. injected with input cells (1×10^7 cells per mouse), and the spleens of recipient mice were isolated after 16 h. Specific lysis was assessed by flow cytometry by the analysis of the $\beta 2m^{-/-}$ ($CFSE^{high}$): *WT* ($CFSE^{low}$) cell ratio in input sample and splenocytes of recipient mice.

RNA isolation and reverse transcription quantitative PCR

RNA from DX5-depleted splenocytes (flow) and DX5 MACS-purified NK cells was isolated using peqGOLD TriFast reagent (PEQLAB), and reverse transcription was performed using iScript First Strand cDNA Synthesis Kit (Bio-Rad). The quantitative PCR was performed with a Stratagene MX3000 instrument (Agilent Technology, Boebelingen, Germany) as described previously (41) using *Ube2d2* as housekeeping gene. The following primers were used: *Ube2d2-forward* 5′-AGG TCC TGT TGG AGA TGA TAT GTT-3′, *Ube2d2-reverse* 5′-TTGGGAAATGAATTG TCA

AGA AA-3′, *Ube2d2-probe* 5′-CCA AAT GAC AGC CCC TAT CAG GGT GG-3′, *Tyk2-forward* 5′-GGG TCA CCT TCA GCC AGA CA-3′, *Tyk2-revers* 5′-GAC CTT AGC CTG TGC ATT GTA GAG T-3′, *Tyk2-probe* 5′-CAC ATC GCA CAC AAA GTC GGC ATC A-3′.

Western blot

Cell lysis, SDS-PAGE, and Western blots were performed as described previously (41). The detection of chemiluminescence was performed using ChemiDoc Touch Imaging System (Bio-Rad) and analyzed by Image Lab software version 5.2 (Bio-Rad). For the analysis of TYK2, cells were lysed in 1× Laemmli sample buffer. The following Abs were used: anti–phospho-STAT3 (Tyr705, no. 9131), anti-STAT3 (no. 9132S), and anti-STAT4 (clone C46B10, no. 2653) from Cell Signaling Technology; anti–phospo-STAT4 (Tyr693, clone 38/pSTAT4) and anti-panERK (clone 16/ERK) from BD Transduction Laboratories. The anti-TYK2 Ab was custom made and described previously (41).

CpG methylation analysis

NK cells were DX5 MACS purified and FACS sorted ($CD3\varepsilon^-NK1.1^+$) using FACS Aria II (BD Biosciences). Genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen). The DNA methylation status of the *Ifng* locus was analyzed by Varionostic GmbH, Germany, using direct pyrosequencing. The following primers were used (see Fig. 6C): 400 bp *Ifng* promoter fragment: forward: 5′-AGAGAATTTTATAAGAATGGTA-TAGGTG-3′, reverse: 5′-Biotin-CATAAAAAAAACTACAAAACCAAA-ATACA-3′; sequencing primer (S)1 (for CpGs −212, −198 and −178): 5′-ATGGTATAGGTGGGTA-3′; S2 (for CpGs −58, −50, −39, and +12): 5′-AAAAAAAATTAAAAAAAAATTTGTG-3′; S3 (for CpGs +91 and +114): 5′-ATGGTATAGGTGGGTA-3′. Methylation levels were analyzed with ProMarkQ24 software. Annotation of the transcriptional start site (TSS) and the translational start site is according to Ensemble genome browser (42) release 90 (ENSMUST00000068592.4) and consistent with a previous report (43).

5-aza-2-deoxycytidine treatment

DX5 MACS-purified IL-2–expanded $Tyk2^{-/-}$ and $Tyk2^{\Delta NK}$ NK cells were treated with 5-aza-2-deoxycytidine (5-Aza) (Sigma-Aldrich) at 10 μM daily for 72 h with an untreated control, as described before (44). Cells were stimulated with anti-NK1.1 or IL-12 as described above, and frequency of IFN-γ^+ NK cells was assessed by flow cytometry. Cell viability was determined with flow cytometry using the LIVE/DEAD fixable dye eFluor 780 (eBioscience).

Statistical analysis

One-way ANOVA with Bonferroni post hoc test, two-way ANOVA with Tukey post hoc test, unpaired *t* test, or log-rank (Mantel–Cox) test (for Kaplan–Meier plot) were performed using GraphPad Prism version 7.0 for Mac (GraphPad Software). Statistical significances are indicated for each experiment (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Results

TYK2 in NK cells is dispensable for NK cell maturation

To analyze NK cell–intrinsic function of TYK2, we generated mice with an NK cell–specific deletion of TYK2 ($Tyk2^{\Delta NK}$) by crossing $Tyk2^{fl/fl}$ mice (34) with *Ncr1-iCre–Tg* mice (35). Deletion of TYK2 in NK cells from $Tyk2^{\Delta NK}$ mice was analyzed at both protein and mRNA level in MACS-enriched NK cells (Fig. 1A, 1B). TYK2 protein was barely detectable in NK cells from $Tyk2^{\Delta NK}$ mice (Fig. 1A), and mRNA levels were reduced to around 15% compared with NK cells from $Tyk2^{fl/fl}$ mice (Fig. 1B). NK cells develop in the BM from NK precursors to immature NK cells and mature NK cells before migrating to the periphery where they undergo full maturation and acquire functional competence (45–47). Four distinct NK cell maturation stages are defined by the cell surface markers CD27 and CD11b: $CD27^-CD11b^-$, $CD27^+$ $CD11b^-$, $CD27^+$ $CD11b^+$, and $CD27^-CD11b^+$ (from least to most mature NK cells). Complete TYK2 deficiency does not affect NK cell development and peripheral NK cell numbers but results in a reduced frequency of the most mature NK cell population (20). We did not observe differences in the maturation of NK cells from $Tyk2^{\Delta NK}$ and $Tyk2^{fl/fl}$ mice (Fig. 1C). In line with this, NK cells from $Tyk2^{\Delta NK}$ mice had similar levels of the inhibitory lectin-like receptor KLRG1 as NK cells from $Tyk2^{fl/fl}$ mice (Fig. 1D, Supplemental Fig. 1A) and only modestly reduced levels of the transcription factor T-bet (Fig. 1E), both of which are highly expressed in the most mature NK cell population (48). Similar to $Tyk2^{-/-}$ mice (20), $Tyk2^{\Delta NK}$

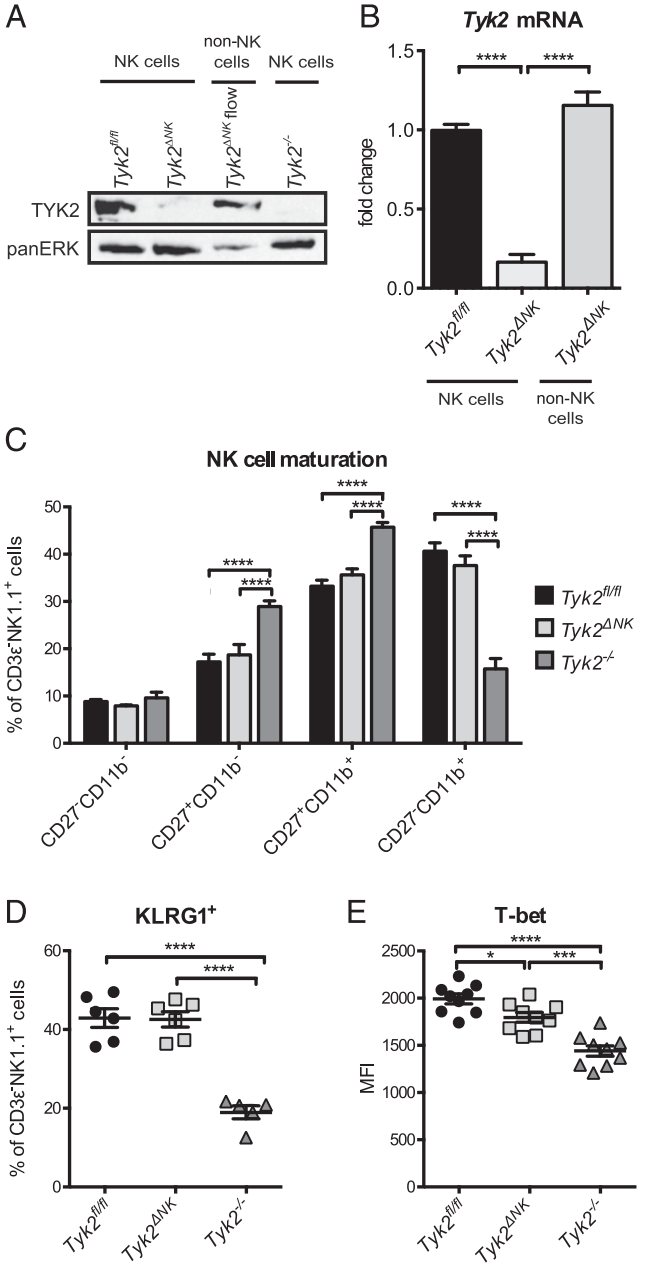

FIGURE 1. NK cells from $Tyk2^{\Delta NK}$ mice fully mature. (**A** and **B**) DX5 MACS-sorted NK cells from $Tyk2^{fl/fl}$ and $Tyk2^{\Delta NK}$ mice and NK cell–depleted splenocytes (flow) were used to analyze the deletion efficiency of TYK2. Directly after isolation (flow) or after 7-d expansion of NK cells in the presence of IL-2, (A) TYK2 protein levels were assessed by Western blot, or (B) RNA was isolated and *Tyk2* mRNA levels were assessed by reverse transcription quantitative PCR. *Tyk2* mRNA expression levels were normalized to the housekeeping gene *Ube2d2* and calculated relative to $Tyk2^{fl/fl}$ cells (set to 1). (A) One out of two independent experiments and (B) mean values ± SEM (n = 2–4 per genotype) are shown. (**C**–**E**) Splenocytes from $Tyk2^{fl/fl}$, $Tyk2^{\Delta NK}$, and $Tyk2^{-/-}$ mice were isolated and analyzed by flow cytometry for levels of (C) the maturation markers CD27 and CD11b, (D) KLRG1, and (E) T-bet in $CD3\varepsilon^-NK1.1^+$ cells. Mean percentages/MFIs ± SEM from two to three independent experiments are shown (n = 5–9 per genotype). *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. MFI, mean fluorescence intensity.

mice did not differ from control mice with respect to the total abundance of splenic NK cells (Supplemental Fig. 1B).

TYK2-deficient NK cells fully mature in a TYK2-sufficient surrounding

Ncr1-driven Cre recombinase expression starts at the immature NK cell stage ($Lin^-CD122^+DX5^-NK1.1^+$) and remains thereafter (35). To exclude the possibility that TYK2-dependent signals prior to Cre recombinase–mediated deletion impact peripheral NK cell maturation, we performed mixed BM chimera experiments. *WT* or *Tyk2*$^{-/-}$ *Ly5.2* BM cells were mixed at a 1:1 ratio with *WT Ly5.1* BM and adoptively transferred into lethally irradiated *Ly5.1* mice. Splenic NK cell maturation was analyzed before (input) and 6 wk after transplantation (Fig. 2A). *Tyk2*$^{-/-}$ BM cells repopulated with the same efficiency as *WT* cells (Supplemental Fig. 2A). Transfer of *Tyk2*$^{-/-}$ BM into *WT* mice restored the frequency of *Tyk2*$^{-/-}$ $CD27^-CD11b^+$ and $KLRG1^+$ NK cells (Fig. 2B, 2C, Supplemental Fig. 2B) and T-bet levels in *Tyk2*$^{-/-}$ NK cells to those observed in *WT* NK cells (Fig. 2D). Thus, TYK2 in the environment rather than in NK cells themselves promotes NK cell maturation.

NK cell maturation is in part driven by TYK2 in CD11c$^+$ cells and can be rescued by IL-15/IL-15Rα treatment of Tyk2$^{-/-}$ *mice*

Next, we sought to determine which cell type requires TYK2 to enable full NK cell maturation. Macrophages/monocytes (49–51), $CD11c^+$ DCs (52), and neutrophils (53) have been implicated in the regulation of NK cell maturation. Hence, we tested a potential role of TYK2 in these cell types by using mice that lack TYK2 in LysM-expressing cells (i.e., monocytes, macrophages, and neutrophils [*Tyk2*$^{\Delta M}$]) or in $CD11c^+$ cells (i.e., mainly DCs [*Tyk2*$^{\Delta DC}$]) (34). NK cells from *Tyk2*$^{\Delta M}$ mice did not display significant differences as compared with those derived from *Tyk2*$^{fl/fl}$ mice (Fig. 3A, 3B). In contrast, the frequency of $CD27^-CD11b^+$ (Fig. 3A) and $KLRG1^+$ (Fig. 3B) NK cells was significantly reduced in *Tyk2*$^{\Delta DC}$ mice, albeit to a lesser extent than in *Tyk2*$^{-/-}$ mice. NK cell homeostasis and maturation critically depends on IL-15 (54, 55). As it has long been suggested that accessory cells use IL-15Rα to *trans*-present IL-15 to developing NK cells (50, 51, 56), we analyzed IL-15Rα surface levels in *Tyk2*$^{fl/fl}$, *Tyk2*$^{\Delta M}$, *Tyk2*$^{\Delta DC}$, and *Tyk2*$^{-/-}$ mice. IL-15Rα levels were strongly reduced on macrophages and DCs but not on monocytes, NK cells, and $CD8^+$ T cells from *Tyk2*$^{-/-}$ compared with *Tyk2*$^{fl/fl}$ mice (Fig. 3C–E, Supplemental Fig. 3A–D). DCs from *Tyk2*$^{\Delta DC}$ mice had a similar reduction in IL-15Rα levels as *Tyk2*$^{-/-}$ mice, suggesting that DC-intrinsic TYK2 promotes IL-15Rα surface expression (Fig. 3C, Supplemental Fig. 3A, 3B). In contrast, macrophages from *Tyk2*$^{\Delta M}$ mice had similar IL-15Rα surface levels as *Tyk2*$^{fl/fl}$ mice, suggesting that cell-extrinsic TYK2 signaling regulates IL-15Rα on macrophages (Fig. 3E). Having established that *Tyk2*$^{-/-}$ mice show reduced IL-15Rα surface levels on macrophages and DCs, we next tested whether treatment with IL-15 rescues the NK cell maturation defects in *Tyk2*$^{-/-}$ mice. Sustained treatment of *Tyk2*$^{-/-}$ mice with IL-15/IL-15Rα complexes restored the abundance of both $CD27^-CD11b^+$ (Fig. 3F) and $KLRG1^+$ NK cells to *WT* levels (Fig. 3G). Taken together, these data indicate that TYK2 in DCs and other cell types drives NK cell maturation and that this function of TYK2 can be compensated for by IL-15/IL-15Rα treatment.

NK cell–intrinsic TYK2 is required for IL-12 signaling and efficient immune defense against L. monocytogenes

Absence of TYK2 in humans and mice results in impaired IL-12 signaling and IFN-γ production in NK cells and T cells (16, 18).

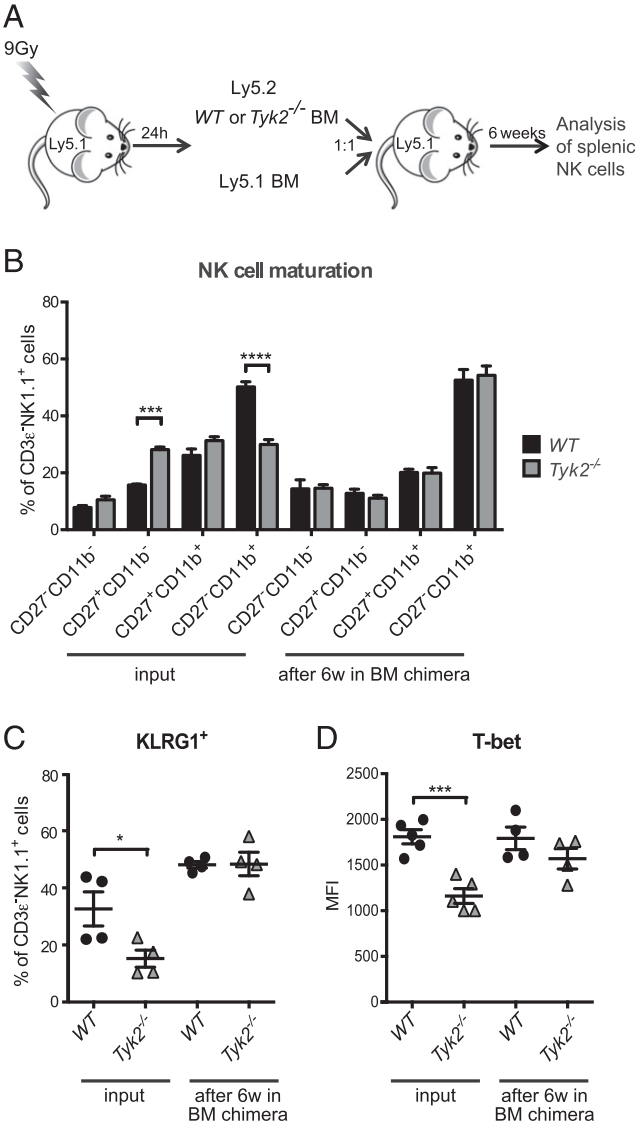

FIGURE 2. TYK2-deficient NK cells fully mature in a TYK2-sufficient surrounding. (**A**) Scheme representing the experimental design of BM chimeras. (**B–D**) BM from *WT* or *Tyk2*$^{-/-}$ mice was adoptively transferred into lethally irradiated *Ly5.1* mice. Splenocytes of donor (input) and recipient mice (after 6 wk in BM chimera) were analyzed by flow cytometry for maturation markers (B) CD27, CD11b, and (C) KLRG1 and (D) T-bet in $Ly5.2^+$ $CD3\varepsilon^-NK1.1^+$ NK cells. Mean percentages/MFI ± SEM from two independent experiments are shown (n = 4–5 per genotype). *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. MFI, mean fluorescence intensity.

In vitro expanded NK cells from *Tyk2*$^{\Delta NK}$ mice showed strongly reduced phosphorylation of STAT3 and STAT4 in response to IL-12 compared with NK cells from *Tyk2*$^{fl/fl}$ mice (Fig. 4A). The defect was less pronounced than in NK cells from *Tyk2*$^{-/-}$ mice (Fig. 4A), which most likely reflects the presence of minor amounts of non–NK cells in MACS-purified and IL-2–expanded NK cell cultures. Ex vivo treatment of NK cells with IL-12 showed a similar impairment of IFN-γ production of NK cells from *Tyk2*$^{\Delta NK}$ and *Tyk2*$^{-/-}$ mice compared with NK cells from *Tyk2*$^{fl/fl}$ mice (Fig. 4B), confirming that IL-12 signaling requires TYK2 in NK cells themselves. To test IL-12 responsiveness of NK cells in vivo, we infected mice with *L. monocytogenes* and analyzed IFN-γ production at day 3 p.i. The frequency of splenic IFN-γ–producing NK cells ($CD3\varepsilon^-NK1.1^+NKp46^+$) was profoundly reduced in *Tyk2*$^{\Delta NK}$ and *Tyk2*$^{-/-}$ compared with *Tyk2*$^{fl/fl}$ mice (Fig. 4C). *Tyk2*$^{-/-}$ mice have

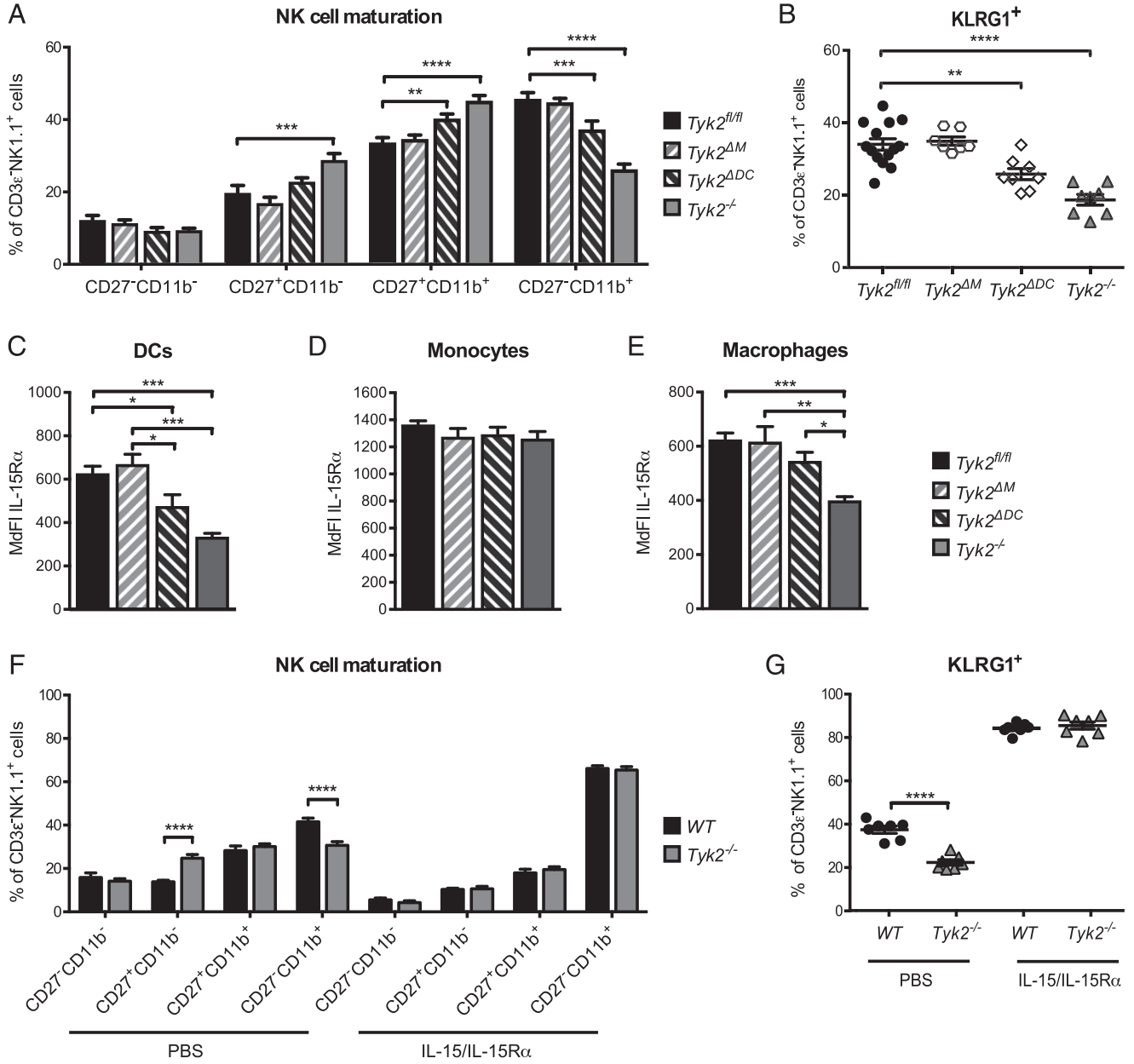

FIGURE 3. TYK2 in CD11c$^+$ cells is required for full NK cell maturation, and IL-15 treatment of *Tyk2*$^{-/-}$ mice rescues NK cell maturation (**A** and **B**) Splenocytes from *Tyk2*$^{fl/fl}$, *Tyk2*$^{\Delta M}$, *Tyk2*$^{\Delta DC}$, and *Tyk2*$^{-/-}$ were isolated, and NK cells (CD3ε$^-$NK1.1$^+$) were analyzed for maturation markers (A) CD27, CD11b, and (B) KLRG1. Mean values ± SEM from two independent experiments per genotype are shown (n = 7–14, *Tyk2*$^{fl/fl}$ are combined littermate controls from *Tyk2*$^{\Delta M}$ and T*yk2*$^{\Delta DC}$ mice). (**C**–**E**) Splenocytes from *Tyk2*$^{fl/fl}$, *Tyk2*$^{\Delta M}$, *Tyk2*$^{\Delta DC}$, and *Tyk2*$^{-/-}$ mice were isolated, and IL-15Rα levels determined by flow cytometry on DCs (CD11c$^+$MHC class II^{high}), monocytes (CD11c$^-$NK1.1$^-$Ly6G$^-$Ly6C$^+$), and macrophages (CD11c$^-$NK1.1$^-$Ly6G$^-$F4/80$^+$). Median fluorescence intensity (MdFI) ± SEM from two to three independent experiments (n = 5–10 per genotype) are shown. (**F** and **G**) *WT* and *Tyk2*$^{-/-}$ mice were treated with rmIL-15/IL-15Rα–Fc complexes every 2–3 d, and after four injections the expression of (F) CD27, CD11b, and (G) KLRG1 on CD3ε$^-$NK1.1$^+$ cells was assessed. Mean values ± SEM from two independent experiments are shown (n = 7 per genotype). For simplicity, significances are only given for the comparison between *Tyk2*$^{fl/fl}$ and the other genotypes in (A) and (B). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

a strongly increased susceptibility to *L. monocytogenes*, which has been associated with an impaired CD8$^+$ T cell cytotoxicity and IFN-γ production (57). *Tyk2*$^{\Delta NK}$ mice succumbed to *L. monocytogenes* significantly earlier and showed a lower survival rate than *Tyk2*$^{fl/fl}$ mice (Fig. 4D). Bacterial burden in spleen and liver was significantly higher in *Tyk2*$^{\Delta NK}$ than in *Tyk2*$^{fl/fl}$ mice, albeit lower than in *Tyk2*$^{-/-}$ mice at day 5 p.i. (Fig. 4E). In line with the differences in bacterial burden, the survival rate of *Tyk2*$^{-/-}$ and *Tyk2*$^{\Delta NK}$ mice was 25 and 75%, respectively, compared with *Tyk2*$^{fl/fl}$ mice at day 5 p.i. (Fig. 4F). Collectively these data indicate that TYK2 in NK cells is required for IL-12 signaling and IFN-γ production by NK cells during *L. monocytogenes* infection and contributes to an efficient immune defense against *L. monocytogenes*.

NK cell–intrinsic TYK2 is dispensable for cytotoxic activity and tumor rejection

We next assessed the ability of NK cells from *Tyk2*$^{\Delta NK}$ mice to lyse tumor cells in vitro. IL-2–expanded NK cells from *Tyk2*$^{\Delta NK}$ mice killed RMA-S and RMA-Rae1 cells as efficiently as those from *Tyk2*$^{fl/fl}$ littermate controls, whereas, consistent with previous reports (19, 20), NK cells from *Tyk2*$^{-/-}$ mice had strongly reduced

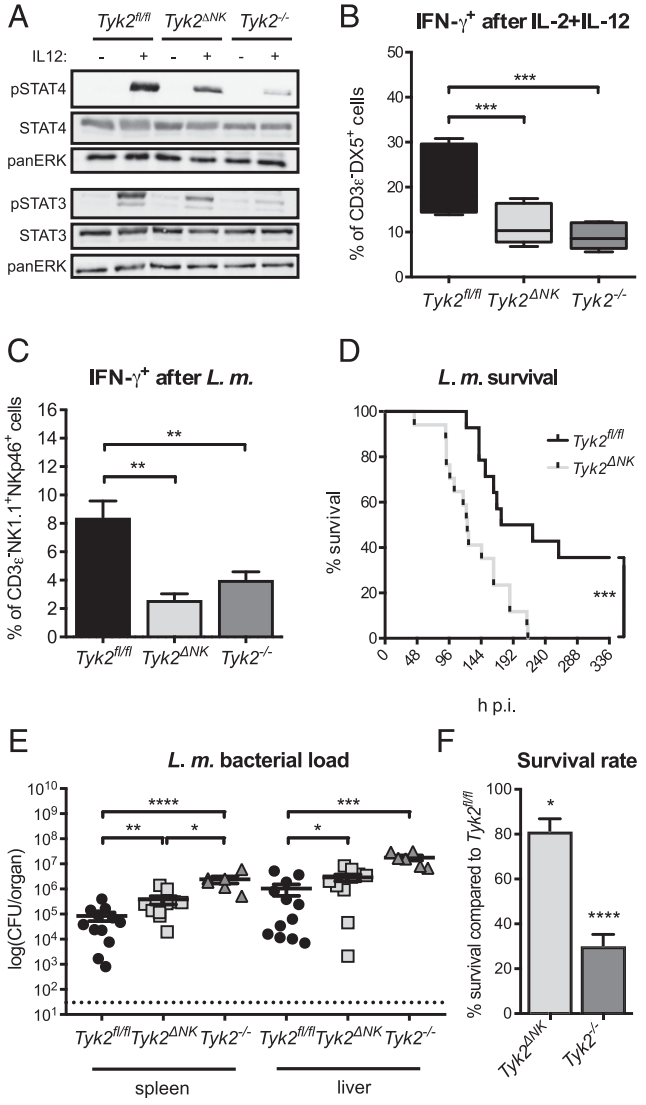

FIGURE 4. NK cell–intrinsic TYK2 is indispensable for the IL-12–mediated response against *L. monocytogenes*. (**A**) DX5 MACS-purified NK cells were expanded for 7 d in the presence of IL-2 and stimulated with IL-12 for 10 min. Levels of phospho- and total STAT4 and STAT3 were assessed by Western blot. Data from one out of two independent experiments are shown. (**B**) Splenocytes from $Tyk2^{fl/fl}$, $Tyk2^{\Delta NK}$, and $Tyk2^{-/-}$ mice were isolated and stimulated with IL-12. Brefeldin A was added after 1 h of incubation, and IFN-γ production by NK cells ($CD3\varepsilon^{-}DX5^{+}$) was assessed after additional 4 h of incubation by flow cytometry. Mean values ± SEM from three independent experiments are shown (n = 6–10 per genotype). (**C**) $Tyk2^{fl/fl}$, $Tyk2^{\Delta NK}$, and $Tyk2^{-/-}$ mice were infected i.p. with *L. monocytogenes* (5×10^5 CFU/mouse). Splenocytes were isolated at day 3 p.i and incubated in the presence of brefeldin A for 4 h. IFN-γ production by NK cells ($CD3\varepsilon^{-}NK1.1^{+}NKp46^{+}$) was assessed by flow cytometry. Mean percentage ± SEM of $IFN\text{-}\gamma^{+}$ NK cells derived from two to three independent experiments are shown (n = 5–9 per genotype). (**D**) $Tyk2^{fl/fl}$ and $Tyk2^{\Delta NK}$ mice were infected i.p. with *L. monocytogenes* (*L.m.*, 5×10^5 CFU/mouse), and survival was monitored for 14 d. Data are derived from two independent experiments (n = 10–17 per genotype). (**E** and **F**) $Tyk2^{fl/fl}$, $Tyk2^{\Delta NK}$, and $Tyk2^{-/-}$ mice were infected i.p. with *L. monocytogenes* (5×10^5 CFU/mouse). (E) Bacterial load in spleen and liver and (F) survival rate compared with $Tyk2^{fl/fl}$ mice ($Tyk2^{fl/fl}$ set to 100%) was determined 5 d p.i. (E) Mean values ± SEM from two independent experiments (n = 6–12 per genotype) are shown. Dotted line represents the detection limit. (F) Mean percentages relative to $Tyk2^{fl/fl}$ mice from two independent experiments are given. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

cytotoxic activity (Fig. 5A, 5B). Cultivation in the presence of IL-15 restored cytotoxic activity of NK cells from $Tyk2^{-/-}$ mice (Fig. 5C), indicating that IL-15 not only restores maturation but also functional defects of $Tyk2^{-/-}$ NK cells. To test NK cell cytotoxic activity in vivo, we injected splenocytes from mice deficient for $\beta 2m^{-/-}$ mice, which are recognized and lysed by NK cells because of low MHC class I (MHC I) expression (58, 59). $\beta 2m^{-/-}$ cells were eliminated in $Tyk2^{\Delta NK}$ mice as efficiently as in $Tyk2^{fl/fl}$ mice, whereas $Tyk2^{-/-}$ showed severely impaired rejection (Fig. 5D). In line with the cytotoxicity assays, $Tyk2^{\Delta NK}$ mice controlled the growth of transplanted RMA-Rae1 tumor cells as efficiently as $Tyk2^{fl/fl}$ mice (Fig. 5E). Consistent with our previous report (20), tumor growth was significantly increased in $Tyk2^{-/-}$ mice (Fig. 5E). NK cell activity is controlled by the balance of signals derived from actR and inhibitory NK cell receptors. Although TYK2 is not involved in signaling downstream of actR (60), such as NK1.1 and Ly49H, $Tyk2^{-/-}$ NK cells have impaired IFN-γ production in response to anti-NK1.1 Ab stimulation (20). Unlike NK cells from $Tyk2^{-/-}$ mice, those from $Tyk2^{\Delta NK}$ mice showed unimpaired anti-NK1.1 Ab–induced IFN-γ production (Fig. 5F). Thus, in contrast to IL-12, actR-induced IFN-γ production does not depend on TYK2 in NK cells themselves. Reminiscent of NK cell maturation and cytotoxicity, IFN-γ production in response to NK1.1 activation was rescued by treatment of $Tyk2^{-/-}$ mice with IL-15/IL-15Rα complexes (Fig. 5G). Having established that TYK2 in NK cells themselves is of little importance for NK cell–dependent target cell killing and tumor surveillance, we next assessed the ability of $Tyk2^{\Delta M}$ and $Tyk2^{\Delta DC}$ mice to kill target cells in vivo and control RMA-Rae1 tumor growth. $Tyk2^{\Delta M}$ and $Tyk2^{\Delta DC}$ mice showed comparably impaired killing of $\beta 2m^{-/-}$ splenocytes, albeit the defect in $Tyk2^{\Delta DC}$ mice was less pronounced than in $Tyk2^{-/-}$ mice (Fig. 5H). $Tyk2^{\Delta M}$ mice were able to control RMA-Rae1 tumor cells as efficiently as $Tyk2^{fl/fl}$ controls, whereas $Tyk2^{\Delta DC}$ mice showed an intermediate tumor growth between $Tyk2^{fl/fl}$ and $Tyk2^{-/-}$ mice (Fig. 5I). We did not observe differences in the production of IFN-γ in response to actR stimulation between NK cells from $Tyk2^{fl/fl}$, $Tyk2^{\Delta M}$, and $Tyk2^{\Delta DC}$ mice (Fig. 4J).

Taken together, these data show that cell-extrinsic TYK2 promotes NK cell cytotoxicity, tumor surveillance, and actR-induced IFN-γ production and that TYK2 signaling in myeloid cells and DCs augments NK cell–dependent target cell killing in vivo.

Impaired actR-induced IFN-γ production by $Tyk2^{-/-}$ NK cells correlates with increased Ifng promoter methylation

Human NK cells acquire the competence to produce IFN-γ in response to actR engagement during maturation through progressive demethylation at the *Ifng* locus (61). To test whether differences in DNA methylation cause the difference in IFN-γ production upon actR stimulation between *WT* and $Tyk2^{-/-}$ NK cells, we expanded NK cells for 72 h in the presence of 5-Aza, a drug that blocks DNA methylation during cell proliferation. *WT* and $Tyk2^{-/-}$ NK cells showed similar survival under these conditions (Supplemental Fig. 4). 5-Aza treatment restored anti-NK1.1 Ab–induced IFN-γ production by $Tyk2^{-/-}$ NK cells to levels produced by *WT* NK cells (Fig. 6A). Importantly, IL-12–induced IFN-γ production remained strongly reduced (Fig. 6B), arguing against a general enhancement of *Ifng* gene induction upon global demethylation. We next analyzed the methylation status of nine CpGs located around the TSS of the *Ifng* gene in NK cells from $Tyk2^{fl/fl}$, $Tyk2^{\Delta NK}$, and $Tyk2^{-/-}$ mice (Fig. 6C). We found a significant increase in methylation of four CpGs (−212, −58, +91, and +114) in NK cells from $Tyk2^{-/-}$ compared with those from $Tyk2^{fl/fl}$ and $Tyk2^{\Delta NK}$ mice (Fig. 6D). Although not conserved in humans, CpGs

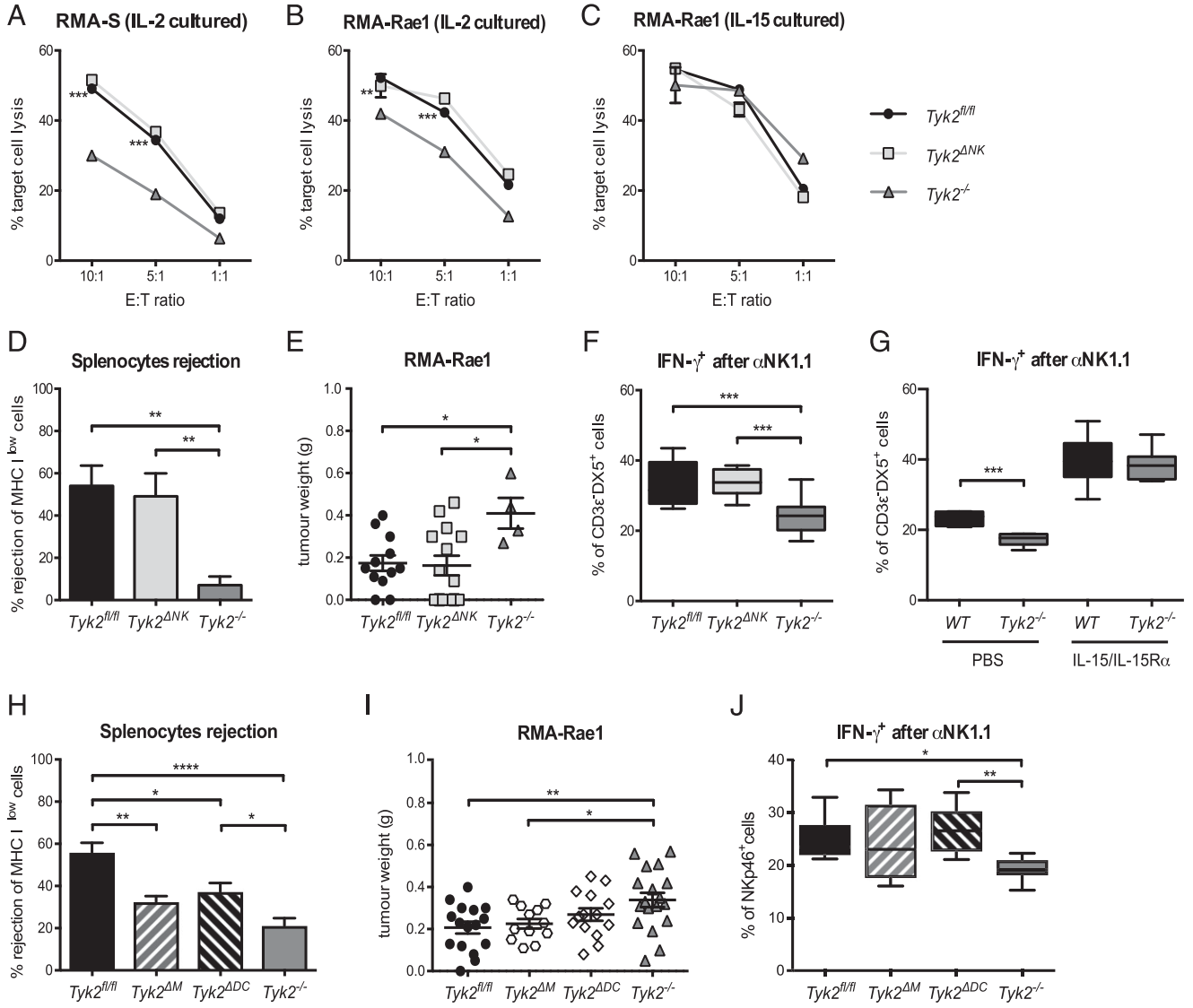

FIGURE 5. *Tyk2*$^{\Delta NK}$ cells show restored cytotoxicity, tumor surveillance, and responses to actR stimulation. DX5 MACS-purified, (**A** and **B**) IL-2–, or (**C**) IL-15–expanded NK cells were incubated with (A) RMA-S or (B and C) RMA-Rae1 cells at the indicated effector:target (E:T) ratios. Specific lysis was assessed after 4 h by flow cytometry. (A–C) One representative out of two independent experiments is shown (with three technical replicates per group). (**D** and **H**) Splenocytes from $\beta 2m^{-/-}$ and *WT* mice were isolated, mixed at an 1:1 ratio, and injected i.v. into (D) *Tyk2*$^{fl/fl}$, *Tyk2*$^{\Delta NK}$, and *Tyk2*$^{-/-}$ mice or (H) *Tyk2*$^{fl/fl}$, *Tyk2*$^{\Delta M}$, *Tyk2*^{DC}, and *Tyk2*$^{-/-}$ mice. The specific rejection of $\beta 2m^{-/-}$ (MHC I^{low}) cells was analyzed after 16 h by flow cytometry, and the mean rejection ± SEM from two independent experiments is shown (n = 5–7 per genotype). (**E** and **I**) 10^6 RMA-Rae1 cells were s.c. injected into (E) *Tyk2*$^{fl/fl}$, *Tyk2*$^{\Delta NK}$, and *Tyk2*$^{-/-}$ mice or (I) *Tyk2*$^{fl/fl}$, *Tyk2*$^{\Delta M}$, *Tyk2*^{DC}, and *Tyk2*$^{-/-}$ mice, and the tumor weight was assessed after 10–13 d. Mean values ± SEM from (E) two independent experiments for *Tyk2*$^{fl/fl}$ and *Tyk2*$^{\Delta NK}$ (n = 12–14 per genotype) and one experiment for *Tyk2*$^{-/-}$ mice (n = 4 per genotype) and (I) two independent experiments (n = 12–16 per genotype) are shown. (**F** and **J**) Splenocytes from (F) *Tyk2*$^{fl/fl}$, *Tyk2*$^{\Delta NK}$, and *Tyk2*$^{-/-}$ mice or (J) *Tyk2*$^{fl/fl}$, *Tyk2*$^{\Delta M}$, *Tyk2*^{DC}, and *Tyk2*$^{-/-}$ mice were isolated and stimulated with anti-NK1.1 Ab. Brefeldin A was added after 1 h of incubation, and IFN-γ production by (F) CD3ε$^-$DX5$^+$ or (J) CD3ε$^-$NKp46$^+$ NK cells was assessed after additional 4 h of incubation by flow cytometry. Mean percentages ± SEM from (F) two to five independent experiments (n = 10–14 per genotype) or (J) four independent experiments (n = 8–15 per genotype) are shown. (**G**) *WT* and *Tyk2*$^{-/-}$ mice were treated with rmIL-15/IL-15Rα–Fc complexes every 2–3 d, and after four injections splenocytes were isolated and stimulated with anti-NK1.1 Ab for 5 h in the presence of brefeldin A. IFN-γ production of NK cells (CD3ε$^-$DX5$^+$) was assessed by flow cytometry. Mean percentages ± SEM from two independent experiments are shown (n = 6–7 per genotype). (D, H, I, and J) *Tyk2*$^{fl/fl}$ are combined littermate controls from *Tyk2*$^{\Delta M}$ and *Tyk2*$^{\Delta DC}$ mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

+91 and +114 are in the same region as CpGs +122 and +128 at human *Ifng* promoter, which undergo demethylation during NK cell maturation (61). Consistent with the maturation-independent low level of methylation of the CpG at position −53 observed in human NK cells (61), CpGs in the proximal *Ifng* promoter (−198, −178, −58, −50, −39) were mostly demethylated in NK cells from *Tyk2*$^{fl/fl}$, *Tyk2*$^{\Delta NK}$, and *Tyk2*$^{-/-}$ mice (Fig. 6D). Collectively, these data suggest that impaired actR-induced IFN-γ production by NK cells from *Tyk2*$^{-/-}$ mice is due to hypermethylation at the *Ifng* gene locus and most likely reflects defective terminal maturation.

Discussion

In this study, we provide evidence that NK cells require at least two distinct TYK2-dependent signaling inputs to be fully functional in both tumor surveillance and antibacterial immunity: an NK cell–extrinsic input that drives NK cell maturation, cytotoxicity, and IFN-γ production in response to actR stimulation and an NK cell–intrinsic input that drives IFN-γ production in response to IL-12 and contributes to the immune defense against bacterial infection.

NK cells from *Tyk2*$^{-/-}$ mice develop normally in the BM but show impaired terminal maturation in the spleen (20). Using mice

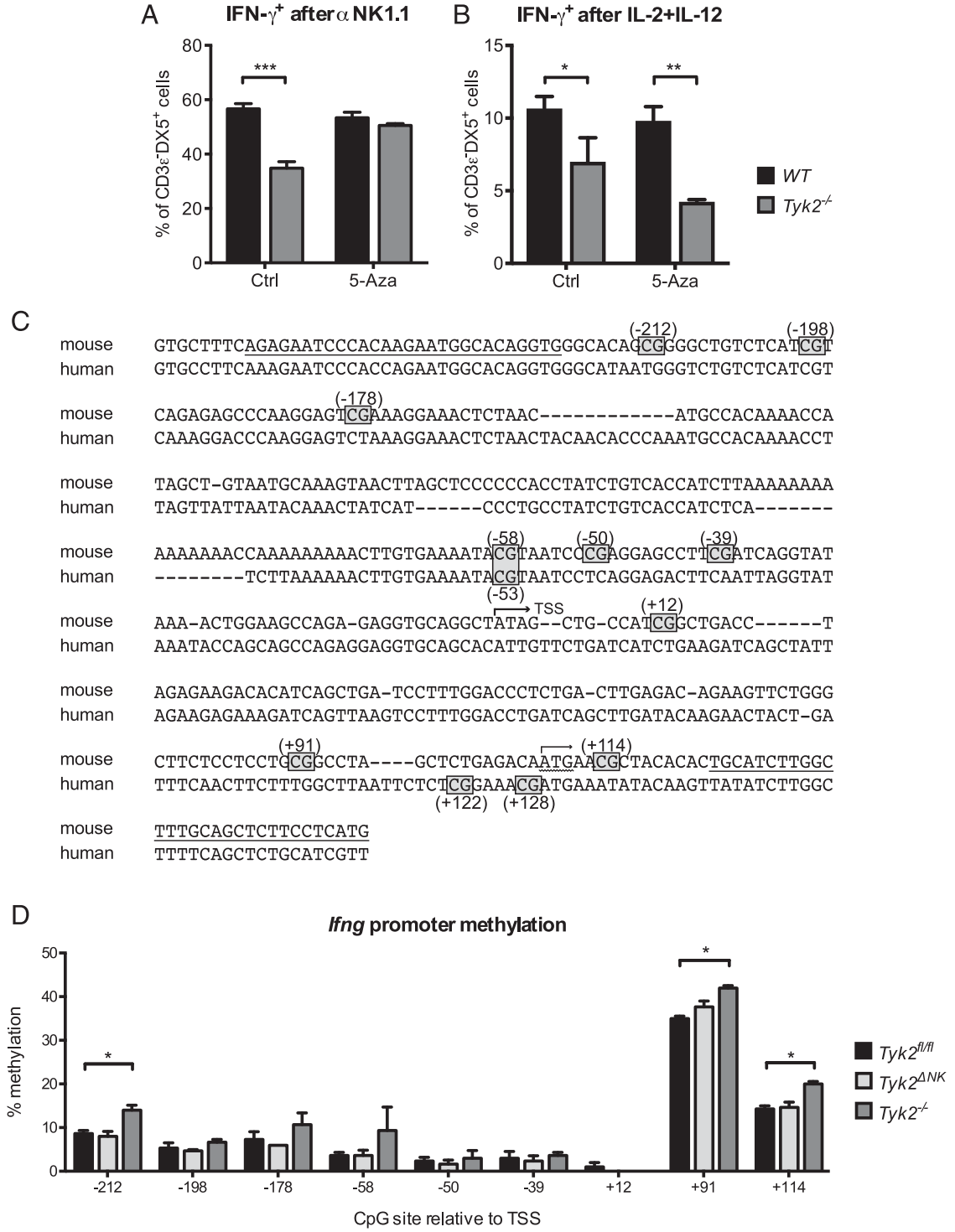

FIGURE 6. DNA demethylation restores IFN-γ production by $Tyk2^{-/-}$ NK cells in response to actR but not IL-12, and $Tyk2^{-/-}$ NK cells have increased methylation at three CpG sites in the *Ifng* gene locus compared with *WT* cells. (**A** and **B**) DX5 MACS-purified NK cells were expanded in the presence of IL-2 for 3 d in the absence (Ctrl) or presence of 5-Aza, stimulated with (A) anti-NK1.1 Ab, or (B) IL-2 and IL-12. Brefeldin A was added after 1 h incubation, and IFN-γ production by $CD3\varepsilon^{-}DX5^{+}$ NK cells was assessed after additional 4 h of incubation by flow cytometry. Mean values ± SEM from (A) two independent experiments (n = 3–4 per genotype and treatment) and (B) two independent experiments (n = 4 per genotype and treatment) are shown. (**C**) Alignment of the mouse and the human *Ifng* promoter sequence around TSS. CpGs are highlighted in gray boxes with the relative position to the TSS indicated in brackets. Underlined sequences indicate the position of the primers used for the amplification of the region of interest. The underlined ATG indicates the translational start site. (**D**) DNA was isolated from FACS-sorted splenic NK cells ($CD3\varepsilon^{-}NK1.1^{+}$) from $Tyk2^{fl/fl}$, $Tyk2^{\Delta NK}$, and $Tyk2^{-/-}$ mice. CpG methylation was analyzed by bisulfite pyrosequencing. Data are shown as mean percentage of methylation ± SEM from three independent experiments (n = 3 per genotype). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

that lack TYK2 specifically in NK cells ($Tyk2^{\Delta NK}$) and mixed BM chimera experiments, we show that TYK2 in accessory cells rather than in NK cells themselves drives splenic NK cell maturation. Several lines of evidence support the idea that IL-15 *trans*-presentation by $CD11c^{+}$ DCs and $LysM^{+}$ cells (i.e., mainly monocytes, macrophages, and neutrophils) is required for peripheral NK cell

homeostasis and terminal maturation into the $CD27^{-}CD11b^{+}$ NK cell stage (49–52). Our finding that lack of TYK2 in $CD11c^{+}$ cells results in a reduction of $CD27^{-}CD11b^{+}$ splenic NK cells further underscores the importance of DCs for peripheral NK cell maturation. In contrast, we did not find evidence for a role of TYK2 in $LysM^{+}$ cells in this process. It is currently unclear which signals drive IL-15/IL-15Rα expression under homeostatic conditions. We show that the absence of TYK2 results in reduced IL-15Rα levels on macrophages and DCs in naive mice. In response to immune challenge, type I IFNs induce IL-15 production and *trans*-presentation from DCs to NK cells (62). It is generally accepted that low levels of type I IFNs are produced under homeostatic conditions (63), prompting the hypothesis that IL-15 *trans*-presentation to NK cells may be driven by type I IFNs even in the absence of immune challenge. This idea is supported by the findings that naive $Stat1^{-/-}$ and $Ifnar1^{-/-}$ mice, but not $Ifnar1^{\Delta NK}$ mice, have less $CD27^{-}CD11b^{+}$ NK cells and that $Stat1^{-/-}$ NK cells fully mature upon transfer into *WT* mice (40, 64). However, further studies will be required to test this possibility and to confirm that TYK2 drives NK cell maturation through its role in the type I IFN signaling cascade.

IFN-γ is a major NK cell effector molecule that is critical for tumor surveillance and immunity against infections. IFN-γ production by NK cells can be triggered by cytokines, such as IL-12 and IL-18, or actR stimulation (65). In support of the well-established role of TYK2 in the IL-12 signaling cascade (18), IFN-γ production in response to IL-12 was similarly impaired in NK cells from $Tyk2^{\Delta NK}$ and $Tyk2^{-/-}$ mice. IFN-γ production by NK cells was also severely impaired in $Tyk2^{\Delta NK}$ mice upon infection with *L. monocytogenes*. Our previous study revealed defects in anti-NK1.1 and anti-Ly49D Ab–induced IFN-γ production in $Tyk2^{-/-}$ NK cells, although TYK2 is not involved in the signaling cascade downstream of NK cell actR (60). Unlike NK cells from $Tyk2^{-/-}$ mice, splenic NK cells from $Tyk2^{\Delta NK}$ mice showed similar IFN-γ production as those from $Tyk2^{fl/fl}$ mice upon stimulation with anti-NK1.1 Ab, indicating that the impact of TYK2 is indirect. Recently the most mature $CD27^{-}CD11b^{+}$ NK cells have been identified as the main producers of IFN-γ in response to actR stimulation (52), suggesting that the impaired anti-NK1.1–induced IFN-γ production in the absence of TYK2 may reflect defects in terminal maturation. However, our finding that NK cells from $Tyk2^{\Delta DC}$ mice have a lower frequency of the $CD27^{-}CD11b^{+}$ cells but show unimpaired actR-induced IFN-γ production argues against this possibility. In human NK cells, terminal differentiation of peripheral NK cells is accompanied with demethylation of the *Ifng* promoter at three CpG sites in the transcribed region (+122, +128, +171), which enables high IFN-γ production in response to actR engagement (61). Our study suggests that such a mechanism is also important in mice, as the impaired maturation of $Tyk2^{-/-}$ NK cells correlated with an increased methylation at CpGs +91 and +114 and impaired IFN-γ production upon actR stimulation. Moreover, treatment of NK cells from $Tyk2^{-/-}$ mice with 5-Aza restored anti-NK1.1 Ab– but not IL-12–induced IFN-γ production. However, more detailed studies will be required to test whether these CpGs are indeed demethylated during murine NK cell maturation and whether this increased methylation in $Tyk2^{-/-}$ NK cells reflects the more immature phenotype.

Furthermore, we show that TYK2 in NK cells is not required for target cell cytotoxicity and NK cell–dependent tumor surveillance. In vitro expanded NK cells from $Tyk2^{\Delta NK}$ mice showed unimpaired cytotoxic activity against RMA-S and RMA-Rae1 target cells, whereas NK cells from $Tyk2^{-/-}$ mice had strongly reduced cytotoxic activity. Similar to what has been shown for NK cells from $Stat1^{-/-}$ mice (66), NK cells from $Tyk2^{-/-}$ mice had restored cytotoxicity against RMA-Rae1 cells when grown in the presence of IL-15 instead of IL-2. It remains to be investigated whether this reflects differences in cellular responses to IL-2 and IL-15 (67) or in signal strength. However, these data further underscore that IL-15 not only restores maturation but also functional competence of NK cells from $Tyk2^{-/-}$ mice. $Tyk2^{\Delta NK}$ mice rejected cells that lack MHC I molecules ($\beta2m^{-/-}$ splenocytes), and controlled s.c. transplanted RMA-Rae1 cells as efficiently as littermate controls. We show that TYK2 in $LysM^{+}$ cells and DCs is required for in vivo killing of $\beta2m^{-/-}$ splenocytes and that TYK2 in DCs contributes to RMA-Rae1 growth control. It remains an open question which external TYK2-dependent signal augments NK cell cytotoxicity and tumor surveillance. Defects in cytotoxicity may, at least in part, be causally linked to defects in terminal maturation or may result from impaired MHC I molecule–dependent NK cell education, although these two processes might not be completely independent (61, 68, 69). Further studies are required to define how TYK2 regulates the interplay between NK cells and other immune and nonimmune cells in both the differentiation of a functionally balanced NK cell pool and in antitumor immunity.

Although not in the focus of our study, we found that TYK2 signaling in NK cells is required for an efficient immune defense against *L. monocytogenes*. This is surprising, as several studies established that NK cells act deleterious rather than protective during systemic *L. monocytogenes* infections (70–72). It seems possible that despite their predominant immunosuppressive phenotype, NK cells have low level of antibacterial activity. This idea is supported by the findings that NK cells produce considerable amounts of IFN-γ upon i.p. *L. monocytogenes* infection (73–76) and modestly reduce bacterial burden upon transfer into $Ifng^{-/-}$ mice (77) and upon local infections (62, 78). Increased sensitivity of $Tyk2^{\Delta NK}$ mice to *L. monocytogenes* infection correlated with an impaired IFN-γ production by NK cells, supporting the notion that the balance between IFN-γ– and IL-10–producing NK cells critically determines their role in antibacterial immunity (79, 80), thus arguing against a previously suggested negative impact of NK cell–derived IFN-γ (72). However, further investigations are required to characterize the impact of TYK2 on NK cell responses other than the production of IFN-γ to understand how NK cell–intrinsic TYK2 improves survival upon *L. monocytogenes* infection.

In summary, we provide evidence that NK cell–extrinsic TYK2 promotes NK cell maturation, cytotoxicity, and tumor surveillance, whereas NK cell–intrinsic TYK2 is needed for an efficient immune defense against *L. monocytogenes* infection. Moreover, we show that TYK2 not only facilitates IFN-γ production through its role in the IL-12 signaling cascade but also through NK cell–extrinsic mechanisms that specifically impact on IFN-γ production in response to NK cell actR stimulation and may involve *Ifng* promoter demethylation. Our findings that cytotoxic defects of NK cells from $Tyk2^{-/-}$ mice can be rescued by IL-15 treatment suggest that unwanted effects of TYK2 inhibitors in tumor therapy may be overcome by boosting NK cell activity. However, more detailed experimentation will be required to better understand how TYK2 regulates NK cell–dependent immunity in vivo and to identify signaling cascades and cellular networks involved.

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Disclosures

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