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TIGIT Safeguards Liver Regeneration Through Regulating Natural Killer Cell-Hepatocyte Crosstalk

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Overactivation of innate immunity, particularly natural killer (NK) cells, is harmful to liver regeneration; however, the molecular mechanisms that limit NK cell overactivation during liver regeneration are still elusive. Here we show that a coinhibitory receptor, T cell Ig and ITIM domain (TIGIT), was selectively up-regulated on NK cells, along with high expression of its ligand, poliovirus receptor (PVR/CD155), on hepatocytes during liver regeneration. The absence of TIGIT impaired liver regeneration *in vivo*, along with overactivation of NK cells and higher NK-derived interferon-gamma (IFN-γ) production. We also show that both depletion of NK cells and deficiency of IFN-γ, but not deficiency of RAG1, rescued impaired liver regeneration caused by the absence of TIGIT. Adoptive transfer of *Tigit*$^{-/-}$ NK cells into NK-deficient *Nfil3*$^{-/-}$ mice sufficiently led to impairment of liver regeneration. On the other hand, silencing PVR in hepatocytes rescued impaired liver regeneration caused by TIGIT deficiency *in vivo*, while blockade of TIGIT in NK-hepatocyte coculture increased IFN-γ production by NK cells *in vitro*. *Conclusion*: TIGIT is a safeguard molecule to improve liver regeneration through negatively regulating NK-hepatocyte crosstalk. This finding suggests a novel mechanism of NK cell self-tolerance towards regenerative hyperplasia of the host. (HEPATOLOGY 2014;60:1389-1398)

The liver is an organ with highly regenerative potential.[1] Liver regeneration is a process of physiologically compensatory hyperplasia. An intrinsic need to compensate for the loss of the hepatic tissue and its function, as well as multiple growth factors and cytokines, such as hepatocyte growth factor (HGF),[2] interleukin (IL)-6,[3] and tumor necrosis factor alpha (TNF-α),[4] have been characterized as important factors that initiate and drive the process until the physiological function of the liver is fully regained. Other factors including interferon-gamma (IFN-γ)[5] and IL-10,[6] on the other hand, were reported to be negative regulators of the process. In recent years, the role of various cellular populations of the innate immune system in liver regeneration have drawn attention,[7-11] posing an extrinsic immune "checkpoint" for the regenerative hyperplasia process in the liver. We previously reported activation of NK cells[12] or NKT cells,[13] in different contexts, impaired liver regeneration. NK cells, in particular, after activated in response to virus/viral analogs, further lost self-tolerance and produced excessive IFN-γ to compromise liver regeneration.[12] This suggests that mechanisms are normally required to preserve NK cell self-tolerance and to limit the innate immune checks for such regenerative hyperplasia process.

NK cells, as a crucial component of the innate immune system,[14,15] are recognized to play an important role in the surveillance against malignant hyperplasia[16,17]; however, NK cells otherwise keep relatively tolerant to tissue/organ regeneration, another kind of host hyperplasia, the underlying mechanisms of which remain unknown. NK cell activation is controlled by synergistic signals from combinations of activating and inhibitory receptors,[18] of which multiple inhibitory receptors that mediate NK cell tolerance have been defined.[19-22] Among them, coinhibitory receptor T cell Ig and ITIM domain (TIGIT), first identified by

Abbreviations: ALT, alanine aminotransferase; IFN, interferon; MNC, mononuclear cell; NK, natural killer; NKT, natural killer T; PHx, partial hepatectomy or partially hepatectomized; PVR, poliovirus receptor; TIGIT, T cell Ig and ITIM domain.

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Yu et al. in 2009,[21] was recently reported by our group to mediate NK cell self-tolerance by interacting with poliovirus receptor (PVR), its ligand, expressed on Kupffer cells, during acute hepatic viral infection,[23] shedding light on role of TIGIT in organ homeostasis, especially in the liver. We wondered whether TIGIT also plays an important role in the regenerative process in the liver. PVR, on the other hand, highly expressed by tumor cells, was reported to be up-regulated at the messenger RNA (mRNA) level in hepatocytes during liver regeneration in rats.[24] However, the physiological function of hepatocyte-expressing PVR on liver regeneration has not been investigated.

We previously demonstrated that NK negatively regulate liver regeneration by producing IFN-γ. This effect was more prominent after MCMV infection, which preactivated NK cells.[12] NK cell activation and IFN-γ production, on the other hand, was reported by us to be inhibited by TIGIT in acute viral hepatitis.[23] In this study, we observed that TIGIT and PVR were selectively up-regulated on NK cells and hepatocytes, respectively, during liver regeneration. TIGIT played important roles in keeping NK cell tolerance by inhibiting NK cell activation and thereafter decreasing its IFN-γ production, through interacting with PVR on hepatocytes, which finally protects liver regeneration.

Materials and Methods

Mice. The 5 to 10-week-old C57BL/6 wild-type mice were purchased from the Shanghai Experimental Animal Center (Shanghai, China). Age-matched *Tigit*$^{-/-}$ C57BL/6 mice, *Nfil3*$^{-/-}$C57BL/6 mice, and *Ifn-*$\gamma^{-/-}$ C57BL/6 (GKO) mice were used, which were kindly provided by Bristol-Myers Squibb, Dr. Tak W. Mak (University of Toronto, Toronto, Ontario, Canada), and Dr. Shaobo Su (Shantou University, Shantou, China), respectively. Age-matched *Rag1*$^{-/-}$ C57BL/6 mice, originally obtained from the Jackson Laboratory (Bar Harbor, ME), were purchased locally from the Model Animal Research Center (Nanjing, 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.

Two-Thirds Partial Hepatectomy (PH). Two-thirds of the liver was surgically removed as described.[25]

Reagents. 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 monoclonal antibody (clone HB170) was purified from the culture supernatant of R4-6A2 cells (ATCC, Manassas, VA). To block TIGIT or to neutralize IFN-γ *in vivo*, 125 μg of anti-TIGIT or anti-IFNγ was intraperitoneally injected, respectively, 1 day before PH and every other day after that. Rat IgG purified from rat serum was used as a control.

Antibody Staining and Flow Cytometry. We purchased eFluor 660-anti-TIGIT and PE-anti-PVR from eBioscience, 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.[26] Hepatocytes and Kupffer cells were isolated using a two-step collagenase perfusion method as described previously.[27]

Cell Depletion. For NK cell depletion, mice were intravenously injected with 30 μg of anti-ASGM1 antibody (Wako, Tokyo, Japan) 24 hours before PH.

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

Cell Sorting and Transfer. A FACSAria cell sorter (BD Biosciences) was used to purify CD3$^-$NK1.1$^+$ NK cells. The purity of sorted NK cell population was >95%, as verified by post-sort flow cytometric analysis. For adoptive transfer of NK cells, 2×10^6 purified CD3$^-$NK1.1$^+$ splenocytes from wild-type (WT) or *Tigit*$^{-/-}$ mice were intravenously injected into *Nfil3*$^{-/-}$ mice 48 hours before PHx.

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Hematoxylin & Eosin Staining. For histological analysis, liver sections were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were mounted on slides, deparaffinized, stained with hematoxylin-eosin (H&E), and examined under light microscopy.

Immunohistochemical Staining. For the bromodeoxyuridine (BrdU) incorporation assay, 50 μg BrdU per gram body weight was injected intraperitoneally 2 hours before harvesting the liver after PHx. Sections from formalin-fixed and paraffin-embedded liver tissue were stained for BrdU using the BrdU In-Situ Detection Kit (BD Biosciences) according to the manufacturer's instructions. Percentage of BrdU-labeled hepatocytes was determined by counting positively stained hepatocyte nuclei and total hepatocyte nuclei in 3 to 6 low-power (10×) microscope fields, and the mean was calculated.

NK-Hepatocyte Coculture System. For the NK-hepatocyte coculture, 10^5 purified NK1.1$^+$CD3$^-$ hepatic NK cells (>95% purity) were cocultured with 2.5×10^4 purified hepatocytes in 96-well U-bottom tissue culture plates in 100 μL Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 10 mM HEPES, 10 mM 2-mercaptoethanol, and 100 IU/mL penicillin/streptomycin. IgG control or rat antimouse TIGIT (13G6, produced in-house) monoclonal antibodies (5 μg/mL) were added to the culture when indicated. The supernatant was harvested 24 hours later for determination of IFN-γ concentration by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Dakewe Biotech, Shenzhen, China).

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

Results

TIGIT on NK cells Is Up-Regulated During Liver Regeneration. We previously showed that TIGIT was up-regulated during NK cell activation in acute viral hepatitis.[23] Since NK cells were also shown to be activated during liver regeneration,[12] we wondered whether TIGIT would be regulated in a similar manner during liver regeneration. Indeed, we observed significant up-regulation of TIGIT expression on hepatic NK cells, but not on splenic NK cells, in mice that received partial hepatectomy (PHx), compared with that in mice receiving a sham operation (Fig. 1A). On the other hand, TIGIT expression on T cells were comparable between PHx and sham-operation (Fig. 1B). Meanwhile, CD226 as well as CD96, although to a less extent, were down-regulated on hepatic NK cells during liver regeneration (Supporting Fig. 1.A,B), which coincided with what we observed in acute viral hepatitis.[23]

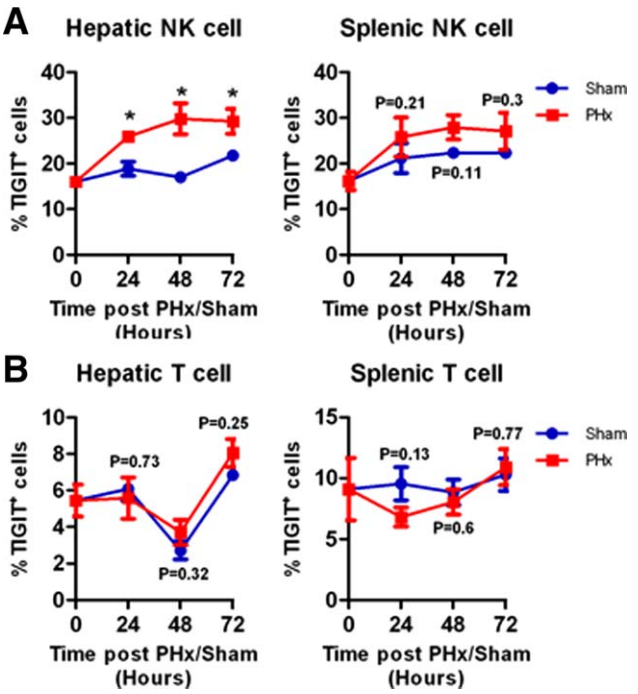

Fig. 1. TIGIT on NK cells is up-regulated during liver regeneration. (A,B) Percentage of TIGIT$^+$ cells in CD3$^-$NK1.1$^+$ NK cells (A) and CD3$^+$NK1.1$^-$ T cells (B) after mice received PHx or sham-operation (n = 5 or 6). Data are representative of at least three independent experiments and are represented as the mean ± SEM. *$P < 0.05$.

TIGIT Is Beneficial to Liver Regeneration. In order to investigate the role of the up-regulated TIGIT expression in liver regeneration, *Tigit*$^{-/-}$ mice were subjected to PHx. Compared to WT mice, the recovery of liver weight to body weight ratio after PHx was suppressed and delayed in *Tigit*$^{-/-}$ mice (Fig. 2A; Supporting Fig. 2), and in mice with TIGIT signaling blockade by monoclonal antibody (mAb) treatment (Supporting Fig. 3A). Also, the incorporation of BrdU by hepatocytes in the absence of TIGIT was significantly reduced (Fig. 2B,C; Supporting Fig. 3B,C), indicating that TIGIT might normally benefit liver regeneration. We previously showed that TIGIT prevented hepatocytes from immune attack in acute viral hepatitis[23]; however, we did not detect serum ALT changes between WT mice and *Tigit*$^{-/-}$ mice during liver regeneration (Fig. 2D). Also, only occasional terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive hepatocytes were observed

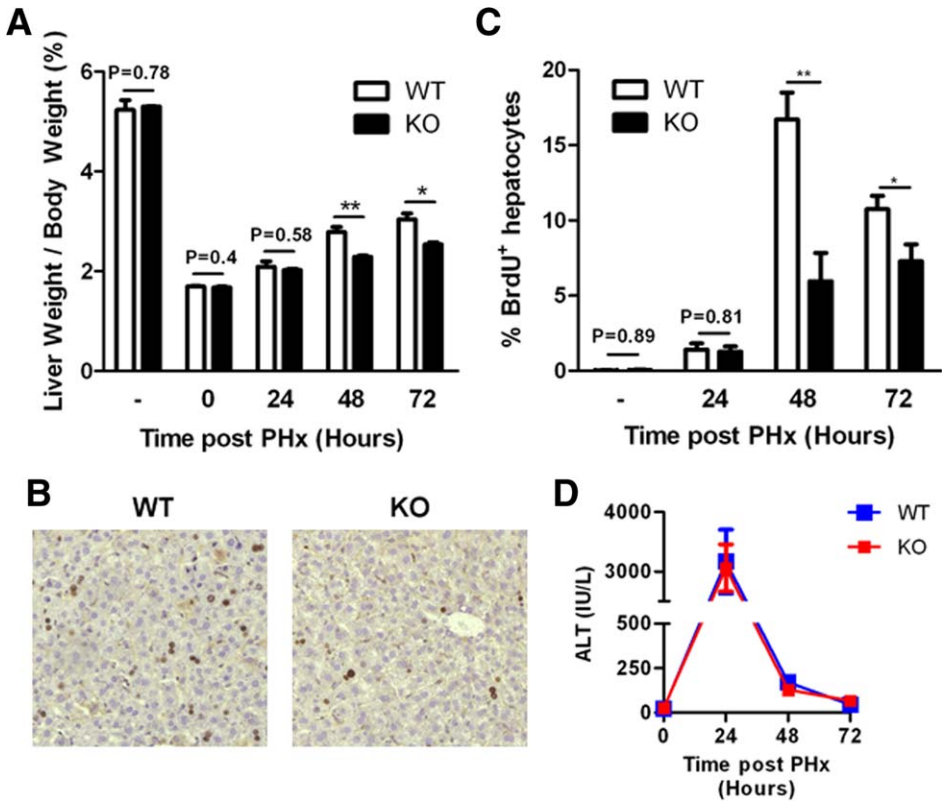

Fig. 2. TIGIT deficiency impairs liver regeneration. (A) Liver weight to body weight ratio of WT or *Tigit*$^{-/-}$ mice are shown before or after PHx (n = 6). (B) Representative BrdU staining of liver sections from WT or *Tigit*$^{-/-}$ mice 48 hours after PHx. (C) Percentages of BrdU-positive hepatocytes from WT or *Tigit*$^{-/-}$ mice before or after PHx (n = 6). (A,C) Data are representative of at least three independent experiments and are represented as the mean ± SEM. $*P < 0.05$, $**P < 0.005$. (D) Serum ALT concentration was measured from WT or *Tigit*$^{-/-}$ mice that received PHx.

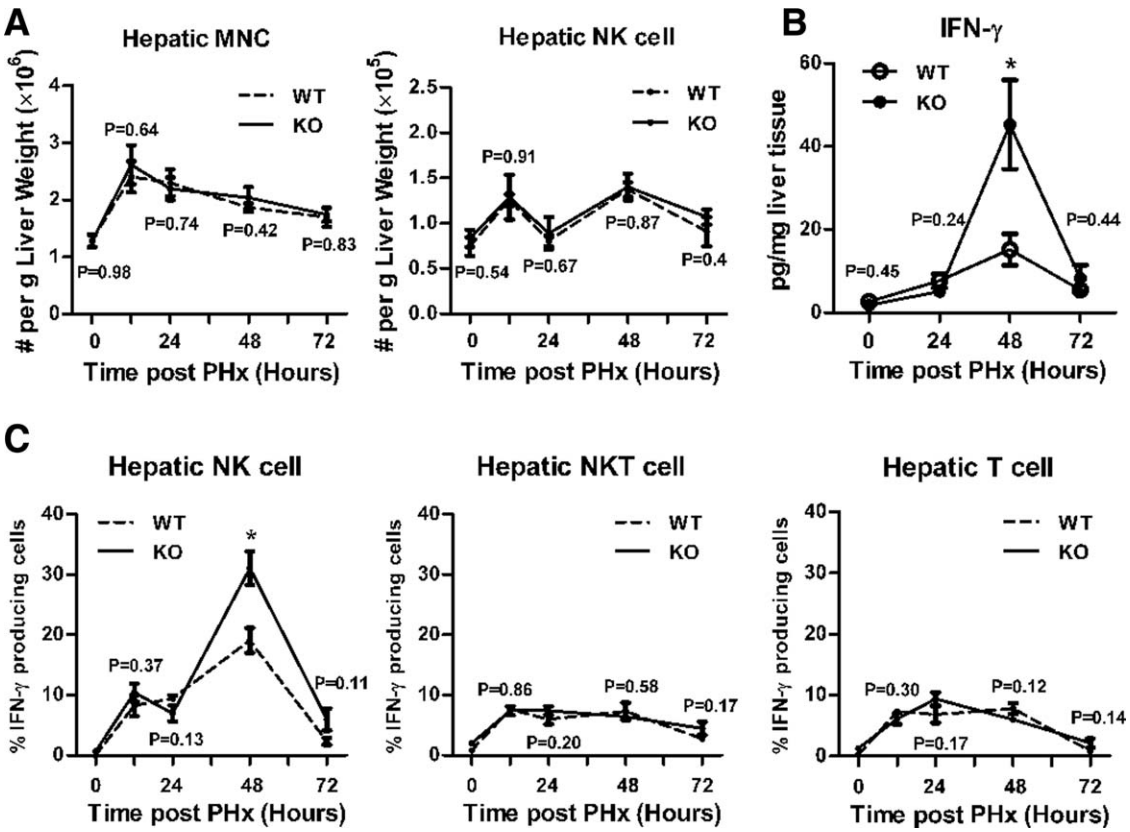

Fig. 3. TIGIT deficiency promotes NK cell activation during liver regeneration. (A) Absolute number of MNCs and CD3$^-$NK1.1$^+$ cells in the liver was evaluated before or after PHx (n = 6). (B) IFN-γ expression in liver tissue was evaluated before or after PHx (n = 6). (C) Percentages of IFN-γ-containing cells in the CD3$^-$NK1.1$^+$ hepatic NK cells, CD3$^+$NK1.1$^+$ hepatic NKT cells, and CD3$^+$NK1.1$^-$ hepatic T cells before or after PHx are shown (n = 6). (A-C) Data are representative of at least three experiments and are represented as the mean ± SEM. $*P < 0.05$.

from 24 to 72 hours post-PH, which was comparable between WT mice and *Tigit*$^{-/-}$ mice (data not shown). These indicated that the impaired liver regeneration of *Tigit*$^{-/-}$ mice might not be due to aggravated hepatocyte injury or apoptosis.

TIGIT Prevents NK Cells From Overactivation in Liver Regeneration. Although neither total lymphocyte nor NK cell infiltration into the liver was altered by TIGIT deficiency (Fig. 3A), we detected higher IFN-γ production in the liver of *Tigit*$^{-/-}$ mice (Fig. 3B), which was reported to inhibit liver regeneration. This higher production of IFN-γ might be contributed by NK cells, since we detected significantly higher intracellular IFN-γ level in hepatic NK cells, but not in hepatic NKT cells or T cells, in *Tigit*$^{-/-}$ mice (Fig. 3C). These suggest that TIGIT inhibits NK cell activation and its IFN-γ production during liver regeneration.

We previously reported that activated NK cells produced IFN-γ and inhibited liver regeneration.[12] We wondered whether TIGIT-mediated inhibition of NK cell activation was critical for liver regeneration. We found that, after depletion of NK cells, both recovery of liver weight (Fig. 4A; Supporting Fig. 4A) and incorporation of BrdU by hepatocytes (Fig. 4B; Supporting Fig. 4B,C) were similar in the presence or absence of TIGIT, indicating that TIGIT-mediated protection of liver regeneration depended on the presence of NK cells. Next, in order to further characterize the role of NK cells in TIGIT-mediated protection of liver regeneration, we used T- and B-deficient *Rag1*$^{-/-}$ mice and NK-deficient *Nfil3*$^{-/-}$ mice[28] (Supporting Fig. 5). We found that, as in WT mice, loss of TIGIT signaling resulted in impaired recovery of liver weight (Fig. 4C) and lower percentage of BrdU-positive hepatocytes (Fig. 4D) in *Rag1*$^{-/-}$ mice, indicating that TIGIT-mediated protection of liver regeneration is not dependent on T cells or B cells. Furthermore, reconstitution of NK-deficient *Nfil3*$^{-/-}$ mice with NK cells from WT or *Tigit*$^{-/-}$ mice showed that TIGIT deficiency in NK cells sufficiently impaired recovery of liver weight (Fig. 4E) and BrdU incorporation by hepatocytes (Fig. 4F). These results collectively suggest that TIGIT prevents NK cells from overactivation and overproduction of IFN-γ to improve liver regeneration.

Hepatocyte PVR Contributes to TIGIT-Mediated Improvement of Liver Regeneration. We previously showed that PVR on hepatocytes was down-regulated during poly I:C/D-GalN-induced acute hepatitis.[23] Here we found that PVR on hepatocytes, but not on Kupffer cells, was up-regulated during liver regeneration (Fig. 5A). In order to study the role of PVR, we hydrodynamically injected a plasmid expressing shRNA targeting PVR (shPVR) to knockdown PVR expression on hepatocytes[23] (Fig. 5B). We found that after silencing PVR, TIGIT deficiency did not significantly impair liver regeneration (WT+shPVR versus *Tigit*$^{-/-}$+shPVR, Fig. 5C-E), as it did without PVR silencing (WT+shNeg versus *Tigit*$^{-/-}$+shNeg, Fig. 5C-E), indicating that TIGIT-sustained normal liver regeneration is dependent on the up-regulated hepatocyte-PVR. These results also demonstrated that silencing PVR partially recovered both liver weight and hepatocyte proliferation in *Tigit*$^{-/-}$ mice (*Tigit*$^{-/-}$+shNeg versus *Tigit*$^{-/-}$+shPVR, Fig. 5C-E), where PVR engagement by activating CD226 might be enhanced, suggesting that activating PVR-binding receptors CD226 might contribute to NK cell activation and compromise liver regeneration, at least in the absence of inhibitory PVR-binding receptor TIGIT. On the other hand, we did not observe any significant changes in liver weight or hepatocyte proliferation after silencing PVR in WT mice, which weakened signaling from both inhibitory TIGIT and activating CD226 (WT+shNeg versus WT+shPVR, Fig. 5C-E), suggesting that opposing signaling from these PVR-binding receptors counterbalanced each other in WT mice, and that TIGIT normally prevents NK cell from overactivity and improves liver regeneration by keeping activating PVR-binding receptors CD226 under control.

TIGIT-PVR Interaction Negatively Regulates NK-Hepatocyte Crosstalk. Given that NK cell activation and subsequent IFN-γ production abrogates liver regeneration and that hepatocyte PVR contributes to TIGIT-mediated improvement of liver regeneration, we wondered whether TIGIT-PVR interaction between NK cells and hepatocytes regulated IFN-γ production by NK cells. We observed the elevated IFN-γ production in hepatic MNC-hepatocytes coculture experiment upon blocking TIGIT (Fig. 6A) when hepatocytes were isolated from mice that received PHx, but not sham-operation (Fig. 6A). Such an effect of blocking TIGIT was not observed when using NK-depleted hepatic MNCs (Fig. 6B). More important, blocking TIGIT enhanced IFN-γ production in hepatocytes coculture with NK cells alone (Fig. 6C). Also, this effect was only observed when hepatocytes were isolated from mice that received PHx, but not sham-operation (Fig. 6C). Given that PVR was up-regulated on hepatocytes in mice that received PHx, but not sham-operation (Fig. 5A), and PVR expression on hepatocytes was critical for TIGIT-mediated improvement of liver regeneration (Fig. 5C-E), the above

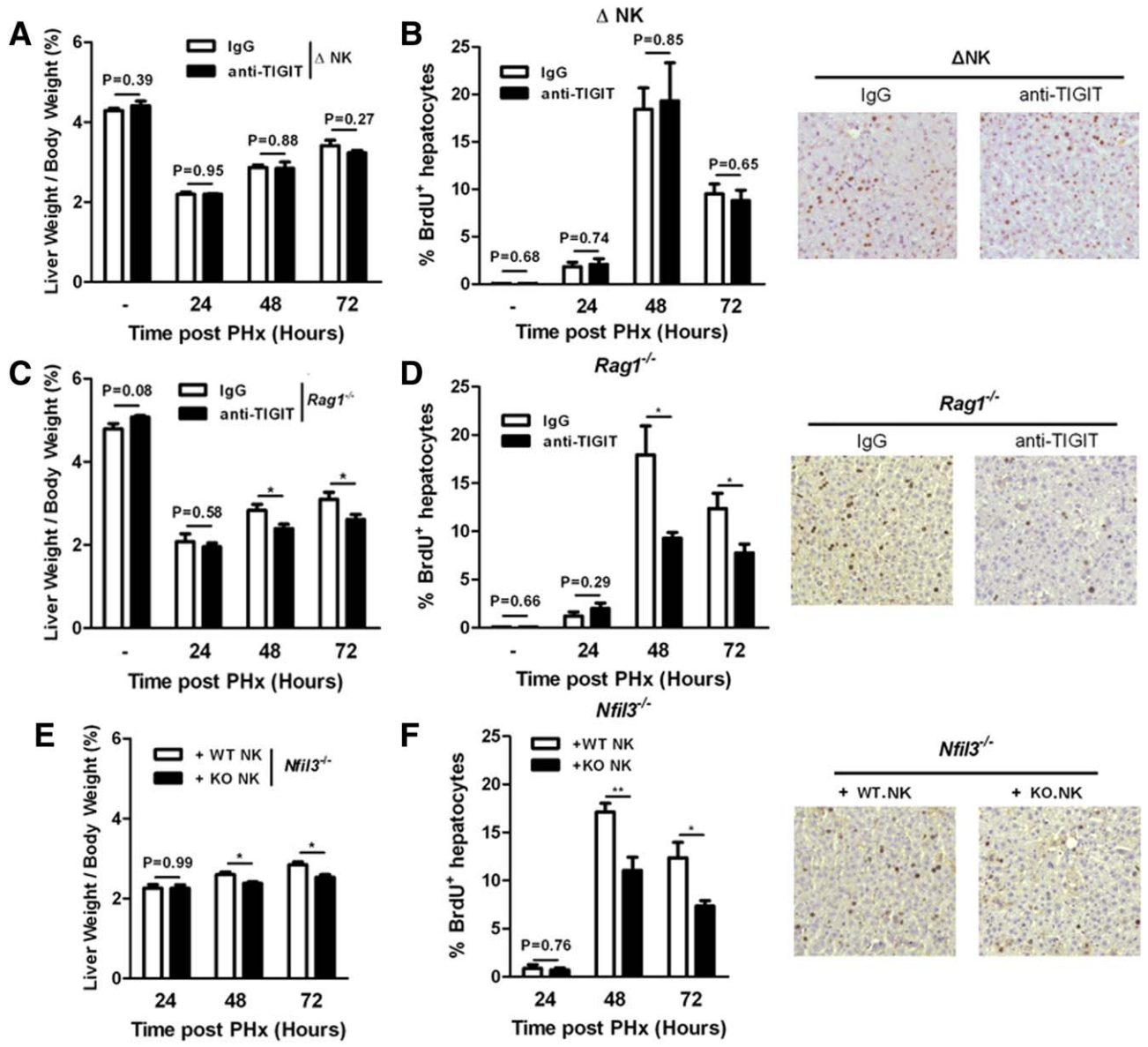

Fig. 4. TIGIT deficiency in NK cells impairs liver regeneration. (A) WT mice were injected with anti-ASGM1 to deplete NK cells, together with anti-TIGIT mAb or control IgG, 24 hours before PHx. Liver weight to body weight ratio was calculated before or after PHx (n = 6). (B) Percentages of BrdU-positive hepatocytes from NK-depleted mice before or after PHx (n = 6) from (A) were calculated (left). Representative BrdU staining of liver sections 48 hours after PHx is shown (right). (C) *Rag1*$^{-/-}$ mice were treated with anti-TIGIT mAb or control IgG 24 hours before PHx. Liver weight to body weight ratio was calculated before or after PHx (n = 6-11). (D) Percentages of BrdU-positive hepatocytes from *Rag1*$^{-/-}$ mice before or after PHx (n = 6-11) from (C) were calculated (left). Representative BrdU staining of liver sections 48 hours after PHx was shown (right). (C,D) Data were pooled from two experiments. (E) CD3^{-}NK1.1^{+} splenocytes from WT or *Tigit*$^{-/-}$ mice were adoptively transferred into *Nfil3*$^{-/-}$ mice 48 hours before PHx. Liver weight to body weight ratio was calculated after PHx (n = 6-10). (F) Percentages of BrdU-positive hepatocytes from *Nfil3*$^{-/-}$ mice after PHx (n = 6-10) from (E) were calculated (left). Representative BrdU staining of liver sections 48 hours after PHx is shown (right). (E,F) Data are pooled from two experiments. (A-F) Data are represented as the mean ± SEM. *$P < 0.05$, **$P < 0.005$.

results collectively indicated that TIGIT-PVR interaction negatively regulates NK-hepatocyte crosstalk and IFN-γ production by NK cells.

TIGIT Protection of Liver Regeneration Is Due to Inhibiting Production of IFN-γ. We showed above that TIGIT inhibited NK cell-derived IFN-γ production during liver regeneration (Fig. 3C) upon interaction with PVR on hepatocytes (Fig. 6C). We then explored the importance of IFN-γ to TIGIT-mediated protection of liver regeneration. We found that in the absence of IFN-γ, blocking TIGIT signaling *in vivo* by mAb treatment failed to impair either liver weight recovery (Fig. 7A; Supporting Fig. 6A) or BrdU incorporation by hepatocytes (Fig. 7B,C; Supporting Fig. 6B,C), further confirming that TIGIT might normally prevent overproduction of NK cell-derived IFN-γ,

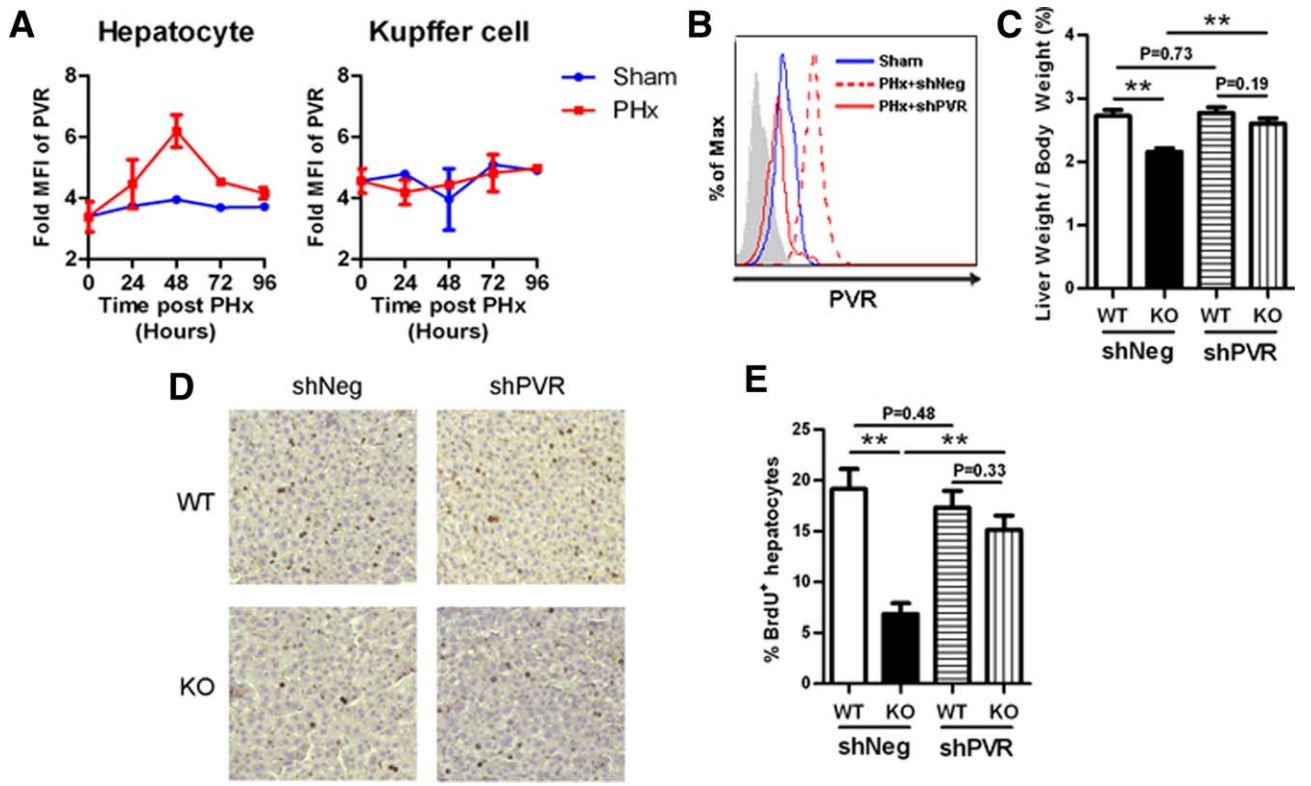

Fig. 5. TIGIT deficiency impairs liver regeneration dependent on PVR on hepatocytes. (A) Mean fluorescence intensity (MFI) of PVR on hepatocytes and Kupffer cells after PHx or sham-operation (n = 3-5) was determined by flow cytometry. Fold MFI was calculated as the ratio of PVR-MFI to the MFI of isotype control. (B) WT mice were hydrodynamically injected with 100 μg plasmids expressing shRNA targeting PVR on hepatocytes or control shRNA 48 hours before PHx. Representative plot is shown for the expression of PVR on hepatocytes 48 hours after PHx or sham-operation. (C) WT or *Tigit*$^{-/-}$ mice were hydrodynamically injected with 100 μg plasmids expressing shRNA targeting PVR on hepatocytes or control shRNA 48 hours before PHx. Liver weight to body weight ratio was calculated 48 hours after PHx (n = 9-12). (D) Representative BrdU staining of liver sections 48 hours after PHx from WT or *Tigit*$^{-/-}$ mice preinjected with shRNA-expressing plasmids. (E) Percentages of BrdU-positive hepatocytes 48 hours after PHx from WT or *Tigit*$^{-/-}$ mice preinjected with shRNA-expressing plasmids (n = 9-12). (C,E) Data were pooled from two experiments and are represented as the mean ± SEM. *$P < 0.05$, **$P < 0.005$.

which directly mediate cell cycle arrest in hepatocytes,[5,12,29] to improve liver regeneration.

Discussion

In this study we investigated the molecular interactions that mediate NK cell self-tolerance during hepatic regenerative hyperplasia. After partial hepatectomy, NK cells in the liver are activated and produce IFN-γ, which negatively regulates liver regeneration.[9,12] This poses an extrinsic "checkpoint" from the innate immune system for hyperplasia events in the body. Therefore, mechanisms exist to protect regenerative hyperplasia from attack by the immune system. Here, our data indicated that up-regulated TIGIT on NK cells are normally required to mediate NK cell self-tolerance and protect regenerative hyperplasia in a manner dependent on up-regulated PVR on hepatocytes during liver regeneration. We provided both *in vivo* and *in vitro* evidence that TIGIT inhibits NK cell activation and IFN-γ production in NK-hepatocyte crosstalk, preventing proliferating hepatocytes from cell cycle arrest by excessively produced IFN-γ.

In the current study we provided evidence of NK-hepatocyte crosstalk. Activation of NK cells upon contact with hepatocytes has long been reported.[30,31] Here we observed the augmented IFN-γ production by NK cells in the absence of TIGIT upon interaction with hepatocytes (Fig. 6), especially those from regenerating livers. Up-regulation of PVR expression on hepatocytes during liver regeneration (Fig. 5A) might explain why the effect of blocking TIGIT in the coculture experiment was more prominent when using hepatocytes from regenerating livers instead of from intact livers. However, we also observed that such hepatocytes from regenerating livers showed enhanced NK-stimulatory effects compared with those from intact livers (Fig. 6), suggesting that there might exist certain stimulatory molecular patterns associated with the proliferating status of hepatocytes, which still needs further studies.

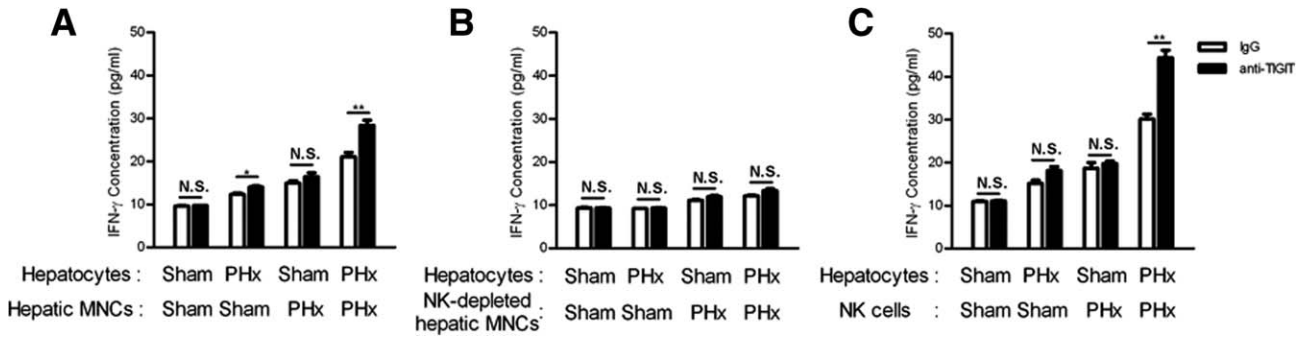

Fig. 6. TIGIT-PVR interaction negatively regulates NK-hepatocyte crosstalk. (A) Hepatic MNCs and hepatocytes from mice that received PHx or sham-operation were coincubated for 24 hours in the presence of anti-TIGIT mAb or control IgG. IFN-γ concentration in the supernatant from was determined by ELISA. (B) Coculture of NK-depleted hepatic MNCs and hepatocytes was performed as in (A). (C) Coculture of MACS-enriched NK cells and hepatocytes was performed as in (A). (A-C) Data are represented as the mean ± SEM. $*P < 0.05$, $**P < 0.005$.

PVR is a multifunctional protein. Besides as a ligand for NK cell receptors, CD226/CD96[32-35] and TIGIT,[21] PVR is involved in cell growth,[36] migration,[37] adhesion,[38] and immunomodulation.[21] In connection with its role in cell growth, a group reported that PVR expression is up-regulated at mRNA levels during liver regeneration in rats.[24] However, its role in such processes is not clear. Here we found that TIGIT protects liver regeneration in a manner depending on the up-regulated PVR expression on hepatocytes (Fig. 5C-E). On the one hand, our data showed that knocking down PVR in hepatocytes to pre-PH level had little effects on liver regeneration in WT mice, suggesting that the hepatocyte-intrinsic functions of PVR (at least for the up-regulated part) might be redundant in this respect. However, whether the basal level of PVR on hepatocytes contributes to liver regeneration still needs further studies by using PVR-deficient mice. On the other hand, however, knocking down PVR in hepatocytes in *Tigit*$^{-/-}$ mice rescued the impaired liver regeneration, indicating that PVR on hepatocytes may overactivate NK cells to break immune tolerance if the safeguard molecule TIGIT is absent. Given that TIGIT-mediated protection of liver regeneration is dependent on the presence of NK cells (Fig. 4A,B; Supporting Fig. 4), CD226 and/or CD96, two other

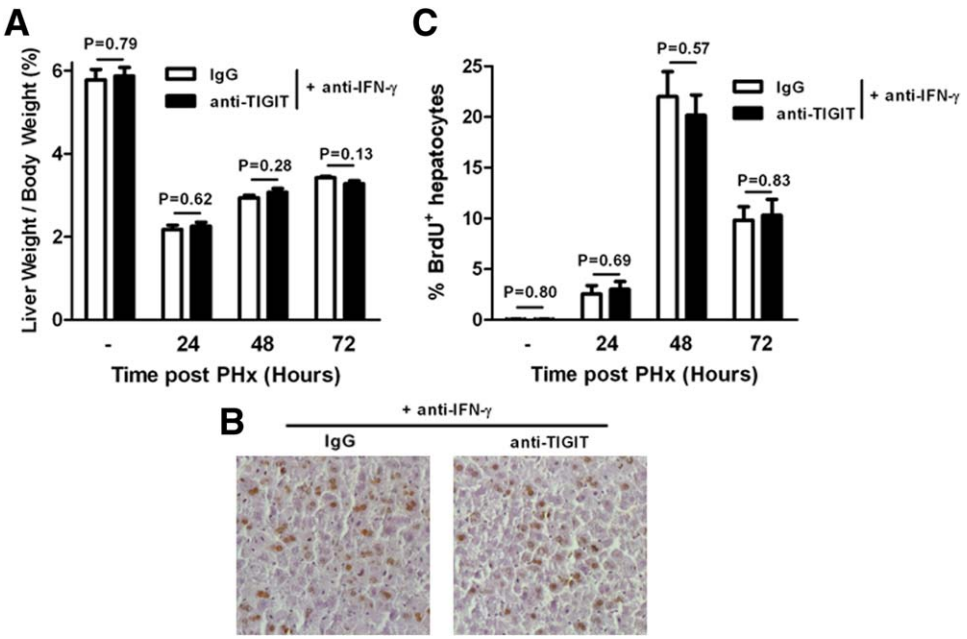

Fig. 7. TIGIT blockade impairs liver regeneration dependent on IFN-γ. (A) WT mice were injected with anti-IFN-γ to neutralize IFN-γ *in vivo*, together with anti-TIGIT mAb or control IgG, 24 hours before PHx, and injected with anti-IFN-γ every other day after the first injection. Liver weight to body weight ratio was calculated before or after PHx (n = 6). (B) Percentages of BrdU-positive hepatocytes from IFN-γ-neutralized mice before or after PHx (n = 6) from (A) were calculated (left). Representative BrdU staining of liver sections 48 hours after PHx is shown (right). Data are represented as the mean ± SEM.

NK cell receptors which also interact with PVR, might be involved in interaction between NK cells and hepatocytes. The actual interaction of the NK cell receptor array of both inhibitory and activating ones in liver regeneration, however, remains to be elucidated.

The role of IFN-γ in regulating liver regeneration has been extensively investigated.[5,29] Instead of inducing apoptosis,[39] IFN-γ was reported to cause cell cycle arrest of hepatocytes through the generation of NO.[29] Specific cellular populations, which are reported to be critical for IFN-γ production during liver regeneration, varied depending on the animal model used, although they mainly belong to the innate immune system. We previously found that IFN-γ produced by activated NK cells inhibits liver regeneration in WT mice. Such an effect is further augmented if the mice were pretreated with MCMV or viral dsRNA analog poly I:C, enhancing NK cell activation and IFN-γ production,[12]wWhereas in HBV transgenic mice it is NKT cells that are critical for IFN-γ production and inhibit liver regeneration.[13]

As reviewed recently,[40] both hypertrophy[41] and hepatocyte proliferation contribute to liver regeneration after 70% PHx. According to previous research[12] and the current study, NK cells regulate liver regeneration by producing IFN-γ to inhibit hepatocyte proliferation, which is proposed to be regulated by TIGIT. At 24 hours post PHx, when hypertrophy peaks but low DNA synthesis/hepatocyte proliferation occurs,[42] low IFN-γ levels might be insufficient to produce significant effects (Fig. 3B). Also at this timepoint, the effects of TIGIT on IFN-γ production and liver mass gain was not significant ("24h" in Figs. 2A-C, 3B,C). Therefore, it is not likely that either NK cell-IFN-γ or TIGIT might affect hypertrophy-induced early liver mass gain. However, whether NK cells or TIGIT regulate hypertrophy in a later phase still needs further investigation, by employing mouse models (e.g., 30% PHx[42]) in which the liver mass gain mostly comes from hypertrophy, so as to exclude the effects of NK-IFN-γ-proliferation pathway on liver mass gain.

On the other hand, after the peak of hepatocyte proliferation, along with the declining production of IFN-γ (Fig. 3B,C), the effect of TIGIT-deficiency on liver regeneration might be gradually diminished in *Tigit*$^{-/-}$ mice, thus possibly allowing the liver in such mice to respond normally to the physiologically compensating need and gradually recover the liver mass. Indeed, although liver regeneration was suppressed in *Tigit*$^{-/-}$ mice around 48 hours post-PH, at later timepoints the liver mass in such mice were getting closer to that in the WT mice (Supporting Fig. 2). This suggests that TIGIT might normally function to protect liver regeneration mainly by regulating NK cell-mediated innate immune response.

Unlike on murine NK cells, TIGIT expression on human NK cells was reported to be constitutive.[21] Besides, human NK cells are highly enriched in the liver (accounting for 30% of all intrahepatic lymphocytes on average).[43] The constitutive expression of TIGIT, as well as the enriched NK cells in human liver, suggests that TIGIT might play an important role in regulating NK cell-mediated hepatic innate immune response in human and might benefit regeneration after liver transplantation or liver injury in patients with liver diseases. Furthermore, TIGIT expression on human NK cells is differentially regulated by different cytokines. While stimulatory IL-2 plus IL-15 up-regulates TIGIT,[21] immune-suppressive TGF-β alone, on the contrary, down-regulates TIGIT.[44] The final pattern of regulation might depend on the cytokine predominance in a specific immune microenvironment, which thereafter might either amplify or diminish the impact of TIGIT-mediated effects. Therefore, the exact mode of TIGIT-mediated protection might be context-dependent, which calls for further investigation.

In conclusion, our study demonstrated the role of TIGIT in NK cells as a safeguard of liver regeneration. We suggested that TIGIT is normally required to mediate NK cell self-tolerance to regenerative hyperplasia, in order to limit innate immunity against the regenerating organ/tissue, especially for the liver, an organ with high regenerative potential. This also sheds light on the mechanisms by which the immune system regulates organ/tissue homeostasis in the body.

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References

1. Michalopoulos GK, DeFrances MC. Liver regeneration. Science 1997; 276:60-66.
2. Ishiki Y, Ohnishi H, Muto Y, Matsumoto K, Nakamura T. Direct evidence that hepatocyte growth factor is a hepatotrophic factor for liver regeneration and has a potent antihepatitis effect in vivo. HEPATOLOGY 1992;16:1227-1235.
3. Cressman DE, Greenbaum LE, DeAngelis RA, Ciliberto G, Furth EE, Poli V, et al. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. Science 1996;274:1379-1383.
4. Akerman P, Cote P, Yang SQ, McClain C, Nelson S, Bagby GJ, et al. Antibodies to tumor necrosis factor-alpha inhibit liver regeneration after partial hepatectomy. Am J Physiol 1992;263(4 Pt 1):G579-585.
5. Sato Y, Tsukada K, Matsumoto Y, Abo T. Interferon-gamma inhibits liver regeneration by stimulating major histocompatibility complex class II antigen expression by regenerating liver. HEPATOLOGY 1993;18: 340-346.

6. Louis H, Van Laethem JL, Wu W, Quertinmont E, Degraef C, Van den Berg K, et al. Interleukin-10 controls neutrophilic infiltration, hepatocyte proliferation, and liver fibrosis induced by carbon tetrachloride in mice. Hepatology 1998;28:1607-1615.
7. Meijer C, Wiezer MJ, Diehl AM, Schouten HJ, Schouten HJ, Meijer S, et al. Kupffer cell depletion by CI2MDP-liposomes alters hepatic cytokine expression and delays liver regeneration after partial hepatectomy. Liver 2000;20:66-77.
8. Kalinichenko VV, Bhattacharyya D, Zhou Y, Gusarova GA, Kim W, Shin B, et al. Foxf1 +/- mice exhibit defective stellate cell activation and abnormal liver regeneration following CCl4 injury. Hepatology 2003;37:107-117.
9. Tamura F, Masuhara A, Sakaida I, Fukumoto E, Nakamura T, Okita K. FK506 promotes liver regeneration by suppressing natural killer cell activity. J Gastroenterol Hepatol 1998;13:703-708.
10. Nakashima H, Inui T, Habu Y, Kinoshita M, Nagao S, Kawaguchi A, et al. Activation of mouse natural killer T cells accelerates liver regeneration after partial hepatectomy. Gastroenterology 2006;131:1573-1583.
11. Dong Z, Wei H, Sun R, Tian Z. The roles of innate immune cells in liver injury and regeneration. Cell Mol Immunol 2007;4:241-252.
12. Sun R, Gao B. Negative regulation of liver regeneration by innate immunity (natural killer cells/interferon-γ). Gastroenterology 2004;127: 1525-1539.
13. Dong Z, Zhang J, Sun R, Wei H, Tian Z. Impairment of liver regeneration correlates with activated hepatic NKT cells in HBV transgenic mice. Hepatology 2007;45:1400-1412.
14. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol 2008;9:503-510.
15. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol 2008;9:495-502.
16. Whiteside TL, Herberman RB. The role of natural killer cells in immune surveillance of cancer. Curr Opin Immunol 1995;7:704-710.
17. Takeda K, Hayakawa Y, Smyth MJ, Kayagaki N, Yamaguchi N, Kakuta S, et al. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat Med 2001;7:94-100.
18. Long EO, Kim HS, Liu D, Peterson ME, Rajagopalan S. Controlling natural killer cell responses: integration of signals for activation and inhibition. Annu Rev Immunol 2013;31:227-258.
19. Karlhofer FM, Ribaudo RK, Yokoyama WM. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. Nature 1992;358:66-70.
20. Brooks AG, Posch PE, Scorzelli CJ, Borrego F, Coligan JE. NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. J Exp Med 1997;185:795-800.
21. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. Nat Immunol 2009;10:48-57.
22. Ndhlovu LC, Lopez-Verges S, Barbour JD, Jones RB, Jha AR, Long BR, et al. Tim-3 marks human natural killer cell maturation and suppresses cell-mediated cytotoxicity. Blood 2012;119:3734-3743.
23. Bi J, Zhang Q, Liang D, Xiong L, Wei H, Sun R, et al. T-cell Ig and ITIM domain regulates natural killer cell activation in murine acute viral hepatitis. Hepatology 2014;59:1715-1725.
24. Erickson BM, Thompson NL, Hixson DC. Tightly regulated induction of the adhesion molecule necl-5/CD155 during rat liver regeneration and acute liver injury. Hepatology 2006;43:325-334.
25. Mitchell C, Willenbring H. A reproducible and well-tolerated method for 2/3 partial hepatectomy in mice. Nat Protoc 2008;3:1167-1170.
26. Wang J, Sun R, Wei H, Dong Z, Gao B, Tian Z. Poly I:C prevents T cell-mediated hepatitis via an NK-dependent mechanism. J Hepatol 2006;44:446-454.
27. Hou X, Zhou R, Wei H, Sun R, Tian Z. NKG2D-retinoic acid early inducible-1 recognition between natural killer cells and Kupffer cells in a novel murine natural killer cell-dependent fulminant hepatitis. Hepatology 2009;49:940-949.
28. Gascoyne DM, Long E, Veiga-Fernandes H, de Boer J, Williams O, Seddon B, et al. The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. Nat Immunol 2009;10: 1118-1124.
29. Brooling JT, Campbell JS, Mitchell C, Yeoh GC, Fausto N. Differential regulation of rodent hepatocyte and oval cell proliferation by interferon gamma. Hepatology 2005;41:906-915.
30. Ochi M, Ohdan H, Mitsuta H, Onoe T, Tokita D, Hara H, et al. Liver NK cells expressing TRAIL are toxic against self hepatocytes in mice. Hepatology 2004;39:1321-1331.
31. Dong Z, Wei H, Sun R, Hu Z, Gao B, Tian Z. Involvement of natural killer cells in PolyI:C-induced liver injury. J Hepatol 2004;41:966-973.
32. Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B, et al. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. J Exp Med 2003;198:557-567.
33. Tahara-Hanaoka S, Miyamoto A, Hara A, Honda S, Shibuya K, Shibuya A. Identification and characterization of murine DNAM-1 (CD226) and its poliovirus receptor family ligands. Biochem Biophys Res Commun 2005;329:996-1000.
34. Fuchs A, Cella M, Giurisato E, Shaw AS, Colonna M. Cutting edge: CD96 (tactile) promotes NK cell-target cell adhesion by interacting with the poliovirus receptor (CD155). J Immunol 2004;172:3994-3998.
35. Seth S, Maier MK, Qiu Q, Ravens I, Kremmer E, Forster R, et al. The murine pan T cell marker CD96 is an adhesion receptor for CD155 and nectin-1. Biochem Biophys Res Commun 2007;364:959-965.
36. Kakunaga S, Ikeda, W, Shingai T, Fujito, T, Yamada A, Minami, Y, et al. Enhancement of serum- and platelet-derived growth factor-induced cell proliferation by Necl-5/Tage4/poliovirus receptor/CD155 through the Ras-Raf-MEK-ERK signaling. J Biol Chem. 2004;279: 36419-36425.
37. Ikeda W, Kakunaga, S, Takekuni K, Shingai, T, Satoh K, Morimoto, K, et al. Nectin-like molecule-5/Tage4 enhances cell migration in an integrin-dependent, Nectin-3-independent manner. J Biol Chem 2004; 279:18015-18025.
38. Sloan KE, Stewart JK, Treloar AF, Matthews RT, Jay DG. CD155/ PVR enhances glioma cell dispersal by regulating adhesion signaling and focal adhesion dynamics. Cancer Res 2005;65:10930-10937.
39. Shinagawa T, Yoshioka K, Kakumu S, Wakita T, Ishikawa T, Itoh Y, et al. Apoptosis in cultured rat hepatocytes: the effects of tumour necrosis factor alpha and interferon gamma. J Pathol 1991;165:247-253.
40. Miyaoka Y, Miyajima A. To divide or not to divide: revisiting liver regeneration. Cell Div 2013;8:8.
41. Nagy P, Teramoto T, Factor VM, Sanchez A, Schnur J, Paku S, et al. Reconstitution of liver mass via cellular hypertrophy in the rat. Hepatology 2001;33:339-345.
42. Miyaoka Y, Ebato K, Kato H, Arakawa S, Shimizu S, Miyajima A. Hypertrophy and unconventional cell division of hepatocytes underlie liver regeneration. Curr Biol 2012;22:1166-1175.
43. Tian Z, Chen Y, Gao B. Natural killer cells in liver disease. Hepatology 2013;57:1654-1662.
44. Sun H, Sun C, Xiao W. Expression regulation of co-inhibitory molecules on human natural killer cells in response to cytokine stimulations. Cytokine 2014;65:33-41.

Supporting Information

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