

NKG2D–Retinoic Acid Early Inducible-1 Recognition Between Natural Killer Cells and Kupffer Cells in a Novel Murine Natural Killer Cell–Dependent Fulminant Hepatitis

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Increasing evidence suggests the contribution of natural killer (NK) cells to pathogenesis of human hepatitis, but the detailed mechanisms have yet to be clearly elucidated. In this study, injection of polyinosinic:polycytidylic acid (poly I:C) and D-galactosamine (D-GalN) was used to establish a novel murine fulminant hepatitis model: results showed that predepletion of either NK cells or Kupffer cells could completely abolish the liver injury. Injection of poly I:C/D-GalN into mice could promote tumor necrosis factor- α production and surface retinoic acid early inducible-1 (Rae1) protein expression by Kupffer cells, which then activated NK cells to produce interferon- γ via NKG2D–Rae1 recognition. NK cell–derived interferon- γ and Kupffer cell–derived tumor necrosis factor- α synergistically mediated the severe liver injury. Moreover, Kupffer cell–derived interleukin-12 and interleukin-18 were also found to improve cross talk between NK cells and Kupffer cells. *Conclusion:* These results provide the first *in vivo* evidence that NKG2D/ligand interaction is involved in the synergic effects of NK cells and Kupffer cells on acute liver injury. (HEPATOLOGY 2009;49:940–949.)

As an organ with predominant innate immunity, the liver harbors distinct resident populations of macrophages/Kupffer cells, natural killer (NK) cells, and natural killer T (NKT) cells, all of which play critical roles in defense of the liver against pathogenic

microbes and tumors.^{1–3} However, increasing evidence suggests the contribution of these innate immune cells to the pathogenesis of hepatitis. For instance, Kupffer cells activated by bacterial endotoxin can produce various inflammatory mediators that may cause damage to hepatocytes.^{4–6} NKT cells are the pivotal mediators in concanavalin A (ConA)-induced or α -galactosylceramide-induced liver damage, via cytokine production and direct cytotoxicity against hepatocytes.^{7–9}

NK cells are also an important population of innate immune cells in the liver, comprising 30%–40% and 10%–20% of total intrahepatic lymphocytes in humans and mice, respectively.^{10,11} Recently, the contribution of NK cells in the pathogenesis of human hepatitis and animal models of liver injury has been reported.^{12–15} Because polyinosinic:polycytidylic acid (poly I:C)-induced NK cell–mediated liver injury is relatively mild, as we previously reported,¹² here we treated mice with D-galactosamine (D-GalN) to sensitize the liver to the damage induced by poly I:C.^{16,17} We found that NK cells, co-operating with Kupffer cells via NKG2D/retinoic acid early inducible-1 (Rae1) recognition after being triggered by Toll-like receptor-3 (TLR3) activation, could mediate severe liver damage in poly I:C/D-GalN–treated mice. These results demonstrate a TLR-triggered innate recognition between NK cells and

Abbreviations: ALT, alanine aminotransferase; APC, antigen-presenting cell; ConA, concanavalin A; DC, dendritic cell; D-GalN, D-galactosamine; IFN- γ , interferon gamma; H&E, hematoxylin-eosin; IgG, immunoglobulin G; mAb, monoclonal antibody; MNC, mononuclear cell; mRNA, messenger RNA; Mult 1, mouse UL16-binding protein-like transcript 1; NK, natural killer; NKT, natural killer T; NPC, nonparenchymal cell; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; poly I:C, polyinosinic:polycytidylic acid; Rae1, retinoic acid early inducible-1; SCID, severe combined immunodeficient disease; TNF- α , tumor necrosis factor alpha; TLR, toll like receptor.

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macrophages, as well as provide a novel NK cell-mediated hepatitis model.

Materials and Methods

Mice. Male C57BL/6 (H-2^b), BALB/c (H-2^d) and BALB/c severe combined immunodeficient disease (SCID) (H2^d) mice, were purchased from Experimental Animal Center, Chinese Science Academy (Shanghai, China). Interferon-gamma knockout mice (IFN- $\gamma^{-/-}$) were kindly provided by Dr. Bing Sun (Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). All mice were maintained in a specific pathogen-free microenvironment, and received care in compliance with the guidelines outlined in the *Guide for the Care and Use of Laboratory Animals*. Mice used were between 4 and 6 weeks of age.

Reagent. Poly I:C and D-GalN (Sigma Chemical Co., St. Louis, MO) were respectively dissolved in the pyrogen-free saline. To induce liver injury, mice were injected intravenously with poly I:C (1 μ g/mouse) and intraperitoneally with D-GalN (10 mg/mouse) at the same time. Recombinant murine tumor necrosis factor alpha (TNF- α) and IFN- γ were purchased from Cytolab, Peprotech Asia. The monoclonal antibodies (mAbs) used for flow cytometry in this study included Cy5-anti-CD3e, phycoerythrin (PE)-anti-CD69, fluorescein isothiocyanate (FITC)-anti-NK1.1, PE-anti-NK1.1, PE-anti-TNF- α , PE-anti-IFN- γ , PE-anti-NKG2D, FITC-anti-F4/80, FITC-anti-DX5, PE Rat immunoglobulin G2a (IgG2a) isotype control, PE Armenian Hamster IgG isotype control (all of above from eBioscience, San Diego, CA), and PE-anti-pan Rael1 (R&D Systems, Minneapolis, MN). Anti-TNF- α (MP6-XT3)-neutralizing mAb was purchased from BD Pharmingen. Functional grade purified anti-mouse NKG2D (blocking) (CX5) and anti-mouse interleukin-12 (IL-12; p40 subunit) (C17.8) mAbs were purchased from eBioscience (San Diego, CA). Anti-IL-18 (93-10C)-neutralizing mAb was purchased from Medical & Biological Laboratories Co., Ltd. Corresponding amount of functional grade purified Rat IgG1 isotype control (eBioscience) was used as negative control for *in vivo* neutralizing/blocking experiments.

Cell Depletion. Anti-NK1.1 mAb (PK136) was obtained from partially purified hybridoma culture supernatant by ammonium sulfate precipitation (American Type Culture Collection, Manassas, VA). For NK cell depletion, mice were injected with 50 μ g of anti-ASGM1 antibody (Wako Co., Tokyo, Japan) or 200 μ g of anti-NK1.1 mAb per mouse 24 hours before challenge. The elimination of NK cells was confirmed by flow cytometry.

For Kupffer cell depletion, mice were injected intravenously with 100 μ L of clodronate-liposomes 48 hours before challenge, as described.^{7,18} Clodronate-liposomes were kindly provided by Dr. N. van Rooijen (Vrije Universiteit, Amsterdam, The Netherlands). Because liposomes themselves interfered with macrophage phagocytosis,¹⁹ saline was used as a negative control.

Analysis of Liver Transaminase Activities. Liver injury was assessed at the indicated time points after poly I:C and D-GalN treatment by measuring serum enzyme activities of alanine aminotransferase (ALT) using commercially available kit (Rong Sheng, Shanghai, China).

ELISA for Cytokine Detection. For determination of hepatic cytokine levels, liver sections were homogenized in extraction buffer containing Triton-X100, and a protease inhibitor cocktail (Complete Mini; Roche, Switzerland). The homogenate was centrifuged at 3000g and 4°C for 15 minutes. The concentration of total protein in the supernatant was measured by BCA Protein Assay Kit (Pierce, Thermo Scientific). The cytokines were detected using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems).

RNA Preparation and Reverse Transcription Polymerase Chain Reaction. Total liver RNA was extracted by using Trizol Reagent (Invitrogen). Cellular RNA (2 μ g) was used for complementary DNA (cDNA) synthesis. Primer sequences were as follows: β -actin, sense, 5'-GGA CTC CTA TGT GGG TGG CGA GG-3', antisense, 5'-GGG AGA GCA TGC CCT CGT AGA T-3'; Mult1, sense, 5'-GGG AGC CTT CCA TCA GC-3', antisense, GTG ACG GGC AAG CAG TA-3'; Rael1, sense, 5'-GCT GTT GCC ACA GTC ACA TC, antisense, 5'-CCT GGG TCA CCT GAA GTC AT.

Quantitative PCR. RNA extraction and cDNA synthesis were similar as the protocol shown in the reverse transcription polymerase chain reaction (RT-PCR) assay. Quantitative PCR was performed using a sequence detector (ABI-Prism 7000; Applied Biosystems) and a SYBR Premix Ex Taq (Takara), according to the manufacturer's instructions. The primer sequences used were as follows: β -actin, sense, 5'-TGG AAT CCT GTG GCA TCC ATG AAA-3', antisense, 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'; TNF- α , sense, 5'-ACT GGC AGA AGA GGC ACT C-3'; antisense, 5'-CTG GCA CCA CTA GTT GGT TG-3'; IFN- γ , sense, 5'-AAC GCT ACA CAC TGC ATC T-3', antisense, 5'-GAG CTC ATT GAA TGC TTG G-3'; IL-18, sense, 5'-ACT GTA CAA CCG CAG TAA TAC-3', antisense, 5'-AGT GAA CAT TAC AGA TTT ATC CC-3'; IL-12, sense, 5'-CAAGAACGAGAGTTGCCTG-3', antisense, 5'-CTCAGATAGCCCATCAC-3'.

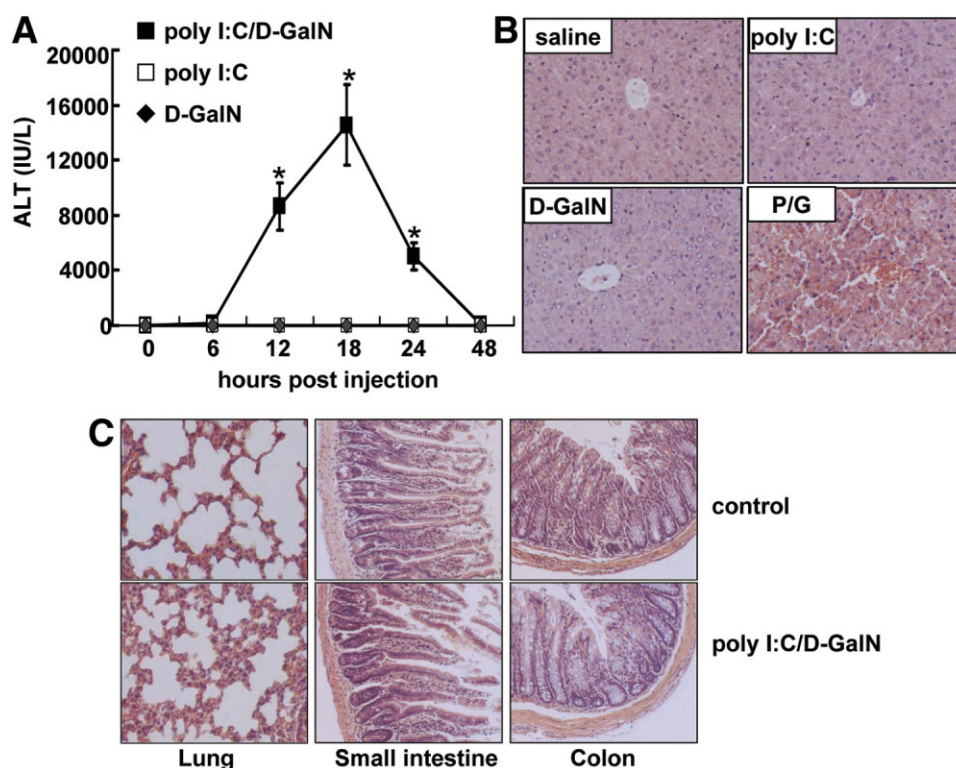


Fig. 1. Poly I:C induces severe liver injury in D-GalN-sensitized mice. (A) B6 mice were injected with poly I:C (1 μ g/mouse) alone, D-GalN (10 mg/mouse) alone, or poly I:C/D-GalN for indicated time periods. Serum ALT levels were measured. The figure shows the results of one representative experiment with four mice per group, and data are shown as mean $\pm$ SEM; *, $P < 0.05$ versus 0 hour time point. (B) B6 mice were killed 18 hours after treatment with saline, poly I:C (1 μ g/mouse) alone, D-GalN (10 mg/mouse) alone, or poly I:C/D-GalN, and liver tissues were fixed and stained with H&E staining (original magnification $\times 100$). (C) Lungs, small intestines, and colons were collected from mice treated with or without poly I:C/D-GalN for 18 hours and stained with H&E staining (original magnification $\times 100$). P, poly I:C; G, D-GalN.

For analysis, all expression levels of target genes were normalized to the housekeeping gene β -actin (Δ Ct). Gene expression values were then calculated based on the $\Delta\Delta$ Ct method as mentioned before,²⁰ using the mean of the respective cytokines in mock-treated mice ("0 h") as a calibrator. Relative quantities (RQs) were determined using the equation: $RQ = 2^{-\Delta\Delta Ct}$.

Hematoxylin & Eosin Staining and Immunofluorescence. For histological analysis, liver sections were fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were affixed to slides, deparaffinized, stained with hematoxylin-eosin (H&E) and examined under light microscopy. For two-color immunofluorescence staining, 10- μ m cryostat sections of livers were thawed onto glass slides, air dried, and fixed in 1:1 acetone-methanol (4°C, 10 minutes). After washing in phosphate-buffered saline (PBS), the sections were blocked with 3% bovine serum albumin/PBS (room temperature, 30 minutes). Incubation was continued with rat anti-mouse FITC-F4/80 mAb directed against murine macrophages and PE-anti-TNF- α mAb dissolved in 3% bovine serum albumin/PBS overnight at 4°C. After rinsing with PBS, the sections were coverslipped with 10% glycerol/

PBS, pH 8.6, and examined by confocal laser-scanning microscopy (Axiovert 100 M, Carl Zeiss, Oberkochen, Germany).

Isolation of Liver Mononuclear Cells. Liver mononuclear cells (MNCs) were isolated essentially as described previously.²¹ Briefly, livers were passed through a 200-gauge stainless steel mesh. The cells were resuspended in 40% Percoll (Sigma) and then gently overlaid on 70% Percoll and centrifuged at 750g for 30 minutes at room temperature. Liver MNCs were collected from the interphase.

Preparation of Mouse Hepatocytes. Hepatocytes were isolated as described previously.^{22,23} Briefly, the liver was sequentially perfused with two solutions. Solution A was composed of original solution (136 mM NaCl, 5.3 mM KCl, 0.5 mM NaH₂PO₄, 0.4 mM Na₂HPO₄, 9.1 mM HEPES, and 4.1 mM NaHCO₃ [pH 7.3]), 0.5 mM ethylene glycol tetraacetic acid and 5 mM D-glucose. Solution B was composed of original solution and 0.05% collagenase IV (Sigma-Aldrich) and 5 mM CaCl₂. Then the viable hepatocytes were separated by 40% Percoll solution with centrifugation at 420g for 10 minutes at 4°C.

Isolation of NK Cells. Liver NK cells were separated

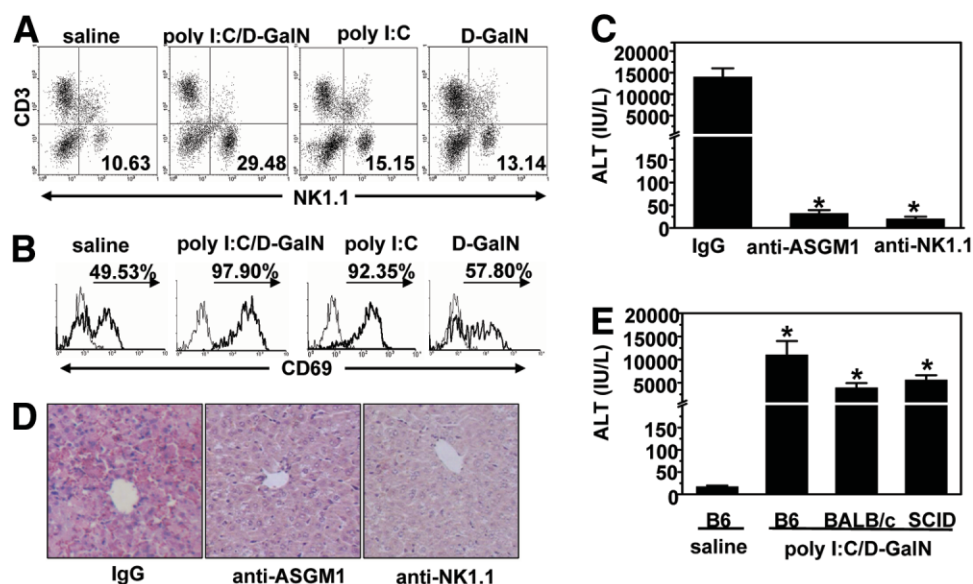


Fig. 2. NK cells play a critical role in poly I:C/D-GalN-induced liver injury. (A, B) B6 mice were treated with poly I:C (1 μ g/mouse) alone, D-GalN (10 mg/mouse) alone, or poly I:C/D-GalN, and sacrificed at 18 hours after challenge. Hepatic mononuclear cells (MNCs) were isolated and analyzed by flow cytometry with anti-NK1.1 and anti-CD3 antibodies. (A) The percentages of NK cells among total hepatic MNCs are shown. (B) The surface expression of CD69 on CD3-NK1.1⁺ cells was also analyzed (bold lines). The thin lines represent negative control stained with Armenian Hamster IgG. These results are representative examples taken from one out three experiments. (C) NK cell depletion was conducted by anti-NK1.1 mAb (PK136) or anti-ASGM-1 mAb. Mice were then treated with poly I:C/D-GalN. Serum ALT levels were measured 18 hours after poly I:C/D-GalN injection. Data are expressed as mean $\pm$ SEM ($n \geq 3$ for each group). *, $P < 0.05$ versus control IgG-treated mice. (D) Liver samples were collected for H&E staining from PK136-treated and anti-ASGM-1-treated mice 18 hours after poly I:C/D-GalN injection (original magnification $\times 100$). (E) C57BL/6, BALB/c, and BALB/c SCID mice were injected with poly I:C/D-GalN or saline. ALT levels were determined 18 hours after injection. Data are expressed as mean $\pm$ SEM from four mice in each group, *, $P < 0.05$ versus saline-treated mice.

by positive magnetic cell sorting using anti-DX5 mAb according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA) from C57BL/6 mice. Approximately 90% of the magnetic cell sorting-purified cells were DX5⁺.

Isolation of Kupffer Cells. Kupffer cells were also isolated using two-step collagenase perfusion method. Solution A was the same as described above. Solution B was added with 0.1% Pronase E (Roche Diagnostics, GmbH,

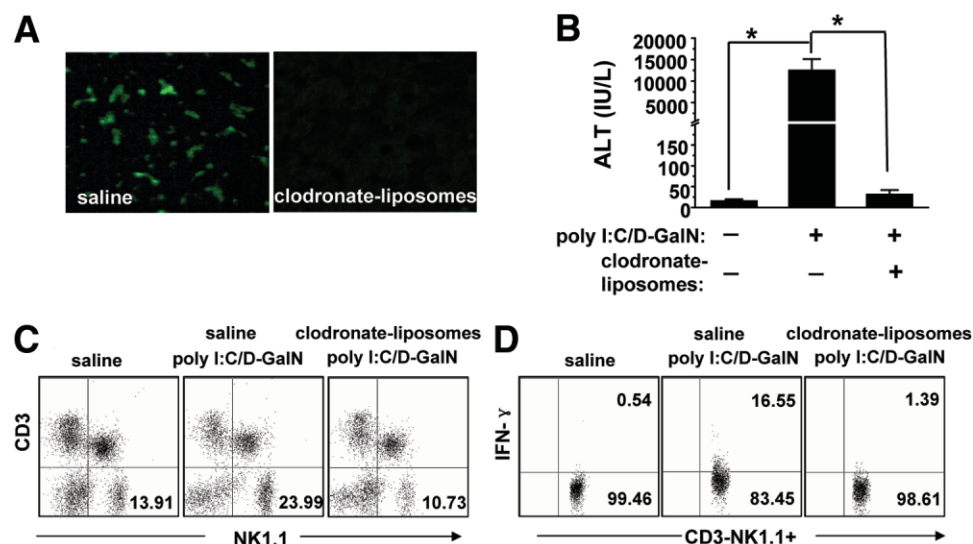


Fig. 3. The presence of Kupffer cells is necessary for Hepatic NK cell activation and accumulation in this hepatitis model. (A) Mice were pretreated with clodronate-liposomes to deplete Kupffer cells, and the efficiency was verified by immunofluorescent staining of liver tissue sections using anti-F4/80 Ab (original magnification $\times 100$). (B-D) After pretreatment with saline or clodronate-liposomes, mice were administered with poly I:C/D-GalN, and sacrificed at 18 hours (B, C) or 6 hours (D) later. Control mice did not receive poly I:C/D-GalN. (B) Serum ALT levels were detected. Data are expressed as mean $\pm$ SEM ($n = 4$ /group). *, $P < 0.05$. (C) The percentages of NK cells (CD3-NK1.1⁺) among total hepatic MNCs were analyzed. (D) Intracellular IFN- γ production by hepatic NK cells (CD3-NK1.1⁺) was examined by flow cytometry.

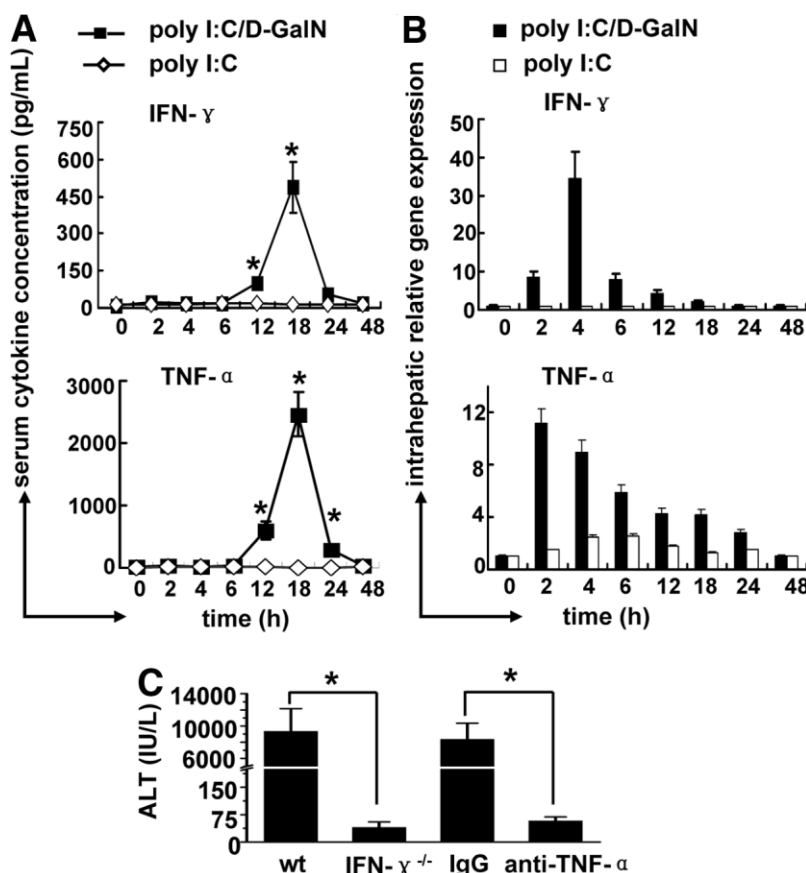


Fig. 4. Systemic and intrahepatic induction of IFN- γ and TNF- α by poly I:C/D-GalN treatment. (A) Serum levels of IFN- γ and TNF- α at indicated time points post poly I:C/D-GalN or poly I:C injection were determined by ELISA. Data are shown as mean $\pm$ SEM; $n \geq 3$; *, $P < 0.05$ versus 0 hour time point. (B) Intrahepatic IFN- γ and TNF- α mRNA expression levels were measured by quantitative PCR after poly I:C/D-GalN or poly I:C alone injection. Results are shown as the relative increase of expression of cytokines compared with β -actin. Gene expression values were then calculated based on the $\Delta\Delta C_t$ method, using the mean of the respective cytokines in mock-treated mice ("0 h") as a calibrator. RQs were determined using the equation: $RQ = 2^{-\Delta\Delta C_t}$. Values are shown as mean $\pm$ SEM from three independent experiments. (C) B6 wild-type (wt) mice and IFN- $\gamma^{-/-}$ mice were treated with poly I:C/D-GalN. Other B6 mice received neutralizing anti-TNF- α mAb or control IgG 0.5 hours before poly I:C/D-GalN injection. Serum ALT levels were measured 18 hours after poly I:C/D-GalN treatment. Data are expressed as mean $\pm$ SEM; $n \geq 3$; *, $P < 0.05$.

Mannheim, Germany) and 20 μ g/mL of deoxyribonuclease (Sangon, Shanghai, China). After perfusion, livers were excised and shaken in Roswell Park Memorial Institute 1640 (RPMI 1640; GIBCO-BRL, Gaithersburg, MD) for 20 minutes. Then the suspension was centrifuged at 50g for 1 minute at 4°C to discard hepatocytes. Resulting suspension was then washed twice at 500g for 8 minutes. The collected pellet was resuspended in PBS and gently layered on a double Percoll gradient (20% and 50%), centrifuging at 800g for 15 minutes. The layer between the 20% to 50% gradient interface was collected. The collected cells were applied to magnetic cell sorting to purify Kupffer cells using anti-F4/80 mAb.

Coculture of Hepatic NK Cells and Kupffer Cells.

NK and Kupffer cells were cocultured for 48 hours at 1:1 ratio in complete medium (RPMI 1640, 10% fetal bovine serum, penicillin, streptomycin) with poly I:C (100 μ g/mL). Blocking antibodies used were as following: anti-NKG2D, anti-IL-12, and anti-IL-18. All mAbs were used at a final concentration of 10 μ g/mL.

Flow Cytometric Analysis. Cells were stained with indicated fluorescence-labeled mAbs for surface antigens according to a standard protocol. For intracellular cytokine staining, after extracellular markers became stained, cells were fixed, permeabilized, and stained with PE-anti-

IFN- γ . The stained cells were analyzed using a flow cytometer (FACScalibur; Becton Dickinson, Franklin Lakes, NJ), and data were analyzed with WinMDI2.9 software.

Statistical Analysis. The results were analyzed by Student *t* test or analysis of variance where appropriate. All data were shown as mean $\pm$ standard error of the mean (SEM). P value < 0.05 was considered to be statistically significant.

Results

Treatment with Poly I:C Induces Severe Liver Injury in D-GalN-Sensitized Mice. In this study, we found that poly I:C could induce severe liver injury in D-GalN-sensitized mice, which differed from the mild liver injury of our previous study, where poly I:C alone was injected.^{12,24} Coadministration with poly I:C and D-GalN induced significant elevation of serum ALT and histological necrosis in the liver, but injection with poly I:C or D-GalN alone did not cause any liver injury (Fig. 1A,B). We also found that the same amount of poly I:C/D-GalN did not induce any injury in other organs, such as the lungs, colon, and small intestine (Fig. 1C).

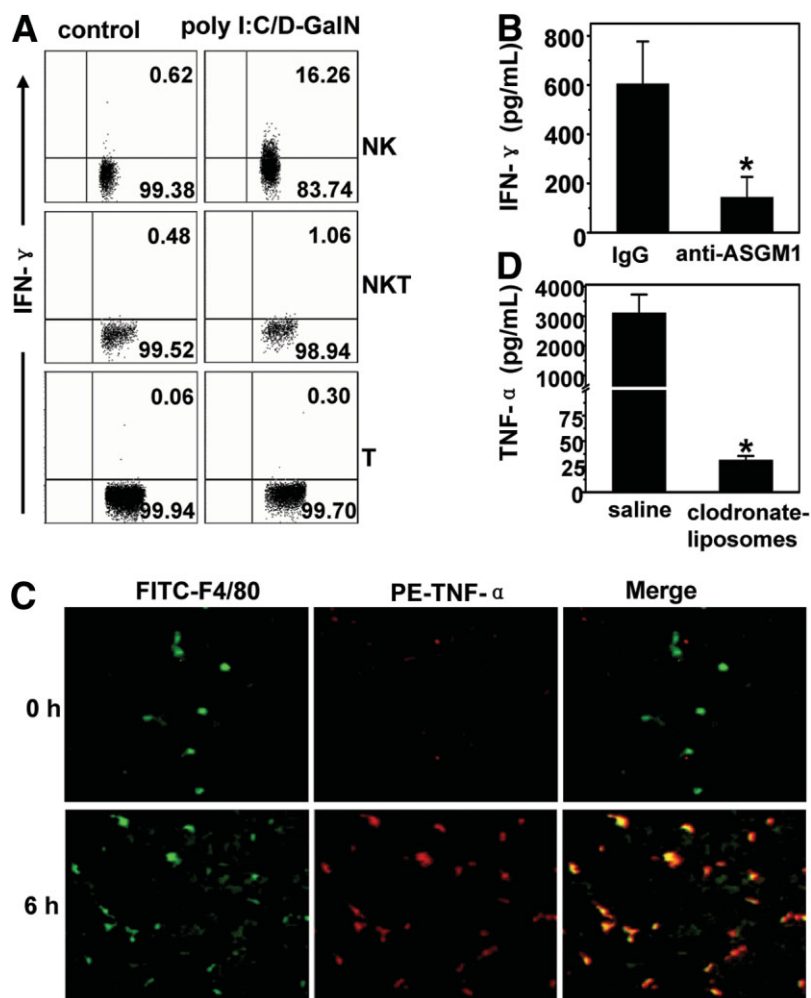


Fig. 5. Poly I:C/D-GalN-induced IFN- γ production by NK cells and TNF- α production by Kupffer cells. (A) Hepatic MNCs were isolated from mice livers 6 hours after poly I:C/D-GalN injection and examined by flow cytometry using anti-NK1.1, anti-CD3 and anti-IFN- γ antibodies. The figures displayed here are representative of the results derived from three independent experiments. (B) Mice were pretreated with anti-AsGM1 mAb or control IgG 24 hours before poly I:C/D-GalN injection. IFN- γ levels in the serum were determined by ELISA 18 hours after poly I:C/D-GalN treatment. Data are shown as mean $\pm$ SEM; $n \geq 3$; *, $P < 0.05$ versus mice pretreated with control IgG. (C) Poly I:C/D-GalN-induced TNF- α expression on liver sections was examined by immunofluorescence 0 and 6 hours after poly I:C/D-GalN injection, in which the antibodies used were PE-anti-TNF- α and FITC-anti-F4/80 (original magnification $\times 100$). (D) Poly I:C/D-GalN was injected into mice pretreated with clodronate-liposomes or saline 48 hours before injection, sera were obtained 18 hours later, and TNF- α levels were determined by ELISA. Data are shown as mean $\pm$ SEM; $n \geq 3$; *, $P < 0.05$ versus mice pretreated with saline.

Poly I:C/D-GalN-Induced Severe Liver Injury Is NK Cell-Dependent. Because we previously found that NK cells could be activated by poly I:C in vivo and played important roles in poly I:C-induced mild liver injury,¹² we examined the role of NK cells in poly I:C/D-GalN coadministration model. The results showed that treatment with poly I:C/D-GalN induced the accumulation and activation of NK cells in the liver (Fig. 2A,B), and depletion of NK cells before poly I:C/D-GalN administration could significantly prevent liver injury (Fig. 2C,D). Further, the severe liver injury in SCID mice triggered by poly I:C/D-GalN (Fig. 2E) proved that NK cells could mediate poly I:C/D-GalN-induced liver injury without the help of B cells and T cells.

NK Cell Activation Depends on the Presence of Kupffer Cells in Poly I:C/D-GalN-Treated Mice. Macrophages express abundant TLRs that enable them to sense the presence of pathogens, and are pivotal in several murine models of hepatitis.^{25,26} To examine the role of Kupffer cells in this model, we depleted Kupffer cells with clodronate-liposomes and verified the effective depletion using immunofluorescent staining with macrophage-spe-

cific mAb anti-F4/80 (Fig. 3A). Poly I:C/D-GalN-induced liver injury was markedly reduced in Kupffer cell-depleted mice (Fig. 3B), indicating that Kupffer cells were indispensable in this model. Further, depletion of Kupffer cells significantly inhibited the accumulation of NK cells in the liver (Fig. 3C), and suppressed production of IFN- γ by NK cells (Fig. 3D). These results suggested that Kupffer cells were involved in the accumulation and activation of NK cells induced by poly I:C/D-GalN.

Poly I:C/D-GalN-Induced Liver Injury Results from the Synergic Effects of NK Cell-Derived IFN- γ and Kupffer Cell-Derived TNF- α .

In accordance to the time course of poly I:C/D-GalN-induced liver injury, cytokine levels in the serum and liver up to 48 hours after coadministration were measured by ELISA and real-time RT-PCR, respectively. The levels of TNF- α and IFN- γ in the serum increased significantly at 18 hours after poly I:C/D-GalN administration (Fig. 4A). IFN- γ and TNF- α messenger RNA (mRNA) levels in liver tissue preceded the serum peak by approximately 14 hours and 16 hours, respectively (Fig. 4B). To evaluate the roles of increased TNF- α and IFN- γ in liver injury, we injected

poly I:C/D-GalN into IFN- $\gamma^{-/-}$ or TNF- α -neutralized mice. The results showed that both TNF- α and IFN- γ were critical in this injury (Fig. 4C). Further, as a supplement to previous conclusion that D-GalN-treated mice were more sensitive to TNF- α -mediated liver injury,²⁷ we proved that D-GalN-treated mice were also very sensitive to IFN- γ -mediated liver injury (Supporting Table 1). Co-administration of IFN- γ and TNF- α could cause much more severe liver injury than either cytokine alone (Supporting Table 2), suggesting that TNF- α and IFN- γ had a synergic effect on liver injury.

To confirm NK cells as a major source of IFN- γ in this model, we first examined the intracellular expression of IFN- γ in different cell subsets of the liver (T cells, NK cells, and NKT cells). As shown in Fig. 5A, after poly I:C/D-GalN injection, IFN- γ was mainly produced by liver NK cells, and NK cell depletion could significantly inhibit IFN- γ production in the serum (Fig. 5B). The immunofluorescent staining of liver tissues showed that TNF- α was produced mainly by Kupffer cells (Fig. 5C). Similarly, Kupffer cell depletion could markedly inhibit TNF- α production in the serum (Fig. 5D).

Crosstalk Between NK Cells and Kupffer Cells Occurs via NKG2D-Rae1 Recognition After Being Triggered by TLR3 Activation. Because NKG2D ligands on murine macrophages could be induced by TLR signaling activation,²⁸ we attempted to determine whether NKG2D-ligand interactions were involved in the cooperation between NK cells and Kupffer cells. Expression of Rae1 and Mult 1, two important ligands of NKG2D, in the liver was investigated by RT-PCR (Fig. 6A). Rae1 expression was markedly increased after poly I:C/D-GalN injection, while Mult 1 expression was not changed. We further found that Rae1 expression was significantly up-regulated on the surface of Kupffer cells but not on hepatocytes (Fig. 6B). Poly I:C/D-GalN injection did not significantly affect NKG2D expression on NK cells. (Fig. 6C). To investigate whether the interaction between Rae1 and NKG2D contributed to the liver damage, we injected mice with NKG2D blocking mAb before poly I:C/D-GalN treatment. Blockade of NKG2D recognition alleviated poly I:C/D-GalN-induced liver injury (Fig. 6D), and reduced levels of IFN- γ in the serum (Fig. 6E). The *in vitro* data also showed that blockade of NKG2D recognition reduced IFN- γ secretion by NK cells when co-incubated with poly I:C-stimulated Kupffer cells (Fig. 6F).

IL-12 and IL-18 Are Also Involved in the Cross Talk Between NK Cells and Kupffer Cells. The critical roles of IL-12 in poly I:C-induced NK cell-mediated liver mild injury have been demonstrated in our previous study.¹² Recently, it was also reported in an *in vitro* study

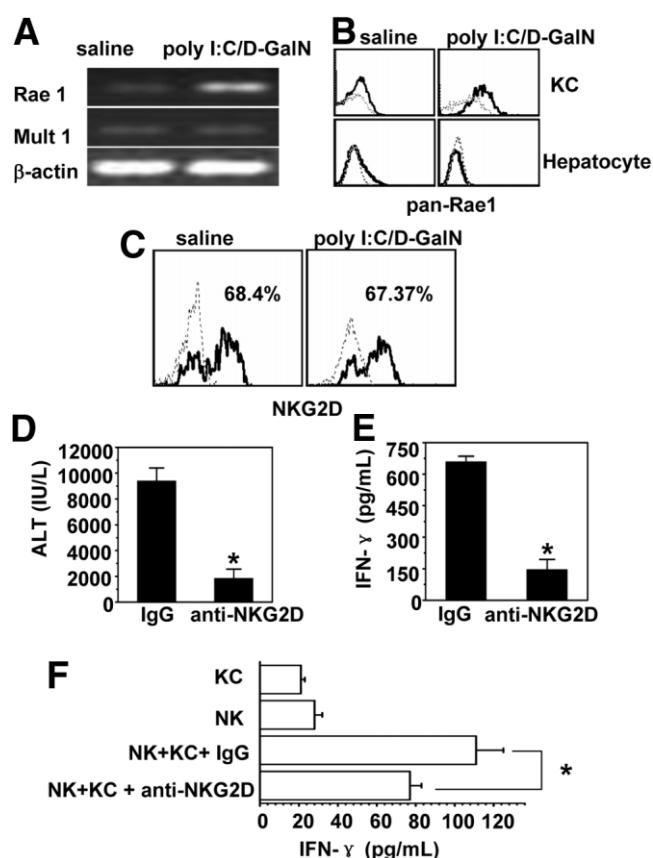


Fig. 6. Rae1 is up-regulated after poly I:C/D-GalN injection and stimulates IFN- γ release. (A) mRNA levels of indicated NKG2D ligands (Rae1 and Mult 1) in the liver of poly I:C/D-GalN-treated mice were analyzed by RT-PCR. (B) Rae1 protein levels on the surface of hepatocytes and Kupffer cells were analyzed by flow cytometry (filled lines). Dotted lines represent staining with isotype matched IgG. (C) Hepatic MNCs from saline-treated or poly I:C/D-GalN-treated mice were analyzed by flow cytometry with anti-NK1.1, anti-CD3, and anti-NKG2D mAbs. The percentage of NKG2D positive subset is indicated. (D, E) Mice were injected with NKG2D blocking mAb or control IgG 24 hours before poly I:C/D-GalN treatment. Sera were collected for ALT determination (D) and cytokine determination (E) 18 hours after challenge. (F) Hepatic NK cells were incubated alone or cocultured with Kupffer cells at a ratio of 1:1 for 48 hours and stimulated by poly I:C (100 μ g/mL). IFN- γ secretion in the supernatant was measured by ELISA. A dose of 10 μ g/mL anti-NKG2D (blocking) mAb was added in the coculture for the blockade. Data are expressed as mean $\pm$ SEM; *, $P < 0.05$.

that human Kupffer cells, after response to TLR ligands, might activate NK cells through IL-12 and IL-18.²⁹ To test whether these cytokines were involved in the cross talk between NK cells and Kupffer cells in this *in vivo* model, we first measured the expression of IL-12 and IL-18 in the liver and the serum upon poly I:C/D-GalN injection. As measured by ELISA, both IL-12 and IL-18 were expressed constitutively in the liver, but markedly increased 12 to 18 hours after injection. Modest induction of IL-18 and IL-12 in the serum was observed 18 hours and 12 to 18 hours after injection, respectively (Fig. 7A). Moreover, blockade of either of the two cytokines *in*

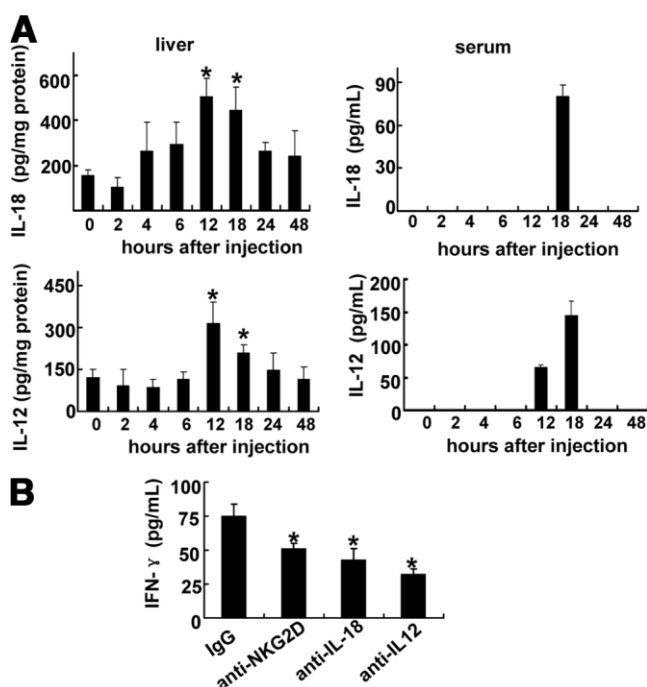


Fig. 7. IL-12 and IL-18 are also involved in the cross talk between NK cells and Kupffer cells. (A) C57BL/6 mice were injected with poly I:C/D-GalN. Sera and livers were harvested at indicated time points. IL-12 and IL-18 levels in the serum and liver homogenates were determined by ELISA. Data are shown as mean $\pm$ SEM; $n \geq 3$; *, $P < 0.05$ versus 0 time point. (B) Freshly isolated hepatic NK cells were cocultured with Kupffer cells at a ratio of 1:1, and stimulated by poly I:C for 48 hours in the presence or absence of the indicated antibody. All mAbs were used at a final concentration of 10 μ g/mL. IFN- γ secretion in the supernatant was measured by ELISA. Data are expressed as mean $\pm$ SEM; *, $P < 0.05$ versus control.

vitro reduced IFN- γ production by NK cells if cocultured with poly I:C-stimulated Kupffer cells (Fig. 7B).

Discussion

Although NK cells are a major component of liver lymphocytes and have been found to be involved in many human liver diseases, the underlying mechanisms of NK cells remain unclear. This is at least partly due to the lack of suitable NK cell-dependent liver injury models. Here, we established a murine NK cell-mediated hepatitis model from which cellular (e.g., Kupffer cell-dependence) and molecular (e.g., NKG2D-ligands recognition) mechanisms underlying the NK cell-mediated fulminant hepatitis are demonstrated.

Notably, our results demonstrated that NK cell-mediated hepatitis induced by poly I:C/D-GalN depended on cooperation with Kupffer cells, among which NKG2D-Rae1 interaction was a critical step. In an *in vitro* study, cross talk has been observed between human NK cells and monocytes mediated by NKp80-AICL innate immune recognition, in which NKp80-AICL interaction pro-

moted secretion of IFN- γ and TNF- α by NK cells and monocytes, respectively.³⁰ In the present study, NKG2D recognition of Rae1 was critical for NK cell-derived IFN- γ production both *in vivo* and *in vitro* (Fig. 6). TNF- α (and possibly IL-12 and IL-18 as described below) and IFN- γ are important activators for NK cells and macrophages,^{31, 32} respectively, and both cytokines regulated each other in respect to their production.^{33, 34} Thus, the cross talk between NK cells and macrophages by innate recognition such as NKG2D-Rae1 might enhance the positive feedback by these cytokines. In addition, IFN- γ acted synergistically with TNF- α to injure the liver (Supporting Table 2), as has been reported in numerous cell death models,^{35, 36} and ConA-induced liver injury.³⁴ Some *in vitro* experiments have already demonstrated that NK cells could kill intracellular pathogen-infected mononuclear phagocytes³⁷ or high doses of lipopolysaccharide-treated macrophages³⁸ via NKG2D-ligand recognition, indicating that the innate recognition between NK cells and macrophages (even Kupffer cells) exists extensively in the innate immune response. This not only prevents against pathogen invasion but also induces immune injury if dysregulated, as indicated in the present study.

Besides Kupffer cells, dendritic cells (DCs) are also one of the major types of antigen presenting cells (APCs) in the liver. Hepatic DCs are rare, comprising less than 1% of hepatic nonparenchymal cells (NPCs) while Kupffer cells comprise approximately 20% of hepatic NPCs. Poly I:C/D-GalN injection did not markedly affect the number of DCs in the liver, and hepatic DCs responded weakly to poly I:C stimulation *in vitro* (data not shown). It has been demonstrated that the interaction between peripheral blood mononuclear cell-derived DCs and NK cells play an important role in the functional regulation of these cells in immunity.^{39, 40} After stimulation by poly I:C, human peripheral blood myeloid DCs could induce NK cells to produce IFN- γ via IL-12 and cell contact.⁴⁰ NK cells could stimulate or inhibit autologous DCs at different NK/DC ratios.³⁹ These studies may help to investigate the interaction between hepatic DCs and NK cells in the future.

It has been reported that D-GalN can augment lipopolysaccharide-induced elevation of serum TNF- α ,⁴¹ but the mechanisms remain unknown. In the present study, D-GalN, which is a known specific hepatotoxin,⁴² worked synergistically with poly I:C to induce the production of IFN- γ as well as TNF- α even though D-GalN itself did not elevate the levels of both cytokines as previously reported^{17, 41, 43} (Fig. 4 and data not shown). Meanwhile, there was only a slight increase in serum IFN- γ after poly I:C injection alone in our previous study¹² as well as in this study. To explore the roles of D-GalN in the

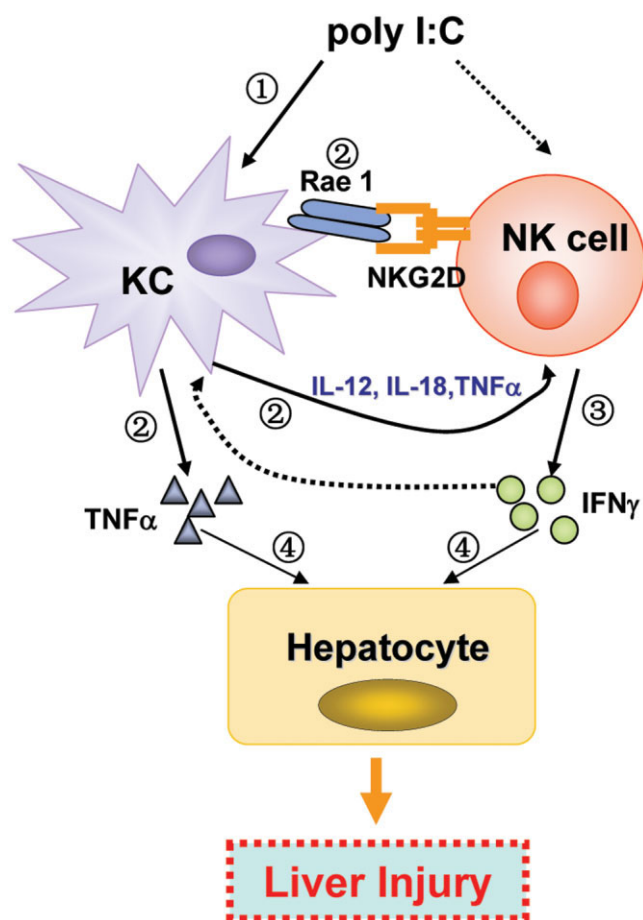


Fig. 8. Model of the cross talk between Kupffer cells and NK cells triggered by poly I:C in mediating liver injury. (1) As a response to stimulation from the TLR3 ligand, (2) Kupffer cells simultaneously express surface Rae1 and produce soluble IL-12, IL-18, and TNF- α . (3) Rae1 on Kupffer cells then directly activates NK cells by Rae1-NKG2D interaction, and Kupffer cell-derived IL-12, IL-18, and TNF- α help to indirectly stimulate NK cells to produce IFN- γ . (4) Finally, NK cell-derived IFN- γ and Kupffer cell-derived TNF- α synergistically induce hepatocyte damage. KC, Kupffer cell.

present hepatitis model, we first added the supernatant of D-GalN-stimulated hepatocytes into the coculture system of Kupffer cells and hepatic NK cells, and found that the supernatant could not augment poly I:C-induced cytokine production (data not shown). Thus, D-GalN-stimulated hepatocytes did not supply any soluble factors for cytokine production by NK/Kupffer cell interaction. Second, as reported previously,^{44,45} D-GalN increased the sensitivity of animals to the hepatotoxic effects of exotoxins. For example, D-GalN sensitized the liver to TNF- α -mediated damage in lipopolysaccharide/D-GalN model,²⁷ in accordance with which we also observed that D-GalN sensitized the liver to IFN- γ -mediated damage (Supporting Table 1).

Interestingly, blockade of NKG2D recognition could not completely abolish the production of IFN- γ both *in*

vivo and *in vitro* (Fig. 6), other factors may influence the interaction between these two cell types. Previous research has demonstrated that both human Kupffer cell-derived IL-12 and IL-18 are important in IFN- γ secretion by NK cells.²⁹ Our results also demonstrated that IL-12 and IL-18 were involved in the cross talk between murine Kupffer cells and NK cells, since injection of poly I:C/D-GalN elevated the levels of IL-18 and IL-12 in the serum and liver, and antibodies to IL-12 or IL-18 inhibited NK cell-derived IFN- γ production *in vitro* (Fig. 7).

In summary, the present work describes an NK cell-mediated murine model of fulminant hepatitis which is a valuable supplement to established Kupffer cell-mediated or NKT cell-mediated hepatitis (e.g., lipopolysaccharide/D-GalN model and ConA model). We also demonstrate that the reciprocal activation of hepatic NK cells and Kupffer cells initiates the liver injury in poly I:C/D-GalN-induced hepatitis (Fig. 8). To our knowledge, this is the first report depicting cellular cross talk between hepatic NK cells and Kupffer cells mediated by NKG2D-Rae1 recognition. Taken together, our findings may provide insight into innate immune recognition, which is pivotal in acute liver injury; further, our results may aid in investigating potential therapeutic strategies against innate immune-mediated injury in disease settings.

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