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T-Cell Ig and ITIM Domain Regulates Natural Killer Cell Activation in Murine Acute Viral Hepatitis

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Uncontrolled natural killer (NK) cell activation during the early response to acute viral infection can lead to severe immunopathology, and the mechanisms NK cells use to achieve self-tolerance in such contexts are currently unclear. Here, NK cells up-regulated a coinhibitory receptor, T-cell Ig and ITIM domain (TIGIT), during challenge with the viral double-stranded RNA (dsRNA) analog poly I:C. Blocking TIGIT by antibody treatment *in vivo* or a genetic deficiency in *Tigit* enhanced NK cell activation and aggravated liver injury in a poly I:C/D-GalN-induced model of acute fulminant hepatitis, suggesting that TIGIT is normally required for protecting against NK cell-mediated liver injury. Furthermore, adoptively transferring $Tigit^{-/-}$ NK cells into NK cell-deficient $Nfil3^{-/-}$ mice also resulted in elevated liver injury. Reconstituting Kupffer cell-depleted mice with poliovirus receptor (PVR/CD155, a TIGIT ligand)-silenced Kupffer cells led to aggravated liver injury in a TIGIT-dependent manner. Blocking TIGIT in an NK-Kupffer cell coculture *in vitro* enhanced NK cell activation and interferon-gamma (IFN-γ) production in a PVR-dependent manner. We also found that TIGIT was up-regulated selectively on NK cells and protected against liver injury in an acute adenovirus infection model in both an NK cell- and Kupffer cell-dependent manner. Knocking down PVR in Kupffer cells resulted in aggravated liver injury in response to adenovirus infection in a TIGIT-dependent manner. *Conclusion*: TIGIT negatively regulates NK-Kupffer cell crosstalk and alleviates liver injury in response to poly I:C/D-GalN challenge or acute adenovirus infection, suggesting a novel mechanism of NK cell self-tolerance in liver homeostasis during acute viral infection. (HEPATOLOGY 2014;59:1715-1725)

Natural killer (NK) cells mediate antiviral innate immune responses by producing cytokines and lysing infected cells upon activation.[1-3] During acute viral infection, activated NK cells help control the early phase of infection and promote further priming of virus-specific $CD8^+$ T-cell immunity. However, uncontrolled NK cell activation can also cause severe immunopathology of the infected tissue that can even lead to fatal organ failure.[4-6] Therefore, a finely tuned and timely regulation of NK cell activation is important to induce optimal NK cell self-tolerance and protect the host against viral infection-induced immune injury. The underlying self-tolerance mechanisms that limit NK cell activation during acute viral infection, however, have rarely been reported.

Macrophages can activate NK cells through the release of soluble cytokines[7-10] and by interaction between cell surface molecules, relying on a complex integration of competing activation and inhibitory signals.[1,11] Crosstalk with macrophages/monocytes has extensively been reported to engage activating receptors on NK cells, including NKG2D,[12-14] NCRs,[15] 2B4,[13,16] CD28,[17] and CD40L.[18] Little, however, is known about the role of inhibitory receptors in regulating NK cell activation during such cellular interactions.

Coinhibitory receptors have recently been recognized as promising therapeutic targets in cancer, as blocking their signaling may enhance antitumor immune responses.[19] One of these, T-cell Ig and ITIM domain (TIGIT), was reported with proof-of-

Abbreviations: ALT, alanine aminotransferase; D-GalN, D-galactosamine; IFN, interferon; NK, natural killer; NKT, natural killer T; PVR, poliovirus receptor; TIGIT, T-cell Ig and ITIM domain.

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concept evidence to be a coinhibitory receptor not only on T cells[20-22] but also on NK cells, where TIGIT could inhibit cytotoxicity from both human and mouse NK cells.[23-25] However, the function and effect of TIGIT on NK cells and NK cell-dependent processes *in vivo* have not yet been reported.

We previously described a mouse model mimicking the acute RNA virus-induced immunopathologic process in the liver in which polyinosinic:polycytidylic acid (poly I:C), an analog of viral double-stranded RNA (dsRNA), triggered NK cell-dependent fulminant hepatitis in D-galactosamine (D-GalN)-sensitized livers. Using this model, we previously demonstrated that the activating NK cell receptor NKG2D is critical for mediating the NK-Kupffer cell interaction that leads to severe liver injury.[14] We also observed that treating mice with a DNA virus-based adenovirus vector induced acute liver injury in an NK cell-dependent manner.[6,26] In this study, we found that the coinhibitory receptor TIGIT negatively regulated NK cell activation *in vivo* by way of interacting with its ligand, poliovirus receptor (PVR), expressed on the surface of Kupffer cells. This interaction subsequently protected mice against poly I:C/D-GalN- or adenovirus-induced liver injury, suggesting a novel NK cell self-tolerance mechanism in liver homeostasis during acute viral infection.

Materials and Methods

Mice. Wild-type (WT) C57BL/6 mice were purchased from the Shanghai Experimental Animal Center (Shanghai, China). C57BL/6 *Tigit*$^{-/-}$ mice were obtained from Bristol-Myers Squibb (New York, NY). C57BL/6 *Rag1*$^{-/-}$ mice, which were originally obtained from the Jackson Laboratory (Bar Harbor, ME), were obtained locally from the Model Animal Research Center (Nanjing, China). C57BL/6 *Nfil3*$^{+/-}$ mice were provided by Dr. Tak W. Mak (University of Toronto, Toronto, Ontario, Canada), and *Nfil3*$^{-/-}$ mice were bred in-house. C57BL/6 GKO (interferon-gamma [IFN-γ]-deficient) mice were provided by Dr. Shaobo Su (Shantou University, Shantou, China). All mice were maintained in a specific pathogen-free facility for use according to the guidelines for experimental animals at the University of Science and Technology of China. Mice were used between 5-10 weeks of age.

Reagents. Poly I:C (High Molecular Weight, Invivogen, San Diego, CA) and D-GalN (Sigma Chemical, St. Louis, MO) were each dissolved in pyrogen-free saline. To induce liver injury, mice were injected intravenously with poly I:C (0.75 μg/mouse for most experiments, or 1 μg/mouse for survival experiments) and intraperitoneally with D-GalN (10 mg/mouse) at the same time. Antimouse TIGIT monoclonal antibody 13G6 was generated by Absea (Beijing, China) and tested for its ability to block TIGIT-PVR interactions *in vitro*. Antimouse IFN-γ was purified from the culture supernatant of GK1.5 cells (TIB-207) (ATCC). To block TIGIT or IFN-γ *in vivo*, 125 μg of anti-TIGIT or anti-IFN-γ was intraperitoneally injected, respectively. Rat IgG purified from rat serum was used as a control.

Antibody Staining and Flow Cytometry. We purchased eFluor 660-anti-TIGIT, PE-anti-PVR, APC-anti-F4/80, and PE-anti-human IgG from eBioscience; PE-anti-pan Rae1 from R&D Systems; and all other antibodies for flow cytometry from BD Biosciences. Prior to staining with antibodies, cells were incubated with rat immunoglobulin for 30 minutes to block Fc receptors. We performed flow cytometry on a FACSCalibur platform (BD Biosciences) and analyzed data with FlowJo software (Tree Star).

Cell Preparation. Liver mononuclear cells (MNCs) were isolated essentially as previously described.[27] Hepatocytes and Kupffer cells were isolated using a two-step collagenase perfusion method as previously described.[14]

Cell Depletion. For NK/Kupffer cell depletion, mice were injected with 30 μg of anti-ASGM1 antibody (Wako, Tokyo, Japan) or 200 μL of clodronate liposomes,[28] respectively, 48 hours before challenge.

Analysis of Liver Transaminase Activity. Liver injury was assessed by measuring the serum enzyme activity of alanine aminotransferase (ALT) using a commercially available kit (Rong Sheng, Shanghai, China).

Cytolytic Assay. Cytotoxicity against primary hepatocytes from D-GalN-treated mice was assessed by a colorimetric assay for detecting lactate dehydrogenase (LDH) according to the manufacturer's instructions (Promega).

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Cell Sorting and Transfer. A FACSAria cell sorter (BD Biosciences) was used to purify $CD3^{-}NK1.1^{+}$ NK cells or $F4/80^{+}$ Kupffer cells. The purity of sorted NK and Kupffer cell populations was >95% or 90%, respectively, as verified by postsort flow cytometric analysis.

Hematoxylin and Eosin Staining. Histological analysis was performed as previously described.[14]

Adenovirus Transduction. Recombinant replication-deficient adenovirus for Kupffer cell transduction was generated using the AdEasy system, as described.[29] For Kupffer cell transduction, adenovirus (multiplicity of infection [MOI] = 500) was added into cell culture for 2 hours at 37°C before replacing the culture medium with fresh medium and allowing the cells to rest for 24 hours.

NK-Kupffer Cell Coculture System. For the NK-Kupffer cell coculture, 10^5 purified $NK1.1^{+}CD3^{-}$ hepatic NK cells were cocultured with 10^5 purified $F4/80^{+}$ Kupffer cells in 96-well U-bottomed tissue-culture plates in 100 μL Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 10 mM HEPES, 10 mM 2-mercaptoethanol, and 100 IU/mL penicillin/streptomycin. Isotype control or the following monoclonal antibodies (10 μg/mL) were added to the culture when indicated: antimouse TIGIT (13G6, produced in-house) or antimouse PVR (3F1, Hycult Biotech). Cells were or were not stimulated with 100 μg/mL poly I:C for 24 hours, and cytokine levels in the supernatant were determined using a CBA kit (BD Biosciences) according to the manufacturer's instructions. After removing the supernatant, NK cells were separated from the culture after a 15-minute incubation in phosphate-buffered saline (PBS) supplemented with 5 mM ethylenediamine-tetraacetic acid (EDTA) for further surface or intracellular staining by flow cytometry, or for use in a cytolytic assay.

Adenovirus-Induced Liver Injury Model. To induce liver injury, 10^{11} virus particles (v.p.) of replication-deficient adenovirus containing the EGFP gene (5+ MMI, Beijing, China) were injected intravenously.

Statistics. Statistically significant differences were determined by Student *t* tests when appropriate. Values of $P < 0.05$ were considered significant.

Results

NK Cells Up-Regulate TIGIT During Recovery From Poly I:C/D-GalN-Induced Fulminant Hepatitis. NK cells were previously shown to express TIGIT. Since TIGIT did not phenotypically or functionally affect NK cells at steady state, as determined by cell frequency (Supporting Fig. 1A), maturation markers (Supporting Fig. 1B), cytotoxicity against susceptible targets (Supporting Fig. 1C,D), and signaling by activating receptors (Supporting Fig. 1E), we wondered whether it played a role during NK cell activation. In order to begin investigating the function of TIGIT on activated NK cells *in vivo*, we employed a mouse model of NK cell-mediated fulminant hepatitis induced by poly I:C/D-GalN previously reported by us[14] and evaluated TIGIT expression on NK cells over time in WT mice. Following injection, we observed dramatic up-regulation of TIGIT expression on both splenic and hepatic NK cells (Fig. 1A,B), but not T cells (Fig. 1C). Both poly I:C and D-GalN contributed to TIGIT up-regulation on NK cells *in vivo* (Fig. 1D). Thus, the dynamic TIGIT expression on NK cells suggests that TIGIT may play a functional role during acute viral immune responses.

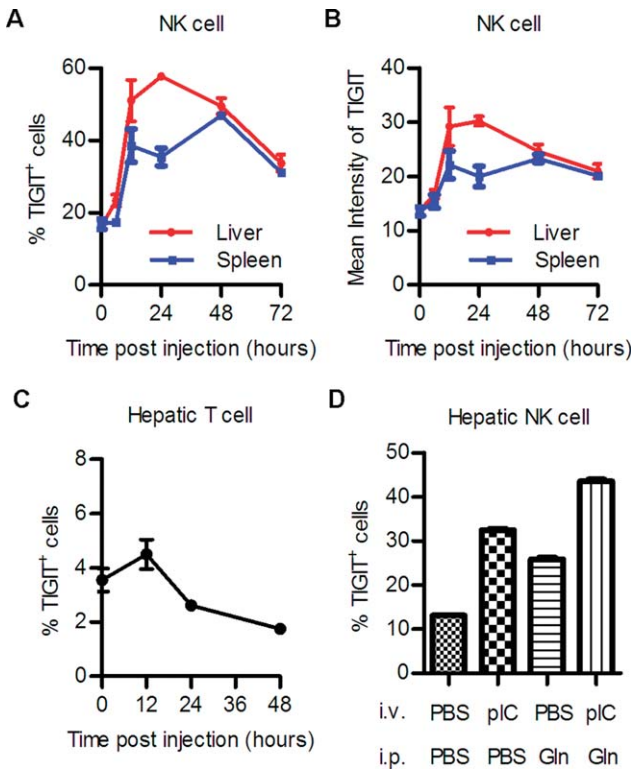

Fig. 1. TIGIT on NK cells is up-regulated in poly I:C/D-GalN-induced fulminant hepatitis. (A,B) Percentage of $TIGIT^{+}$ cells (A) and MFI of TIGIT expression (B) in $CD3^{-}NK1.1^{+}$ hepatic and splenic NK cells after poly I:C/D-GalN injection at the indicated timepoints. (C) Percentage of $TIGIT^{+}$ cells in $CD3^{+}NK1.1^{-}$ hepatic T cells after poly I:C/D-GalN injection at the indicated timepoints. (D) Percentage of $TIGIT^{+}$ cells in $CD3^{-}NK1.1^{+}$ hepatic NK cells 12 hours after poly I:C and/or D-GalN injection. (A-D) Data are representative of at least three independent experiments and are represented as the mean ± SEM.

Similar to TIGIT, the activating receptors CD226 and CD96 can also bind PVR. While CD226

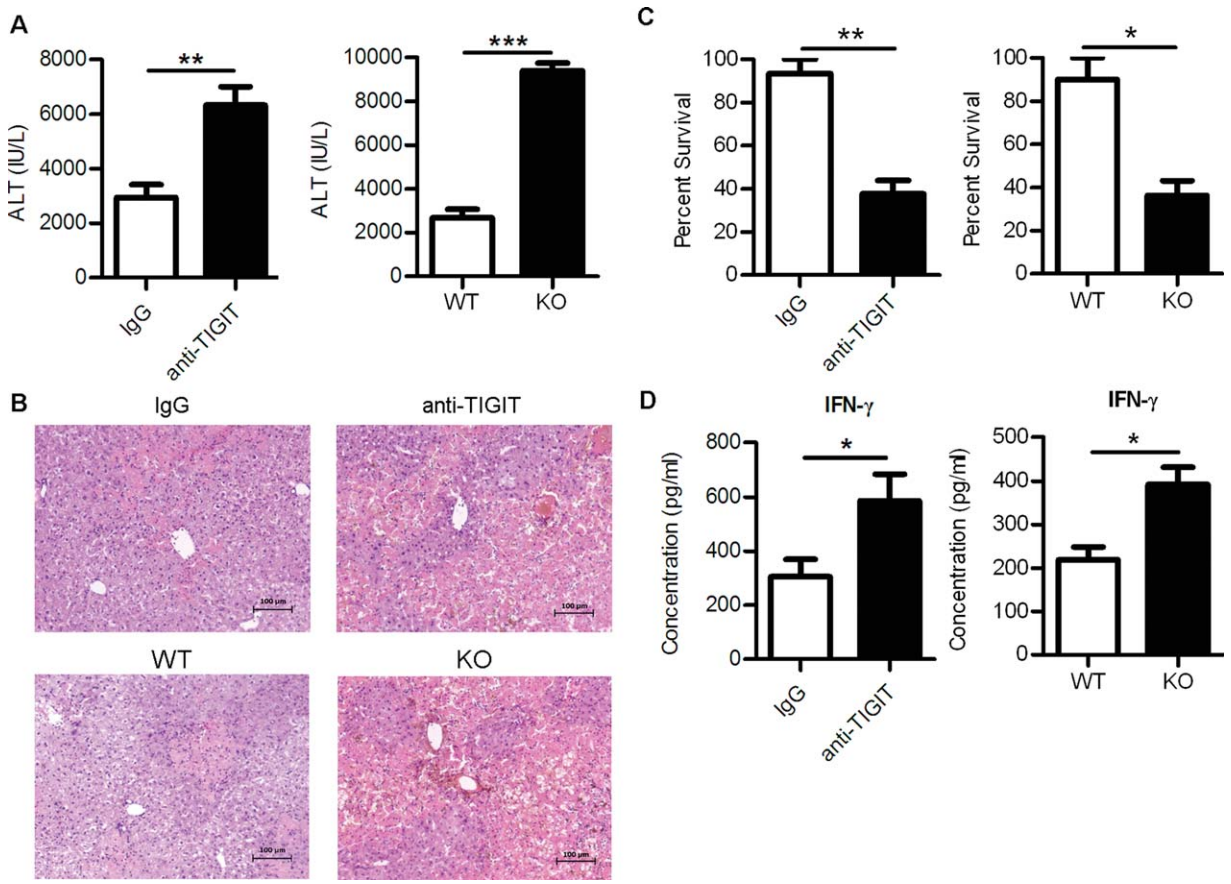

Fig. 2. TIGIT deficiency promotes poly I:C/D-GalN-induced fulminant hepatitis. (A) (Left) Mice were injected with anti-TIGIT mAb or control IgG 12 hours before poly I:C/D-GalN injection. (Right) WT or *Tigit*$^{-/-}$ mice were injected with poly I:C/D-GalN. Serum ALT concentration was measured 18 hours later (n = 5-7). Data are representative of at least three independent experiments and are represented as the mean ± SEM. $^{**}P < 0.005$, $^{***}P < 0.001$. (B) Liver samples were collected from mice in (A) for H&E staining 18 hours after poly I:C/D-GalN injection. (C) (Left) Mice were injected with anti-TIGIT mAb or control IgG 12 hours before receiving a high dose of poly I:C/D-GalN injection. (Right) WT or *Tigit*$^{-/-}$ mice were injected with a high dose of poly I:C/D-GalN. Survival rates at 24 hours postinjection from three independent experiments (n = 7-10 for each experiment) are represented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.005$. (D) Serum IFN-γ concentration was measured from mice in (A) 18 hours after poly I:C/D-GalN injection. Data are represented as the mean ± SEM. $^{*}P < 0.05$.

expression was comparable, CD96 expression was slightly lower on *Tigit*$^{-/-}$ NK cells both in steady state and after poly I:C/D-GalN injection (Supporting Fig. 2A,B). We also observed down-regulation of CD226 and CD96 on hepatic NK cells following poly I:C/D-GalN injection (Supporting Fig. 2B), similar to the phenomenon reported during T follicular helper (Tfh) cell differentiation,[30] possibly suggesting that TIGIT was the dominant PVR-interacting receptor in these contexts.

TIGIT Protects Against Poly I:C/D-GalN-Induced NK Cell-Mediated Fulminant Hepatitis. To determine whether the up-regulated TIGIT expression on NK cells may play a functional role in NK cell-mediated fulminant hepatitis, we used an anti-TIGIT antibody to block TIGIT binding to its receptor or performed this model in *Tigit*$^{-/-}$ mice. Compared to WT mice, blocking TIGIT signaling *in vivo* by mAb treatment or a genetic deficiency in *Tigit* led to significant elevation in serum ALT levels and histological necrosis in the liver after poly I:C/D-GalN injection (Fig. 2A,B) as well as a lower survival rate after injection of high-dose poly I:C/D-GalN (Fig. 2C). Since NK cells play a critical role in pathogenesis by producing IFN-γ in this model, we also found that blocking TIGIT signaling resulted in significantly higher serum IFN-γ levels (Fig. 2D). These results show that TIGIT deficiency or blockade promotes poly I:C/D-GalN-induced fulminant hepatitis, suggesting that TIGIT plays a critical role to inhibit immune-mediated liver injury during acute hepatitis.

Although blocking TIGIT signaling did not increase mononucleocyte or NK cell infiltration into the liver (Fig. 3A), we observed higher CD69 expression and IFN-γ production by NK cells in the absence of TIGIT (Fig. 3B,C), consistent with the elevated serum

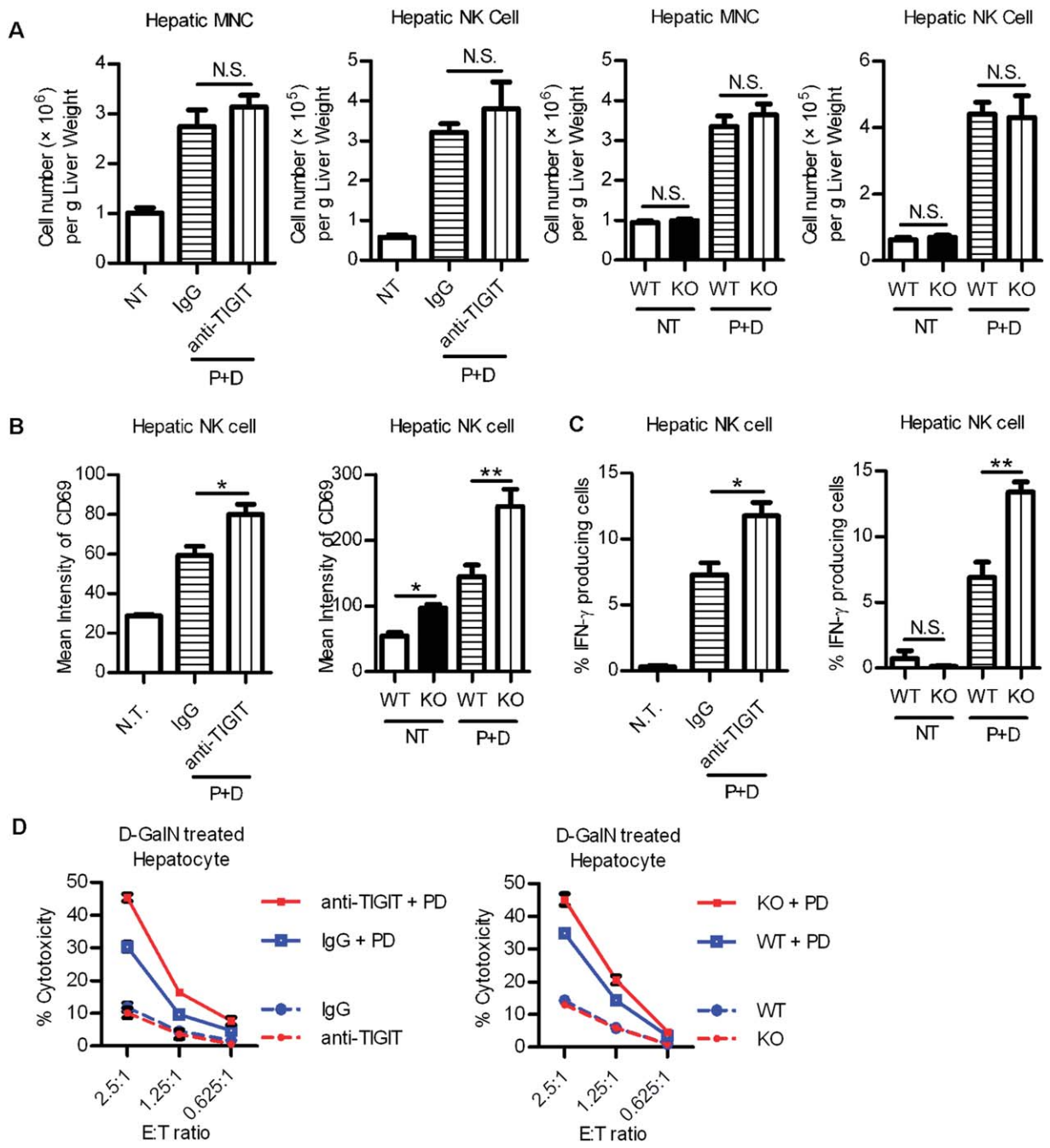

Fig. 3. TIGIT deficiency promotes NK cell activation in poly I:C/D-GalN-challenged mice. (A) Absolute number of MNCs and $CD3^{-}NK1.1^{+}$ cells in the liver was evaluated 18 hours after poly I:C/D-GalN injection from (left) mice pretreated with anti-TIGIT mAb or control IgG and (right) WT or *Tigit*$^{-/-}$ mice. (B) CD69 MFI on the $CD3^{-}NK1.1^{+}$ hepatic NK cells shown in (A). (C) Percentage of IFN-γ-containing cells in the $CD3^{-}NK1.1^{+}$ hepatic NK cells shown in (A). Data are representative of at least three experiments and are represented as the mean ± SEM (n = 3). $*P < 0.05$. (D) $CD3^{-}NK1.1^{+}$ hepatic cells purified from (A) or without poly I:C/D-GalN injection were cocultured in a 4-hour cytotoxicity assay at the indicated ratio with primary hepatocytes from mice treated for 12 hours with D-GalN (n = 3). Data are represented as the mean ± SEM.

IFN-γ levels in the absence of TIGIT and the critical role of NK cells in liver pathology. An *ex vivo* cytotoxic assay against primary hepatocytes from D-GalN-treated mice also showed elevated NK cell cytotoxic function from mice with TIGIT signaling blockade (Fig. 3D). In contrast to the effect on NK cells, we did not observe elevated T or NKT cell activation in the absence of TIGIT as determined by CD69 and IFN-γ expression (Supporting Fig. 3); for IFN-γ production in particular, we only detected baseline IFN-γ levels in NKT cells after poly I:C/D-GalN injection, while above-baseline IFN-γ levels in T cells were not observed until 18 hours postinjection (Supporting Fig. 3A). Thus, these results suggest that TIGIT functions

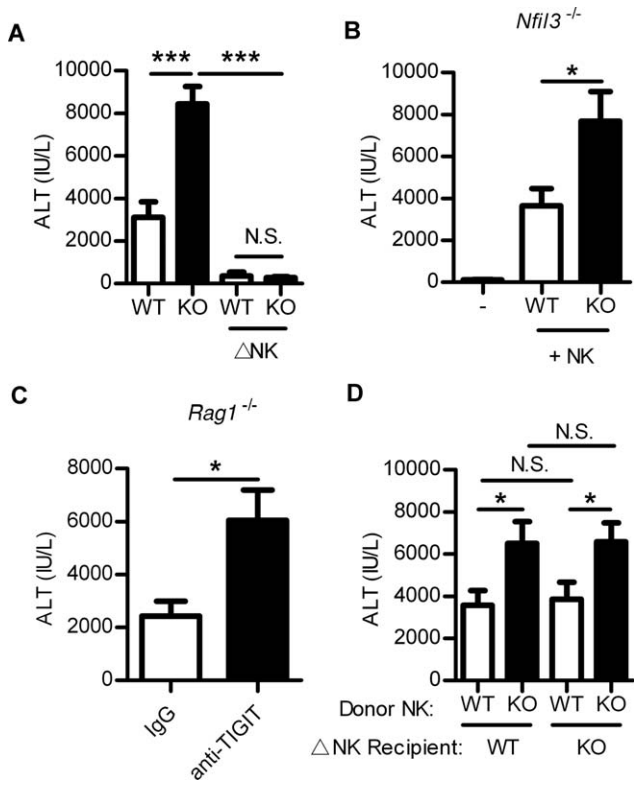

Fig. 4. TIGIT deficiency in NK cells promotes poly I:C/D-GalN-induced liver injury. (A) WT or *Tigit*$^{-/-}$ mice were injected with anti-ASGM1 to deplete NK cells 24 hours before poly I:C/D-GalN injection. Serum ALT concentration was measured 18 hours later (n = 6). (B) CD3^{-}NK1.1^{+} splenocytes from WT or *Tigit*$^{-/-}$ mice were adoptively transferred into *Nfil3*$^{-/-}$ mice 24 hours before poly I:C/D-GalN injection. Serum ALT concentration was measured 18 hours later (n = 3 for nontransfer control; n = 6 for the other groups). (C) *Rag1*$^{-/-}$ mice pretreated with anti-TIGIT mAb or control IgG were injected with poly I:C/D-GalN. Serum ALT concentration was measured 18 hours later (n = 6). (D) WT or *Tigit*$^{-/-}$ mice were injected with anti-ASGM1 to deplete NK cells 24 hours before intrahepatic injection with CD3^{-}NK1.1^{+} hepatic cells from WT or Tigit$^{-/-}$ mice, which was immediately followed by poly I:C/D-GalN injection. Serum ALT concentration was measured 18 hours later (n = 7). (A-D) Data are representative of two independent experiments and are represented as the mean ± SEM. *$P < 0.05$. ***$P < 0.001$.

to inhibit activation of NK, but not T or NKT, cells during acute liver injury.

TIGIT on NK Cells Protects Against Poly I:C/D-GalN-Induced NK Cell-Mediated Fulminant Hepatitis. Since we observed elevated NK cell activation in the absence of TIGIT, we wondered whether TIGIT expressed on NK cells accounted for the inhibition of acute liver injury. Indeed, we observed that aggravated liver injury in the absence of TIGIT was dependent on NK cells, as NK cell depletion with an anti-ASGM1 antibody *in vivo* significantly reduced serum ALT levels in both WT and *Tigit*$^{-/-}$ mice (Fig. 4A). Furthermore, TIGIT on NK cells was required to suppress liver injury, as transferring WT NK cells into NK-deficient *Nfil3*$^{-/-}$ mice[31] showed significantly lower liver injury after poly I:C/D-GalN injection compared to transferring *Tigit*$^{-/-}$ NK cells (Fig. 4B). Although anti-ASGM1 can reportedly deplete cells other than NK cells,[32] our collective observations in both anti-ASGM1-treated mice and *Nfil3*$^{-/-}$ mice indicate that TIGIT on NK cells protects against poly I:C/D-GalN-induced liver injury.

Since T and NKT cells also expressed TIGIT, we next determined whether TIGIT-mediated protection was also dependent on these cells. Similar to immunocompetent mice, immunodeficient *Rag1*$^{-/-}$ mice treated with anti-TIGIT blocking mAb *in vivo* showed significantly higher serum ALT levels than control *Rag1*$^{-/-}$ mice in response to poly I:C/D-GalN (Fig. 4C), suggesting that the adaptive immune system is dispensable for TIGIT-mediated protection against liver injury. Furthermore, adoptive transfer of donor NK cells from WT or *Tigit*$^{-/-}$ mice into NK-depleted recipients showed that TIGIT expressed on NK cells, but not any other cell, mediated protection against acute liver injury (Fig. 4D).

PVR Expressed on Kupffer Cells Contributes to TIGIT-Mediated Inhibition of NK Cells. We next explored the potential underlying mechanisms of TIGIT-mediated inhibition of NK cells. We first observed that TIGIT-independent NK cell activation signals were unaffected in the absence of TIGIT, including NKG2D signaling (Supporting Fig. 4) and proinflammatory cytokine responses (e.g., IL-12) (Supporting Fig. 5).

D-GalN-induced dysregulation of protein synthesis in hepatocytes[33] down-regulated various surface molecules, including PVR (Supporting Fig. 6A), although total TIGIT ligands did not significantly decrease (Supporting Fig. 6A). Hydrodynamic injection of short hairpin RNA (shRNA) targeting PVR (Supporting Fig. 6.B,C) or blocking TIGIT in NK cell-D-GalN-sensitized hepatocytes cytotoxicity assay *in vitro* (Supporting Fig. 6D) showed that TIGIT-ligand interactions between NK cells and hepatocytes in these contexts were not significant.

Kupffer cells are the resident macrophage population in the liver. Contact-dependent interaction between NK cells and macrophages is well documented. We previously showed that NK-Kupffer cell crosstalk was critical for the pathogenesis of poly I:C/D-GalN-induced liver injury. Here, contrary to a recent report,[34] we found that PVR expression on Kupffer cells was stable upon *in vivo* poly I:C/D-GalN challenge (Supporting Fig. 7A,B). Moreover, Kupffer cells were required for both poly I:C/D-GalN-induced liver injury and TIGIT-mediated protection (Fig. 5A). PVR-knocked down Kupffer cells (Supporting Fig. 8A) transferred into Kupffer cell-depleted WT or *Tigit*$^{-/-}$ mice (Fig. 5B) caused aggravated liver injury and

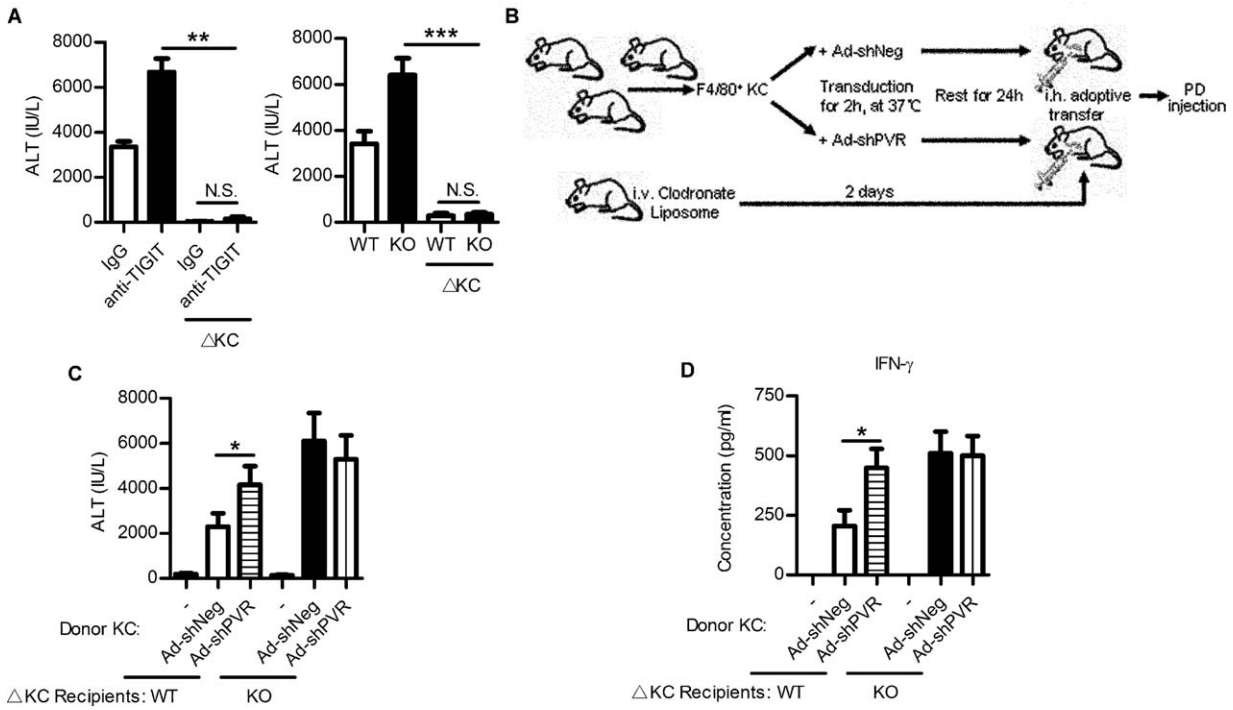

Fig. 5. PVR on Kupffer cells contributes to attenuation of hepatitis by TIGIT. (A) (left) Mice were intravenously injected with 200 μL of clodronate liposomes to deplete Kupffer cells. Mice were treated 24 hours later with anti-TIGIT mAb or control IgG, followed by poly I:C/D-GalN injection another 24 hours later. (Right) WT or *Tigit*$^{-/-}$ mice were intravenously injected with 200 μL of clodronate liposomes to deplete Kupffer cells followed by poly I:C/D-GalN injection an additional 24 hours later. Serum ALT concentration was measured 18 hours later. Data are representative of least three independent experiments and are represented as the mean ± SEM. $^{**}P < 0.005$. (B) The protocol for adoptively transferring adenovirus-transduced Kupffer cells into Kupffer-depleted hosts, as occurred in (C,D). (C) Purified F4/80^{+} Kupffer cells were infected with adenovirus (MOI = 500) overexpressing shRNA targeting PVR or control shRNA for 2 hours at 37°C. Cells were washed and incubated for 24 hours before being intrahepatically injected into WT or *Tigit*$^{-/-}$ mice, which were pretreated 48 hours earlier with 200 μL of clodronate liposomes, and then immediately injected with poly I:C/D-GalN. Serum ALT concentration was measured 18 hours later. (D) Serum IFN-γ concentration from the experiment in (C) was determined by enzyme-linked immunosorbent assay (ELISA). (C,D) Data are representative of two independent experiments and are represented as the mean ± SEM. $^{*}P < 0.05$.

increased serum IFN-γ levels compared to control Kupffer cells in WT, but not *Tigit*$^{-/-}$, mice (Fig. 5C,D). These data indicate that PVR on Kupffer cells protects against poly I:C/D-GalN-induced liver injury in a TIGIT-dependent manner, while TIGIT provides protection in a manner dependent on PVR expression by Kupffer cells. We also ruled out the possibility that lowered PVR expression on Kupffer cells by adenoviral transduction might affect its TIGIT-independent functions, including migration, cytokine production, and conjugation formation with NK cells (Supporting Fig. 8B-D). Collectively, these results suggest that the TIGIT-PVR interaction between NK and Kupffer cells is critical for limiting the extent of liver injury in acute fulminant hepatitis.

TIGIT-PVR Interaction Negatively Regulates NK-Macrophage Crosstalk and NK Cell Activation. Consistent with the attenuated liver injury by the TIGIT-PVR interaction shown above, NK cell activation determined by CD69, IFN-γ expression, and cytotoxicity against D-GalN-treated hepatocytes (Fig. 6A-D) was significantly elevated in NK-Kupffer cell coculture experiments upon blocking TIGIT or PVR with their respective mAbs. Following PVR blockade in addition to TIGIT, IFN-γ production by NK cells was not significantly changed, although slightly decreased (Fig. 6B,C), while CD69 expression and cytolytic activity of NK cells were significantly decreased (Fig. 6A,D), suggesting the involvement of activating PVR-binding receptors CD226 and/or CD96 in NK cell activation in this context. In contrast to PVR, CD112, possibly another TIGIT ligand, did not show similar effects (Supporting Fig. 9). These results indicate that the TIGIT-PVR interaction is involved in NK-macrophage crosstalk and negatively regulates NK cell activation.

Crosstalk between human NK cells and local macrophages in the liver has been documented,[35] and human NK cells constitutively expressed TIGIT.[23] Here, human TIGIT also regulated human NK cell activation in crosstalk with autologous macrophages (Supporting Fig. 10A), similar to mouse-derived cells. Blocking TIGIT in these cocultures enhanced the

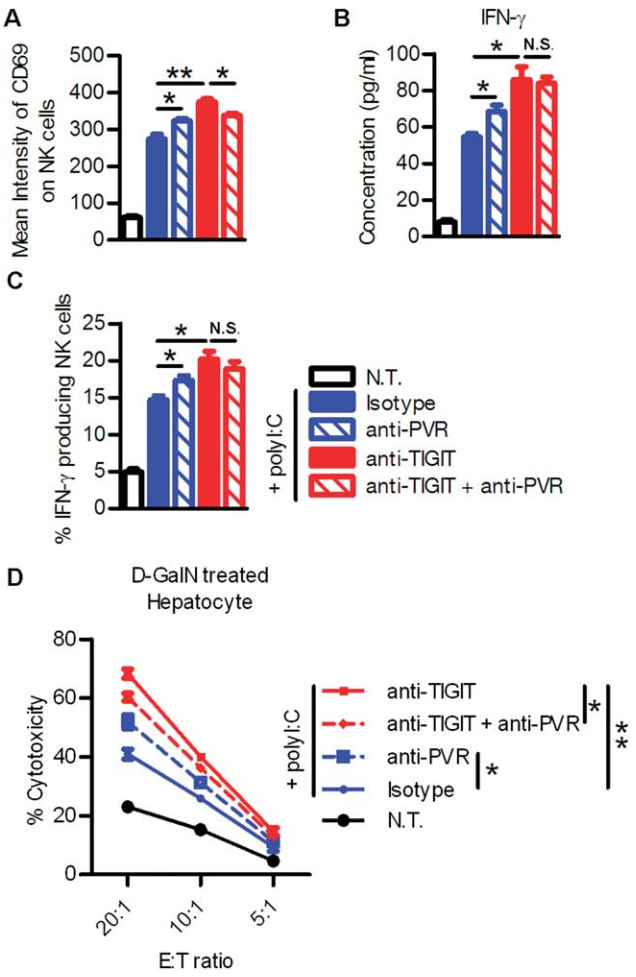

Fig. 6. TIGIT-PVR interaction negatively regulates NK-macrophage crosstalk. (A) Purified $CD3^{-}NK1.1^{+}$ hepatic NK cells were incubated with purified $F4/80^{+}$ Kupffer cells in the presence of poly I:C and the indicated antibodies for 24 hours. CD69 MFI was determined on $NK1.1^{+}$ NK cells. (B) IFN-γ concentration in the supernatant from (A) was determined by ELISA. (C) Percentage of IFN-γ^{+} cells in $NK1.1^{+}$ NK cells from (A). (D) $NK1.1^{+}$ NK cells isolated from (A) were added into a 4-hour cytotoxicity assay against primary hepatocytes from D-GalN-treated mice at the indicated ratios. Statistically significant differences were determined for the 20:1 ratio. (A-D) Experiment performed in triplicate and data are representative of least three independent experiments; data are represented as the mean ± SEM. $*P < 0.05$, $**P < 0.005$.

subsequent cytolytic activity against human hepatocyte L-02 (Supporting Fig. 10B), while additional TIGIT blockade in a subsequent cytolytic assay against L-02 did not further enhance cytolytic activity (Supporting Fig. 10B). Thus, TIGIT regulates NK-macrophage crosstalk, but not NK-hepatocyte interactions, in both mice and humans.

We showed in a previous study that poly I:C/D-GalN-induced liver injury required IFN-γ. Here, TIGIT inhibited IFN-γ production by NK cells in the same model (Figs. 3C, 6C). We next found that neither liver injury nor TIGIT-mediated protection were observed in the absence of IFN-γ in *Tigit*$^{-/-}$ mice *in vivo*, or in the absence of TIGIT in IFN-γ-deficient GKO mice (Fig. 7A). In contrast, plasmid-mediated IFN-γ overexpression restored liver injury, but not TIGIT-mediated protection, in GKO mice (Fig. 7B), consistent with the effect of IFN-γ on liver injury being downstream of TIGIT-mediated IFN-γ regulation. These data confirm the data in our previous study showing that IFN-γ is necessary and sufficient for poly I:C/D-GalN-induced liver injury and collectively suggest that TIGIT-mediated suppression of IFN-γ production is critical for its role in protecting the host from liver injury.

TIGIT Protects Against Adenovirus-Induced NK Cell-Mediated Liver Injury. Adenovirus is a liver-tropic DNA virus widely used for gene therapy in clinical trials.[36] A major role for NK cells in adenovirus-induced acute hepatitis has been described.[37] We therefore wondered whether TIGIT also similarly inhibited NK cells in this independent model of acute viral infection. We observed a robust up-regulation of TIGIT expression on NK cells, but not on T cells (Fig. 8A). We also observed that TIGIT was highly expressed on Tfh cells before adenovirus infection, as previously reported[31] (Supporting Fig. 11A,B), and that following infection it was down-regulated (Supporting Fig. 11B). Next, we found that liver injury was enhanced in the absence of TIGIT in both an NK cell- and Kupffer cell-dependent manner (Fig. 8B) and that silencing PVR in Kupffer cells aggravated liver injury in WT, but not *Tigit*$^{-/-}$, mice (Fig. 8C). Indeed, these features are similar to what we observed in the poly I:C/D-GalN model, suggesting a common mechanism in which the TIGIT-PVR interaction regulates NK cell activation during NK-Kupffer cell crosstalk and prevents excessive immunopathology in acute viral hepatitis.

Discussion

To our knowledge, this study is the first to report that TIGIT regulates NK cell activation *in vivo* and that the TIGIT-PVR interaction regulates NK-macrophage crosstalk in mice. Normally, maximal NK cell activation in response to acute viral infection tends to be excessive for clearing infected cells and properly initiating immune responses, resulting in bystander damage of the infected tissue. Thus, inhibitory mechanisms are required to restrain NK cell activity to prevent fatal organ failure while maintaining control over the extent of infection. Our data demonstrate that TIGIT expression is up-regulated selectively on NK cells during viral dsRNA analog or adenovirus challenge and that TIGIT inhibits NK cell activity by interacting with PVR on

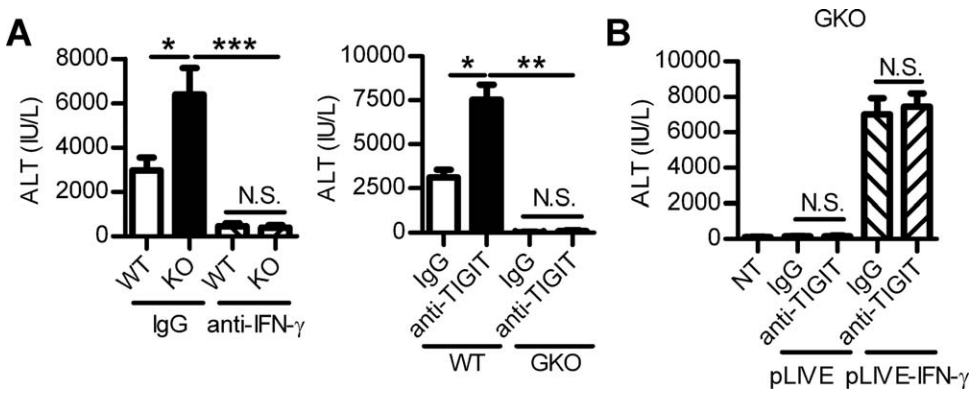

Fig. 7. IFN-γ is necessary and sufficient for the liver injury induced by poly I:C/D-GalN downstream of TIGIT. (A) WT or *Tigit*$^{-/-}$ mice were injected with anti-IFN-γ mAb or control IgG 12 hours before poly I:C/D-GalN injection (left, n = 6). WT or GKO mice were injected with anti-TIGIT mAb or control IgG 24 hours before poly I:C/D-GalN injection (right, n = 3-4). Serum ALT concentration was measured 18 hours later. Data are represented as the mean ± SEM. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$. (B) GKO mice were hydrodynamically injected with plasmid overexpressing IFN-γ or control plasmid 24 hours before being injected with anti-TIGIT mAb or control IgG. Mice were injected 24 hours later with poly I:C/D-GalN. Serum ALT concentration was determined 18 hours later (n = 5). Data are representative of two independent experiments and are represented as the mean ± SEM.

Kupffer cells, protecting the mice from additional liver injury. Collectively, these data suggest that the TIGIT-PVR interaction plays a regulatory role on NK cell activation in both viral and virus-like innate responses.

Our data provides evidence of NK-Kupffer cell interaction *in vivo*, which is further supported by NK-Kupffer cell coculture experiments *in vitro*. Although TIGIT-PVR interaction might also mediate crosstalk between NK cells and other cell types in the liver (e.g., hepatocytes, dendritic cells [DC]), its role in mediating NK-Kupffer cell crosstalk had a major physiological impact (Fig. 5). For NK-hepatocyte crosstalk, a TIGIT-ligand interaction might not be significant (Supporting Fig. 6D). While DCs are well documented to activate NK cells and are targets of clodronate liposome-directed cell depletion,[38] livers contain small numbers of DCs[39] and their interaction with NK cells is unlikely to have a significant effect.

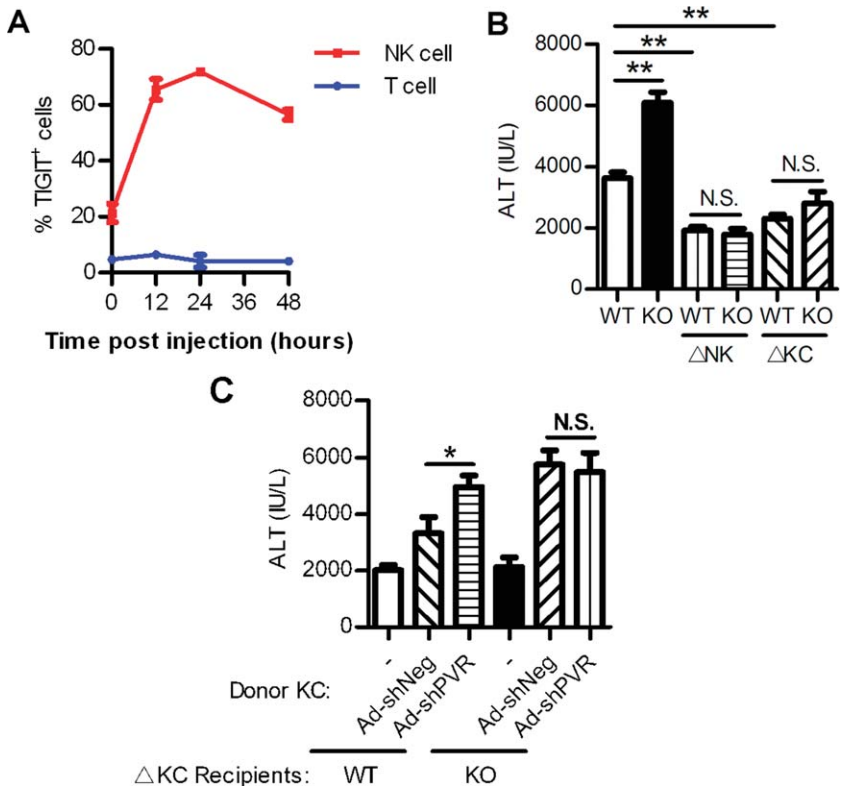

Fig. 8. TIGIT deficiency aggravates adenovirus-induced liver injury. (A) Percentage of TIGIT$^+$ cells in CD3$^-$NK1.1$^+$ hepatic NK cells and CD3$^+$NK1.1$^-$ hepatic T cells after 10^{11} v.p. adenovirus injection at the indicated timepoints. (n = 3) (B) WT or *Tigit*$^{-/-}$ mice were injected with anti-ASGM1 or clodronate liposomes to deplete NK cells or Kupffer cells, respectively. Two days later, mice were injected with 10^{11} v.p. adenovirus. Serum ALT concentration was measured 24 hours postinjection (n = 6). (C) Purified F4/80$^+$ Kupffer cells were infected with adenovirus (MOI = 500) overexpressing shRNA targeting PVR or control shRNA for 2 hours at 37°C. Cells were washed and incubated for 24 hours before being intrahepatically injected into WT or *Tigit*$^{-/-}$ mice, which were pretreated 48 hours prior with 200 μL of clodronate liposomes, and then immediately injected with 10^{11} v.p. adenovirus. Serum ALT concentration was measured 24 hours later (n = 6). (A-C) Data are represented as the mean ± SEM. *$P < 0.05$, **$P < 0.005$.

While multiple activating receptors might be involved in the NK-macrophage interaction,[13,15-18] we showed here that TIGIT on NK cells inhibits NK cell activation and IFN-γ production by interacting with its ligand (e.g., PVR) on macrophages both in mice (Fig. 6) and in humans (Supporting Fig. 10), providing insight into the molecular mechanisms that negatively regulate NK-macrophage crosstalk. However, the clinical significance of TIGIT-regulated NK cell-macrophage crosstalk in hepatic viral/viral-like innate responses in humans still requires further investigation.

Besides TIGIT-PVR,[20,23] CD226/CD96-PVR interactions have been reported.[40-42] CD226/CD96-PVR interactions also contribute to NK cell activation in NK-Kupffer cell crosstalk, as evidenced by a significant decrease in CD69 expression and cytotoxicity of NK cells following PVR blockade in addition to TIGIT (Fig. 6A,D). This effect might come from the stimulatory interactions of CD226 and CD96 with PVR, since blocking PVR does not just block the inhibitory interaction with TIGIT causing elevated activation, but also blocks PVR interactions with CD226/CD96 leading to decreased activation. However, the overall effects of PVR-TIGIT/CD226/CD96 interactions were shown to be protective/inhibitory, as enhanced NK cell activation was observed after silencing PVR in Kupffer cells *in vivo* (Fig. 5C,D) or blocking PVR alone in NK-Kupffer cell coculture (Fig. 6), which impaired both TIGIT-PVR and CD226/CD96-PVR interactions, suggesting that inhibition might be more significant in the activation-inhibition interplay between TIGIT-PVR and CD226/CD96-PVR interactions. On the other hand, *Tigit*$^{-/-}$ NK cells expressed comparable CD226 and slightly lower CD96 (Supporting Fig. 2A,B), possibly due to a need to balance the activation-inhibition interplay between the PVR-binding receptors when TIGIT signaling is weaker or missing, which, together with the up-regulation of TIGIT (Fig. 1A,B) and down-regulation of CD226/CD96 (Supporting Fig. 2B) following poly I:C/D-GalN injection, might function as self-protective host mechanisms in response to viral/viral-like challenge.

PVR has multiple functions.[43-46] In particular, PVR was recently reported to modulate DC activity upon interacting with CD226/TIGIT on T cells,[20] thus acting as both a receptor and a ligand. The possible contribution of downstream signaling of PVR on Kupffer cells to TIGIT/PVR-mediated regulation (Figs. 5C,D, 6) still need further investigation.

One group recently reported that mouse TIGIT inhibits NK cell cytotoxic function against a murine fibroblast cell line but not against some highly susceptible target cells,[25] and our group also observed the latter finding (Supporting Fig. 1C,D). They suggested that the difference was due to differing PVR expression levels on target cells. Although we found that TIGIT did not inhibit NK cell cytotoxicity against low-PVR-expressing hepatocytes (Supporting Fig. 6B,D), we also provided evidence that blocking TIGIT enhanced NK cell activation upon interaction with macrophages (Fig. 6), which also had low PVR expression (Supporting Fig. 8A). This discrepancy suggests that complicated mechanisms other than PVR expression levels on target cells alone affect the importance of TIGIT-mediated inhibition of NK cell activation during crosstalk with certain target cells, although this remains to be tested.

In conclusion, this report provides the first demonstration of TIGIT-mediated inhibition of NK cell activity *in vivo* in murine acute viral and virus-like hepatitis. Our data also provide evidence that the TIGIT-PVR interaction negatively regulates NK cells during NK-macrophage crosstalk. The TIGIT-PVR interaction has been suggested to represent an alternative pathway of "self" recognition[23]; here, we further suggest the importance of this type of recognition as a potential mechanism for achieving self-tolerance in virus-induced innate immune responses to limit immune-mediated damage of host tissues.

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