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Increased Killing of Liver NK Cells by Fas/Fas Ligand and NKG2D/NKG2D Ligand Contributes to Hepatocyte Necrosis in Virus-Induced Liver Failure

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The role of liver NK cells in virus-induced severe viral hepatitis and, subsequently, hepatic failure is not well defined. In this study, we investigated the role of liver NK cells in the development of hepatocyte necrosis in fulminant hepatic failure (FHF) and acute-on-chronic liver failure (ACLF) because of viral infection. A mouse model of FHF induced by murine hepatitis virus strain 3 (MHV-3) was used to study the role of liver NK cells. Samples from patients with hepatitis B virus-related ACLF (HBV-ACLF) were examined. After MHV-3 infection, the number of NK cells in livers of BALB/cJ mice increased markedly, peaked at 48 h postinfection, and remained at a high level until sacrifice. In peripheral blood, spleen, and bone marrow, this number decreased significantly. Expression of CD69, cytotoxic activity, and intracellular IFN- γ and TNF- α production by liver NK cells at 48 h postinfection were all significantly upregulated. Depletion of NK cells 24 h post-MHV-3 infection increased the mice survival from 0 of 18 (0%) to 4 of 18 (22.2%). Highly activated liver NK cells were cytotoxic to MHV-3-infected hepatocytes and this effect was markedly inhibited by anti-Fas ligand (FasL) plus anti-NKG2D mAbs. Furthermore, the accumulation of hepatic NK cells and increased expression of FasL and natural cytotoxicity receptors (NKp30 and NKp46) on the peripheral NK cells from patients with HBV-ACLF were correlated with disease progression. These results indicate NK cells play a pivotal role in the pathogenesis of FHF and HBV-ACLF, in which process Fas/FasL and NKG2D/NKG2D ligand pathway contribute to the liver NK cell-mediated hepatocyte injury. *The Journal of Immunology*, 2010, 184: 466–475.

It is a challenge to treat patients with severe chronic hepatitis B (CHB) who undergo acute deterioration during the chronic phase and subsequently die of acute-on-chronic liver failure (ACLF). Fulminant hepatic failure (FHF) and ACLF because of hepatitis B virus (HBV) infection are the most common severe diseases requiring immediate hospitalization in China and many other Asian countries (1–5). The immunological mechanisms of virus-induced hepatic failure are still not fully defined. The role of immune cells, especially HBV-Ag-specific CTL, is crucial in understanding HBV-related ACLF (HBV-ACLF) (2, 6, 7), whereas the roles of innate immune cells, especially NK cells, remain obscure. NK cells are the first cellular responders after viral in-

fection and they provide a first line of defense against viral infection (8). NK cells not only kill virus-infected cells directly but also produce a large amount of cytokines with direct antiviral activity such as IFN- γ , TNF- α , and immunoregulating cytokines (such as IL-3, GM-CSF, and M-CSF) (9). Recently, NK cells were identified as adaptive immune response regulators by cross-talking with dendritic cells and T cells (10, 11). In addition, compared with the peripheral lymphatic system, NK cells are abundant in the liver and account for around one-third of intrahepatic lymphocytes (12, 13), implying a special role of this cell type in liver biology.

There is increasing evidence implicating liver NK cells in liver damage. Liver NK cells kill freshly isolated hepatocytes by a TRAIL/TRAIL receptor pathway in polyinosinic-polycytidylic acid-induced mouse liver injury (14) and induce severe liver injury through NKG2D/NKG2D ligand (NKG2DL) recognition after low-dose ConA stimulation in HBV transgenic mice (15). These findings can help to understand the role of liver NK cells in the pathogenesis of FHF, but the liver injuries in these models are all induced by chemical agents, and viral replication is absent. To explore the role of liver NK cells in the mechanism of virus-induced liver failure, a mouse model of FHF induced by murine hepatitis virus strain 3 (MHV-3) was established and patients with HBV-ACLF were also investigated. In the current study, MHV-3 induced activation and accumulation of liver NK cells with increased effector activity (i.e., enhanced release of IFN- γ and TNF- α and killing of MHV-3-infected hepatocytes through the Fas/Fas ligand [FasL] and NKG2D/NKG2DL pathways). Furthermore, the accumulation of hepatic NK cells and the upregulation of natural cytotoxicity receptors (NCRs) (NKp30 and NKp46) on peripheral NK cells in patients with HBV-ACLF were correlated with disease progression (as evaluated by total bilirubin level, prothrombinase time [PT], and prothrombinase activity [PTA]).

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Abbreviations used in this paper: ACLF, acute-on-chronic liver failure; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BM, bone marrow; CHB, chronic hepatitis B; FasL, Fas ligand; FHF, fulminant hepatic failure; HBeAg, hepatitis B e Ag; HBV-ACLF, hepatitis B virus-related ACLF; HCV, hepatitis C virus; MHV-3, mouse hepatitis virus strain 3; MNC, mononuclear cell; NCR, natural cytotoxicity receptor; NKG2DL, NKG2D ligand; PT, prothrombinase time; PTA, prothrombinase activity.

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Materials and Methods

All animal studies were carried out according to the guidelines of the Chinese Council on Animal Care and approved by the Tongji Hospital of Tongji Medical School Committees on Animal Experimentation. Informed consent was obtained from human study participants, and the research protocol was reviewed and approved by the institutional review board of Tongji Hospital, Wuhan, People's Republic of China.

Animal model

Six- to 8-wk-old female BALB/cJ mice were purchased from the Laboratory Animal Center in Wuhan University (Wuhan, China), and all mice were maintained under specific pathogen-free and controlled conditions at the Institute of Infectious Disease of Tongji Hospital (Wuhan, China). MHV-3 was obtained from the American Type Culture Collection, and plaques purified on monolayers of DBT cells were titered on L2 cells using a standard plaque assay. MHV-3 was reconstituted in sterile PBS at a concentration of 500 PFU/ml. Mice were injected i.p. with MHV-3 (100 PFU per mouse) in a total volume of 200 μ l.

Studies in patients with HBV

Blood samples and needle biopsy of liver tissues were obtained from patients with HBV-ACLF and mild CHB at the Department of Infectious Disease, Tongji Hospital (Wuhan, China). Biochemical, histological, and clinical features were used to define patients with mild CHB and HBV-ACLF. ACLF was diagnosed according to the consensus recommendations of the Asian Pacific Association for the study of the liver about ACLF (3). Individuals with concurrent hepatitis C virus (HCV), hepatitis G virus, HIV infections, and autoimmune liver diseases were excluded. The clinical characteristics of all patients in this study are shown in Table I for whom the liver biopsy were performed and Table II for whom the blood samples were collected.

Cells isolation and quantitation

Mouse livers were weighed and dissected, pressed through a 200-gauge stainless steel mesh, suspended in RPMI 1640 medium containing 5% FBS, centrifuged at 30g for 3 min. The suspension was transferred and centrifuged at 500g for 10 min. The pellet was resuspended in 40% Percoll (GE Healthcare, Uppsala, Sweden) solution in RPMI 1640 medium, layered onto 70% Percoll solution, and centrifuged for 20 min at 800g. The mononuclear cells (MNCs) were collected from the interphase. The MNCs in blood, spleen, and bone marrow (BM) were isolated by Nycoprep 1.077A lymphocytes separation medium (Axis-Shield, Oslo, Norway). The cells were washed and resuspended with PBS containing 0.1% BSA and 0.01% sodium azide (FACS buffer). The percentage of NK cells in different tissues were analyzed by FACS. The isolated cells from blood, liver, spleen, and BM were suspended in 500 μ l, 500 μ l, 5 ml, and 5 ml of FACS buffer, respectively, and then the number of cells in 100 μ l was determined by FACS that was multiplied by 5, 5, 50, or 50 to assume the total cell number of different tissues. The absolute number of NK cells was subsequently determined by the percentage of NK cells and the total cell number.

Flow cytometric analysis

For phenotyping mouse NK cell surface markers, MNCs from the liver, spleen, BM, and blood were stained with the following mAbs: FITC-conjugated anti-

Table II. Characteristics of the patients who had blood samples taken

Characteristic	Mild CHB	HBV-ACLF
Number of patients	15	14
Age (y) ^a	23.0 (20.0–28.0)	36.5 (32.3–43.8)
Male gender (n %)	10 (67.7%)	14 (100%)
HB surface Ag ⁺ (%)	100	100
HBeAg ⁺ (%)	100	64.3
Anti-HB core Ag ⁺ (%)	100	92.9
HBV DNA log ₁₀ (copies/ml) ^a	7.8 (7.3–8.2)	4.3 (3.8–5.7)
Serum ALT (IU/l) ^a	178.0 (107.5–236.5)	72.5 (54.0–114.0)
Serum total bilirubin (μ mol/l) ^a	14.5 (12.6–17.7)	289.7 (250.6–313.8)
PT ^a	11.8 (11.5–12.2)	27.0 (19.5–33.0)
PTA ^a	100.0 (96.0–111.0)	25.5 (22.3–36.8)

^aMedian (interquartile range).

CD45 (clone 30-F11), PE-conjugated anti-pan-NK cells (clone DX5), PE-Cy5.5-conjugated anti-CD3 (clone 500A2), FITC-conjugated anti-DX5, PE-conjugated anti-CD69 (clone [1H]2F3), PE-conjugated anti-CD95 ligand (FasL) (clone MFL3), PE-conjugated anti-TRAIL (clone N2B2), PE-conjugated anti-NKG2D (clone CX5), PE-conjugated Armenian hamster IgG isotype control, and PE-conjugated rat IgG2a isotype control, which were obtained from eBioscience (San Diego, CA). Goat anti-mouse NKp46/NCR1 Ab, APC-conjugated donkey anti-goat Ab and goat IgG control were obtained from R&D Systems (Minneapolis, MN). The expression of NCRs (NKp30 and NKp46) and FasL on human peripheral NK cell (CD3⁺CD16⁺CD56⁺ and CD3⁺CD16⁺CD56⁺) was analyzed by the following mAbs: FITC-conjugated anti-CD3 (clone HIT3A), APC-conjugated anti-CD56 (clone B159), APC-Cy7-conjugated anti-CD16 (clone 3G8), PE-conjugated anti-NKp30 (clone p30-15), PE-conjugated anti-NKp46 (clone 9E2/NKp46), and PE-conjugated mouse IgG1 isotype control (clone MOPC-21), which were obtained from BD Bioscience (San Jose, CA). PE-conjugated anti-FasL (clone NOK-1) was obtained from Caltag Laboratories (Burlingame, CA). Stained cells were analyzed by the BD Calibur or BD FACSARIA flow cytometer (BD Bioscience). Lymphocytes were firstly gated according to forward scatter and side scatter; then the NK cells (CD3⁺CD16⁺CD56⁺ and CD3⁺CD16⁺CD56⁺) were gated and the percentages of NK cells expressing NKp46⁺, NKp30⁺, or FasL⁺ were determined individually.

In vivo depletion of NK cells

NK cells were depleted at 24 h post-MHV-3 infection by the i.v. injection of 50 μ g of anti-ASGM-1 Ab (Wako Pure Chemical, Osaka, Japan). Non-depleted (control) mice were treated instead with normal rabbit IgG (Wako Pure Chemical) and sterile PBS. The depletion was >90% as determined by FACS. The survival of mice subsequently were observed.

Purification of murine hepatic NK cells

Murine hepatic NK (CD3⁺DX5⁺) cells were purified by magnetic cell sorting. In brief, CD3⁺DX5⁺ T cells were first deleted by positive sorting with CD5 magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and liver NK (CD3⁺DX5⁺) cells were then positively sorted by DX5 (anti-CD49b) magnetic microbeads (Miltenyi Biotec). The sorted cells were >95% pure. The viability was >95% by the trypan blue exclusion test.

Isolation and culture of hepatocytes

Hepatocytes were isolated from the liver of MHV-3-infected or noninfected BALB/cJ mice by a two-step hepatic portal vein perfusion technique. In brief, after the induction of anesthesia with pentobarbital sodium (400 mg/kg i.p.), the peritoneal cavity was opened, and the liver was perfused in situ via the portal vein for 5 min at 37°C with calcium-free HEPES buffer and for 10 min with HEPES containing 50 mg/100 ml collagenase D and 70 mg/100 ml CaCl₂ buffer. The perfusion rate was set at 5 ml/min for both solutions. The viability of hepatocytes was >90% by the Trypan blue exclusion test. Isolated hepatocytes were cultured in the hepatocyte growth medium: PRIM 1640 medium, supplemented with 10% FBS and 100 mg/l insulin-transferrin-selenium (Life Technologies, Rockville, MD), 100 U/ml penicillin/streptomycin, 20 mg/l hepatocyte growth-promoting factors, and 5 mg/l dexamethasone.

Table I. Characteristics of the patients who had a liver biopsy

Characteristic	Mild CHB	HBV-ACLF
Number of patients	10	20
Age (y) ^a	23.0 (20.0–28.0)	36.5(32.3–43.8)
Male gender (n %)	10 (67.7%)	14 (100%)
HB surface Ag ⁺ (%)	100	100
HBeAg ⁺ (%)	100	64.3
Anti-HB core Ag ⁺ (%)	100	92.9
HBV DNA log ₁₀ (copies/ml) ^a	7.8 (7.3–8.2)	4.3 (3.8–5.7)
Serum ALT (IU/l) ^a	178.0 (107.5–236.5)	72.5 (54.0–114.0)
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PT ^a	11.8 (11.5–12.2)	27.0 (19.5–33.0)
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^aMedian (interquartile range).

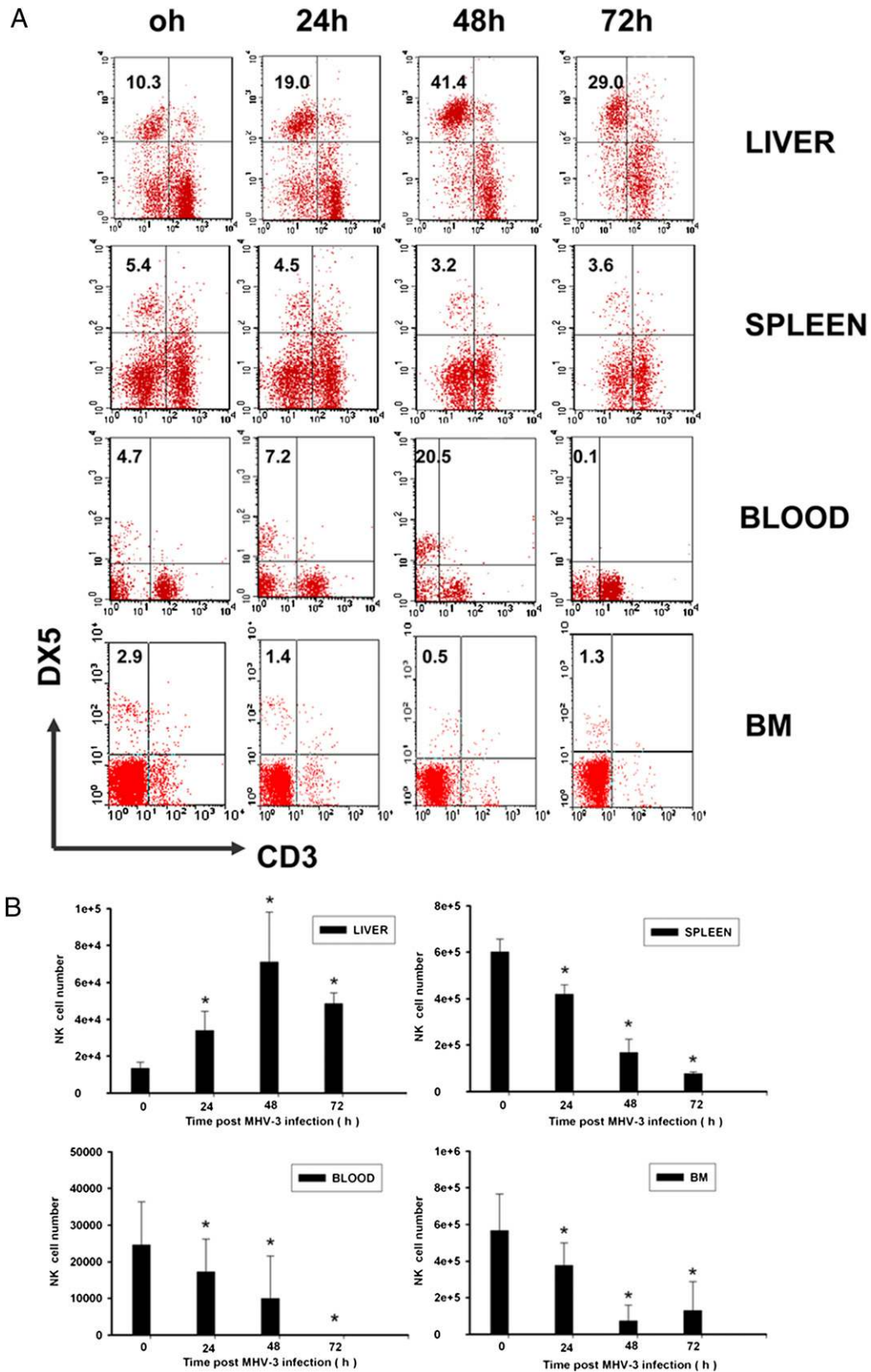


FIGURE 1. Hepatic recruitment of NK cells post-MHV-3 infection in BALB/cJ mice. MNCs from the liver, spleen, blood, and BM at 0, 24, 48, and 72 h post-MHV-3 infection were prepared and stained with FITC-conjugated anti-CD45, PE-conjugated anti-pan-NK cells (DX5), and PE-Cy5.5-conjugated anti-CD3 mAbs. Cells were gated according to forward scatter and side scatter. Then CD45⁺ cells were gated, and NK (CD3⁻DX5⁺) cells were defined. The percentage and total number of isolated NK cells were determined by FACS. **A**, A representative FACS analysis of different tissues-derived NK cells. The results presented represent one of five independent experiments. **B**, Time course study of the total number of different tissues-derived NK cells post-MHV-3 infection. The results presented are the mean \pm SD. For each time point, five mice were analyzed. * $p < 0.05$ as comparison with uninfected groups.

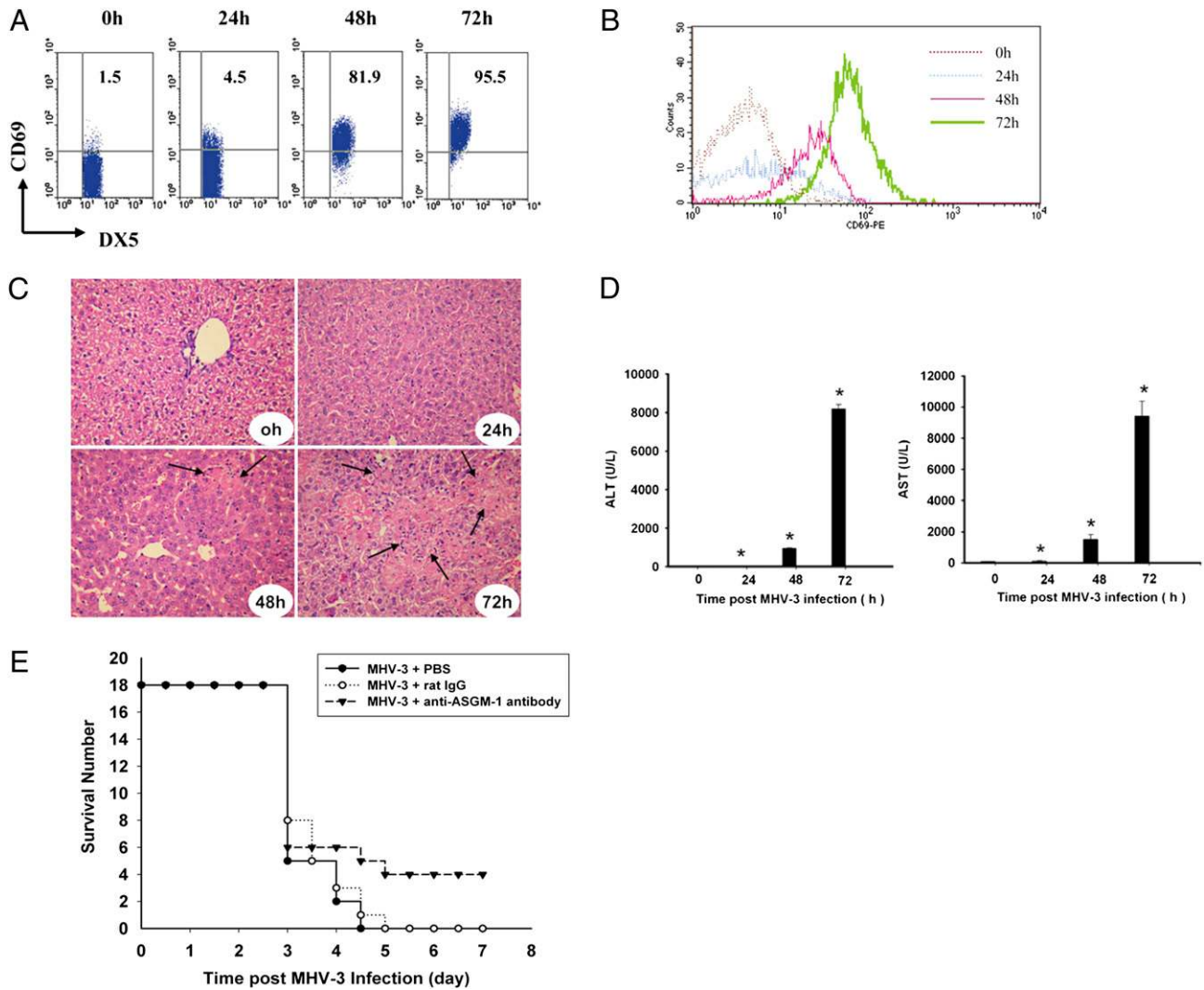


FIGURE 2. MHV-3 infection leads to remarkably enhanced activation of NK cells in liver in correlation with liver injury in BALB/cJ mice. *A*, A representative FACS analysis of the dynamic expression of CD69 on liver NK cells (CD3⁺ DX5⁺) post-MHV-3 infection. *B*, Time course study of liver NK cells activation in the form of histogram post-MHV-3 infection. The results in (*A*) and (*B*) are representative for three independent experiments. *C*, Histological examination of liver tissue at 0, 24, 48, and 72 h post-MHV-3 infection in BALB/cJ mice (H&E staining, original magnification $\times 200$). Small and discrete foci of necrosis with sparse polymorphonuclear leukocyte infiltrates were observed at 24 h post-MHV-3 infection. From 48 to 72 h, the area of these lesions enlarged and became confluent necroses. Arrows represent areas of hepatocyte necrosis. *D*, Time course study of serum ALT and AST levels post-MHV-3 infection in BALB/cJ mice. Results are shown as means \pm SD from five mice in each group. * $p < 0.05$ as comparison with uninfected groups. *E*, Survival rates after depletion of NK cells. To deplete NK cells, anti-mouse asialo GM1 serum (anti-AsGM1; 50 μ g, i.v.) was injected at 24 h after MHV-3 infection and the survival rate of mice was observed.

NK cell cytotoxicity assay against YAC-1 cells

Cytotoxicity of liver NK cells against a NK-sensitive cell line YAC-1 was measured by the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) according to manufacturer's instructions.

NK cell cytotoxicity assay against MHV-3-infected and noninfected hepatocytes

Freshly isolated hepatocytes from BALB/cJ mice that were uninfected or infected with MHV-3 for 48 h were used as target cells. As effector cells, liver NK cells were isolated and purified from BALB/cJ mice at 48 h postinfection. After the hepatocytes (10^4 cells/well) had been cultured with liver NK cells at a series ratio of E:T for 4 h at 37°C in a 5% CO₂ incubator, alanine aminotransferase (ALT) activity in each supernatant was assayed by standard enzymatic methods. Percent cytotoxicity was calculated as (experimental ALT – spontaneous ALT) \times 100/(total ALT – spontaneous ALT). In some experiments, the assay was performed in the presence of 10 μ g/ml of anti-FasL (clone MFL3), 20 μ g/ml anti-NKG2D blocking mAb (clone CX5) or isotype control from eBioscience.

Intracellular cytokine staining

Liver lymphocytes from BALB/cJ mice that were uninfected or infected with MHV-3 48 h were stimulated by PMA/ionomycin/monensin for 4 h. After blocking with anti-CD16/32 mAb (clone 2.4G2), cells were incubated with FITC-conjugated anti-pan-NK (clone DX5) and PE-Cy5.5-conjugated anti-CD3 mAbs (clone 500A2) at 4°C for 30 min, washed twice with PBS, fixed, permeabilized, and stained with PE-conjugated anti-IFN- γ (clone XMG1.2), APC-conjugated anti-TNF- α (clone MP6-XT22), and PE-conjugated anti-perforin mAbs (clone eBioOMAK-D) from eBioscience.

Immunohistochemistry staining

Expression of Fas/FasL on liver tissue. Paraffin-embedded livers obtained from either BALB/cJ mice that were uninfected or infected with MHV-3 for 48 h or patients with mild CHB or HBV-ACLF were sectioned (4 μ m), and the sections were treated with 0.03% H₂O₂/NaNO₃ to block endogenous peroxidase, treated with 10% normal goat serum (30 min) to block non-specific staining, incubated with primary Abs or negative control with rabbit serum (4°C overnight in a humidity chamber), washed in PBS,

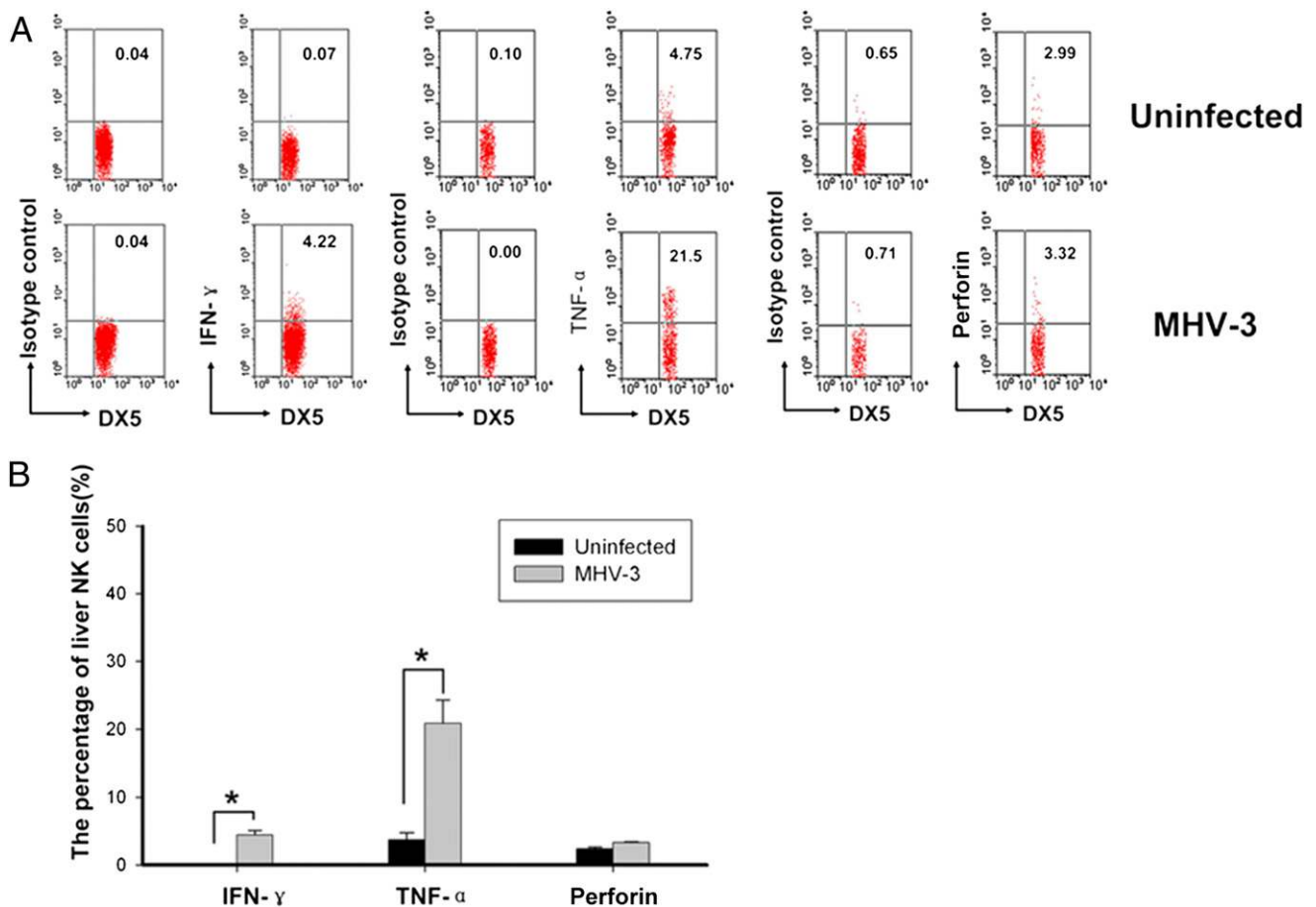


FIGURE 3. Enhanced production of IFN- γ and TNF- α , but not perforin from liver NK cells post-MHV-3 infection. Liver lymphocytes from BALB/cJ mice (uninfected or infected with MHV-3, 48 h) were prepared and examined by FACS using PE-conjugated anti-DX5, PE-Cy5.5-conjugated anti-CD3, PE-conjugated anti-IFN- γ , APC-conjugated anti-TNF- α , and PE-conjugated antiperforin. *A*, A representative frequency analysis of IFN- γ , TNF- α , and perforin-secreting NK cells by FACS assay. *B*, The percentage of liver NK cells secreting IFN- γ , TNF- α , and perforin at 48 h post-MHV-3 infection. The results presented are the mean \pm SD of three independent experiments. * $p < 0.05$ as comparison with uninfected groups.

incubated with peroxidase-coupled goat anti-rabbit Ab (30 min), and viewed after staining with 3,3,2-diaminobenzidine solution.

Dual immunohistochemical staining of CD3 and CD57. Needle biopsy liver tissues were obtained from 20 patients with HBV-ACLF, 10 patients with mild CHB, 3 patients with autoimmune hepatitis (AIH), and 3 health controls. Patient characteristics are noted in Table I. Sections prepared from formalin-fixed, paraffin-embedded liver samples were stained with mAbs specific for CD3 (clone PS1) and CD57 (clone NK-1) that were from the Zymed Laboratories (San Francisco, CA) and then secondary Abs were labeled with alkaline phosphatase or HRP, respectively, using the polymer double staining kit (Zymed Laboratories).

Statistical analysis

Statistical analysis of NK cells number, ALT level, and expression of Nkp30, Nkp46, and FasL was performed by Student *t* test or one-way ANOVA post hoc test ANOVA. $p < 0.05$ is considered significantly different. For the dual immunohistochemical staining, Image-Pro Plus 6.0 was adopted to quantitatively calculate the percentage of liver NK cells.

Results

Hepatic accumulation of NK cells in MHV-3 infected BALB/cJ mice

MNCs from the liver, blood, spleen, and BM were stained with FITC-conjugated anti-CD45, PE-conjugated anti-pan-NK cells (DX5) and PE-Cy5.5-conjugated anti-CD3 mAbs. Cells were gated according to forward scatter and side scatter. Then CD45⁺ cells were gated, and NK (CD3⁺DX5⁺) cells were defined. The proportion and number of NK cells in the liver, blood, spleen, and BM were measured by FACS

analysis. As shown in Fig. 1A and 1B, the proportion and number of NK cells in the liver were markedly increased and peaked at 48 h postinfection compared with normal control (43.2% versus 10.02%, 7.11×10^4 versus 1.34×10^3), and remained high thereafter. In contrast, the proportion and number of NK cells in the spleen (4.32% versus 5.72%, 1.68×10^5 versus 6.01×10^5) and BM (0.74% versus 2.27%, 7.4×10^4 versus 5.68×10^5) were dramatically decreased. Although the proportion of peripheral NK cells increased significantly (18.01% versus 4.82%), the absolute number decreased markedly (9.94×10^3 versus 2.46×10^4). These results suggest that NK cells migrate to the liver from the blood, spleen, and BM post-MHV-3 infection.

Activation of NK cells in liver correlated with massive liver injury in infected BALB/cJ mice

To investigate whether the NK cells (recruited to the liver) were activated, expression of CD69 on liver NK cells was measured at 0, 24, 48, and 72 h postinfection. The proportion of liver NK cells expressing CD69 increased significantly ($5.9 \pm 1.7\%$ at 24 h; $80.3 \pm 6.3\%$ at 48 h; $95.5 \pm 3.1\%$ at 72 h) (Fig. 2A, 2B), suggesting a rapid and dramatic activation of these cells. Increased levels of serum ALT and aspartate aminotransferase (AST) (Fig. 2D) and severe hepatocyte injury (Fig. 2C) were observed at 48–72 h postinfection, which indicated a close association between activation of hepatic NK cells and liver dysfunction. Depletion of NK cells at 24 h post-MHV-3 infection led to survival of 4 of 18 (22.2%) mice compared with 18 of

18 (100%) mice that died of FHF in the controls (Fig. 2E), further supporting the important contribution of NK cells to liver injury.

IFN- γ and TNF- α but not perforin contribute to MHV-3-induced liver failure

Intracellular staining confirmed that the production of IFN- γ and TNF- α by liver NK cells increased at 48 h postinfection (Fig. 3A, 3B), suggesting an important involvement of IFN- γ and TNF- α in MHV-3-induced FHF. However, perforin expression by liver NK cells was unaffected by infection, implying the minimal contribution of perforin-mediated lysis to hepatocyte post-MHV-3 infection in BALB/cJ mice.

Enhanced cytotoxicity of liver NK cells against YAC-1 cells and MHV-3-infected hepatocytes

In our assay, liver lymphocytes from mice that were uninfected or infected for 48 h were used as effectors and YAC-1 cells were used as targets. The cytotoxicity of liver NK cells was dramatically enhanced at 48 h postinfection when the E:T ratios were 12.5:1 and 25:1 (Fig. 4A).

To assess cytotoxicity against MHV-3-infected and uninfected hepatocytes in vitro, a hepatic NK cell cytotoxicity assay was performed with spleen NK cells as control. The effectors (NK cells purified by magnetic cell sorting of liver and spleen lymphocytes from mice 48 h postinfection) and targets (hepatocytes from infected 48 h postinfection and uninfected mice) were used at a ratio of 12.5:1. As shown in Fig. 4B, the cytotoxicity of liver NK cells directed against infected hepatocytes was significantly greater than that directed against uninfected hepatocytes, and spleen NK cells displayed low cytotoxicity.

Fas/FasL and NKG2D/NKG2DL pathways triggered by liver NK cells contribute to the killing of infected hepatocytes

To further detect the possible mechanism involved in the hepatocyte killing, the expression of NK cell surface markers were analyzed by flow cytometry. As shown in Fig. 5A and 5B, the expression of the FasL on liver NK cells dramatically increased, whereas the expression of NKp46, NKG2D, and TRAIL was not upregulated 48 h postinfection. But notably, the expression of two NK cell NCRs (NKp46 and

NKG2D) remained at relative high level postinfection. Similar expression was also found in peripheral NK cells (Fig. 5C). Immunohistochemistry revealed an increase in Fas expression on hepatocytes postinfection (Fig. 5D). These results strongly suggest the possibility that a Fas/FasL pathway triggered by liver NK cells may contribute to hepatocyte necrosis in MHV-3-induced FHF. To further determine the mechanism of toxicity, two neutralizing Abs (anti-FasL and anti-NKG2D mAb) were applied in a hepatocyte cytotoxicity assay. Liver NK cells 48 h postinfection were used as effectors, and uninfected or infected hepatocytes 48 h postinfection were used as targets. Treatment with anti-FasL mAb inhibited cytotoxicity directed to hepatocytes. Unexpectedly, treatment with anti-NKG2D mAb also inhibited cytotoxicity to some extent postinfection even though expression of NKG2D was not increased (Fig. 5E). Taken together, these data demonstrate that both Fas/FasL and NKG2D/NKG2DL pathways triggered by liver NK cells contribute to the killing of infected hepatocytes.

Increased frequency of liver CD3⁻CD57⁺ NK cells, enhanced hepatic FasL and expression of NCRs (NKp30 and NKp46) on the peripheral NK cells in patients with HBV-ACLF

To investigate the role of liver NK cells in HBV-ACLF, immunohistochemistry was used to analyze the frequency of NK cells (CD3⁻CD57⁺) in livers of 20 patients with HBV-ACLF (Fig. 6, D–H), 10 patients with mild CHB (Fig. 6C), three patients with AIH (Fig. 6I), and three healthy controls (Fig. 6J). NK cells (CD3⁻CD57⁺) increased dramatically in the livers of patients with HBV-ACLF compared with patients with mild CHB. In addition, expression of NK cells NCRs (NKp30 and NKp46) on the peripheral NK cells was also upregulated in patients with HBV-ACLF (Fig. 7B–D). These data collectively suggested a pivotal involvement of NK cells in the pathogenesis of human severe viral hepatitis. In similarity to the mice with FHF, the hepatic FasL expression was also enhanced significantly in HBV-ACLF patients (Fig. 7A).

Discussion

The most common cause of FHF and ACLF are viruses and drug-induced hepatotoxicity. HBV infection is the leading cause of

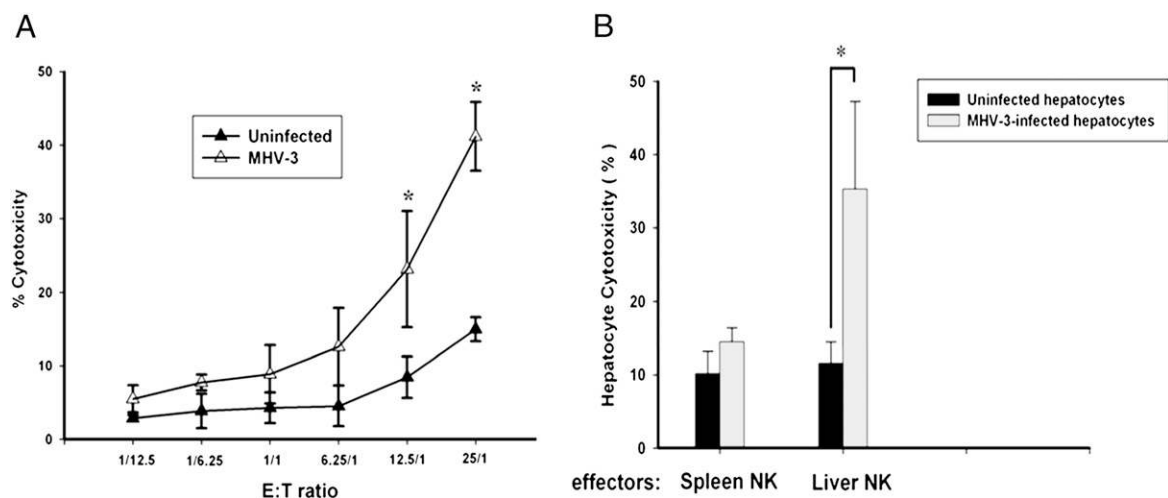


FIGURE 4. Liver NK cells had remarkably enhanced cytotoxicity against YAC-1 cells and MHV-3-infected hepatocyte. *A*, Cytotoxicity of liver NK cells from MHV-3 infected BALB/cJ mice against YAC-1 cells. Liver lymphocytes from BALB/cJ mice (48 h post-MHV-3 infection, $n = 5$) were isolated and used as effectors. YAC-1 cells were used as target cells. The cytotoxicity assay was conducted at the indicated E:T ratios. The results presented are the mean \pm SD of three independent experiments performed in triplicate. $*p < 0.05$ as comparison with uninfected groups. *B*, Cytotoxicity of liver NK cells against MHV-3-infected hepatocytes. Liver and spleen NK cells purified by magnetic cell sorting from isolated lymphocytes in the livers and spleens of mice with MHV-3 infection at 48 h were used as effectors. Hepatocytes were isolated from uninfected and infected BALB/cJ mice and used as target cells. A hepatocyte toxicity assay was performed at an E:T ratio of 12.5:1. The cytotoxicity was evaluated by a 4-h ALT release assay. The results presented are the mean \pm SD of three independent experiments performed in triplicate. $*p < 0.05$ as comparisons with uninfected hepatocytes groups.

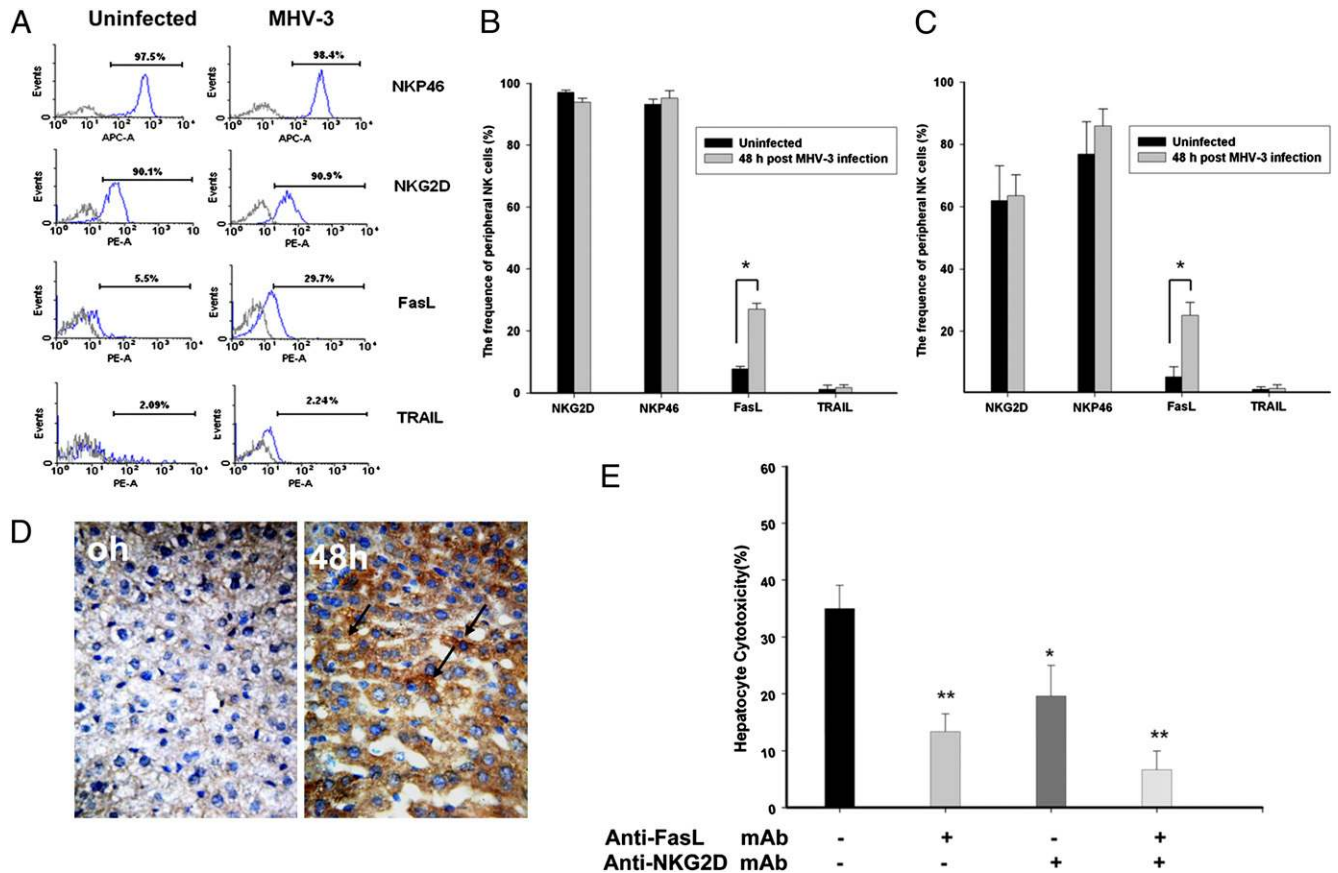


FIGURE 5. Fas/FasL and NKG2D/NKG2DL pathways triggered by liver NK cells contribute to the killing of infected hepatocytes post-MHV-3 infection. **A**, A representative FACS analysis of the expression of NKP46, NKG2D, FasL, and TRAIL on the liver NK cells post-MHV-3 infection. **B**, A statistic analyses of the percentage of NKP46⁺, NKG2D⁺, FasL⁺, and TRAIL⁺ liver NK cells post-MHV-3 infection. **C**, A statistic analyses of the percentage of NKP46⁺, NKG2D⁺, FasL⁺, and TRAIL⁺ peripheral NK cells post-MHV-3 infection. The results presented are the mean \pm SD of three independent experiments. **D**, Expression of Fas on hepatocytes from BALB/cJ mice with MHV-3 infection at 48 h was analyzed by immunohistochemical staining (original magnification $\times 400$). Arrows denote the positive cells for Fas expression. **E**, Blockage of Fas/FasL and NKG2D/NKG2DL inhibited the toxicity of liver NK cells to MHV-3 infected hepatocytes. NK cells purified by magnetic cell sorting (purity $>95\%$) from livers of BALB/cJ mice ($n = 20$) with MHV-3 infection at 48 h was used as effectors and MHV-3-infected hepatocytes was used as targets. Cytotoxicity assays were performed at an E:T ratio of 12.5:1 in the presence or absence of anti-FasL mAb, anti-NKG2D mAb, and isotype. The results presented are the mean \pm SD of three independent experiments performed in triplicate. * $p < 0.05$; ** $p < 0.01$ as comparison with none-blocking isotype control.

severe viral hepatitis in developing countries (1–5). The host immune response to virus plays a critical role in the pathogenesis of HBV infection (16, 17), whereas the HBV Ag-specific CTL is an critical determinant of viral clearance (18, 19) and hepatocyte damage in FHF (2, 6). However, the role of the innate immune system of the liver, especially liver NK cells in severe viral hepatitis is not well understood. Recent reports have shown that liver NK cells may contribute to recurrent inflammation by TRAIL-mediated death of hepatocytes in patients with hepatitis B e Ag (HBeAg)-positive CHB viral infection (20). To understand the contribution of liver NK cells to the development of hepatocyte necrosis and FHF, we counted NK cells and found that the number in the liver increased markedly, peaked at 48 h post-MHV-3 infection, and stayed at a high level, whereas the number in the blood, spleen, and BM decreased significantly. Previous studies have demonstrated that acute infection with MHV-3 in susceptible mice is associated with profound cell depletion in the spleen, BM, and thymus. Depletion was attributed to a productive viral infection of thymic stromal cells, pre-B, and B cells in the BM (21). However, whether NK cells or T cells in the spleen and BM also undergo virus-induced apoptosis post-MHV-3 infection is unclear. To ascertain the cause of NK cells depletion in the spleen and BM, postinfection BM- and spleen-NK cell apoptosis was measured by

the annexin assay. No evidence for increased NK cell death was observed (date not shown), which differed from in vitro results (22). We found that MHV-3 infection recruits a larger number of NK cells from the spleen and BM into the liver. Postinfection, we found markedly increased expression of CD69 on liver NK cells, significantly enhanced target cell killing to YAC-1, and increased production from the liver NK cells of IFN- γ and TNF- α [potent inhibitors of viral replication, promoters of fgl2/fibroleukin leading to apoptosis, and factors in the pathogenesis of murine and human fulminant hepatitis (23–26)]. These findings suggest that highly activated liver NK cells and increased levels of cytokines act synergistically to induce BALB/cJ mice FHF post-MHV-3 infection. Previous studies have shown that with intracellular staining, the expression of perforin in human NK cells (CD3⁻CD16⁺CD56⁺ and CD3⁻CD16⁻CD56⁺) from healthy individual peripheral blood was as high as $>30\%$ (27). However, in the normal mice of different strains, perforin expression in NK cells (CD3⁻DX5⁺ and CD3⁻NK1.1⁺) was as low as $<5\%$ (28). Forty-eight h after stimulation by IL-12, IL-18, or IFN- α in vitro, the perforin expression was 6–15% and as high as $>40\%$ by IL-2 or IL-15. Thus, different cytokines exert various ability to stimulate perforin production by NK cells (28). These reflect the variety of perforin expression from NK cells in different murine hepatitis

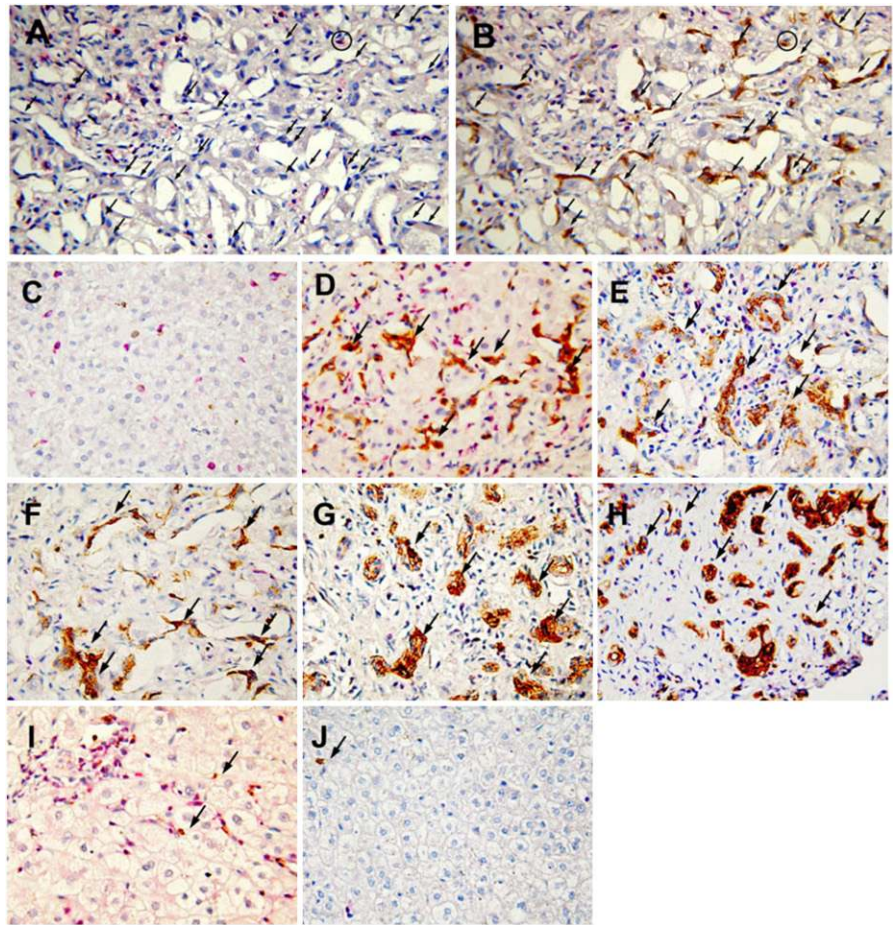
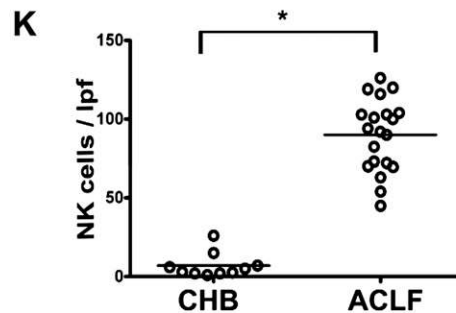


FIGURE 6. Increased frequency of NK cells ($CD3^+CD57^+$) in the livers of patients with HBV-ACLF. Needle biopsy liver tissues were obtained from informed patients (20 patients with HBV-ACLF, 10 patients with mild CHB, and 1 patient with AIH). One healthy liver tissue section from a donor was also used as control. Single staining for CD3 (A) (original magnification $\times 200$) and dual staining of CD3 and CD57 (B) (original magnification $\times 200$) are represented in the serial sections of liver sample in a patient with HBV-ACLF. Arrows denote NK cells only positive for CD57 (brown substrate). The open circle shows a NK T cell, which is simultaneously stained with red substrate (CD3) and brown substrate (CD57). Detection of NK cells in the liver from patients with HBV-ACLF (D–H, original magnification $\times 200$), mild CHB (C, original magnification $\times 200$), AIH (I, original magnification $\times 200$), and healthy control (J, original magnification $\times 200$) were performed by dual immunohistochemical staining of CD3 and CD57. Quantization of intrahepatic NK cells demonstrated a significant increase in patients with HBV-ACLF (K). $*p < 0.05$ as compared with patients with mild CHB.



models (in vivo and in vitro). In this study, we first explored the perforin expression of NK cells in a fulminant hepatitis model induced by MHV-3 in BALB/cJ mice. The 3.32% perforin expression in NK cells in the current study suggested the minimal contribution of perforin in NK cell mediated hepatocyte injury in MHV-3-induced FHF.

The next supportive finding was recruitment of NK cells to the liver and hepatic activation closely correlated with the severity of hepatocyte injury at 48–72 h postinfection as displayed by increased levels of serum ALT and AST, and pathological changes. To further dissect the importance of NK cells in liver injury, a depletion of NK cells was performed in BALB/cJ mice under MHV-3 infection. A previous study showed significantly higher virus titers in liver and more extensive hepatitis in MHV infection when depleting NK cells before MHV-3 infection (29). Similar results were also observed in our study (data not shown). However, NK cell depletion at 24 h post-MHV-3 infection resulted in an increased rate of mice survival. These data indicate the hypothesis that normal NK cells are important in the defense against

MHV-3 infection, whereas excessively activated NK cells may be fatal (i.e., aggravate hepatocyte injury). In agreement with this hypothesis, we found that liver NK cells from BALB/cJ mice 48 h postinfection were more cytotoxic against MHV-3-infected hepatocytes. This result provides direct evidence that liver NK cells participate in lymphocyte-mediated liver injury in MHV-3-induced FHF.

Furthermore, measurement of NKp46 and NKG2D, FasL, and TRAIL on liver NK cells from MHV-3-infected BALB/cJ mice showed that the expression of FasL (but not TRAIL, NKp46, and NKG2D) was highly upregulated at 48 h postinfection. In addition, Fas was increased on liver hepatocytes postinfection. These data suggest that the Fas/FasL pathway may contribute to NK cell-induced hepatocyte injury in MHV-3-induced FHF. However, because of the consistent high-level expression of NKG2D and NKp46 on liver NK cells, regardless of MHV-3 infection status, we cannot exclude the possibility that liver NK cells induce hepatocyte injury through specific recognition between NK receptors and their ligands.

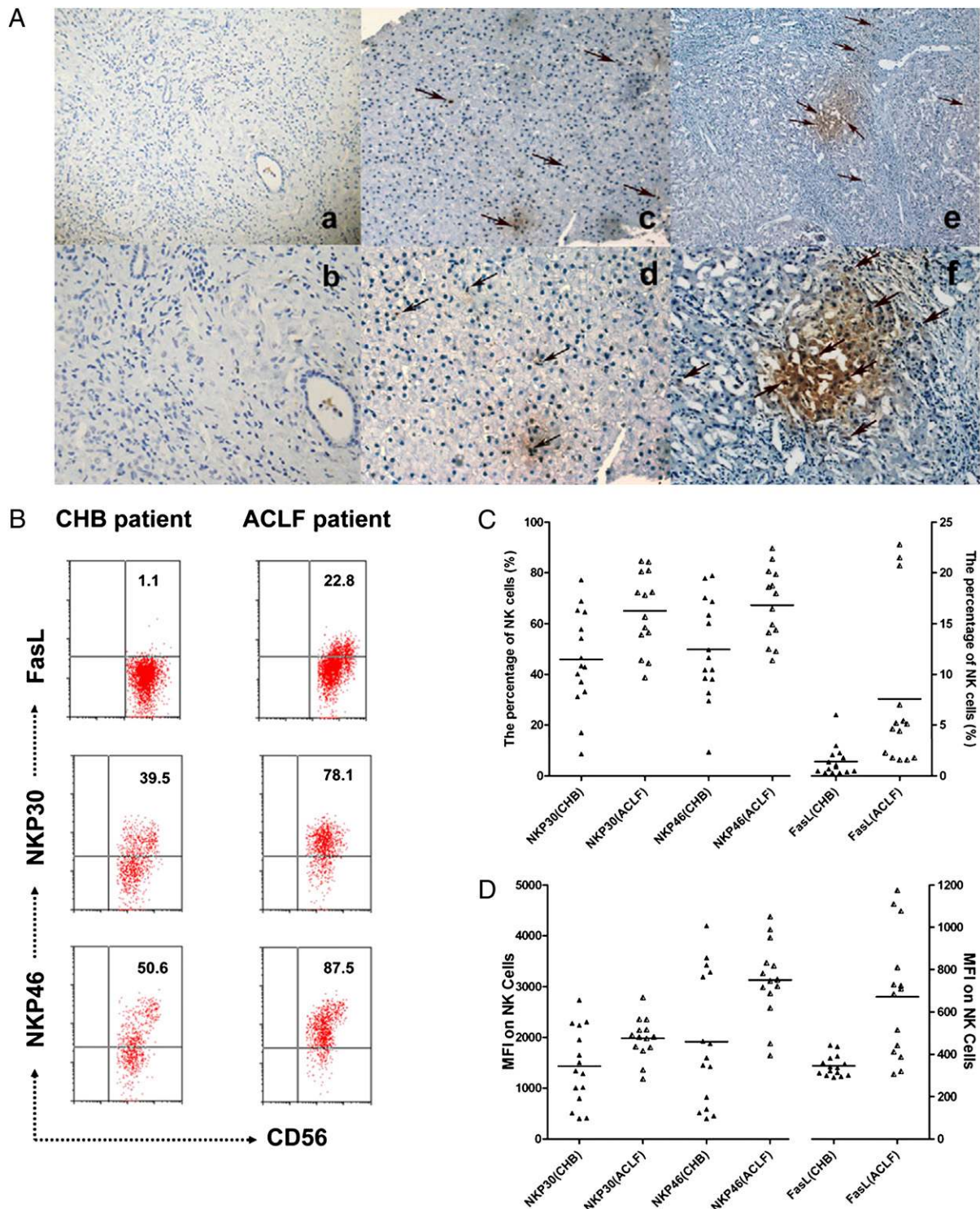


FIGURE 7. Upregulated expression of NCRs (NKp30 and NKp46) and FasL on the peripheral NK cells from patients with HBV infection. **A**, FasL expression in the livers of mild CHB patients and ACLF patients. Needle biopsy liver tissues were obtained from informed patients (Table I, 20 patients with HBV-ACLF and 10 patients with mild CHB). Panels *a* (original magnification $\times 200$) and *b* (original magnification $\times 400$) presented experimental negative controls with rabbit serum as first Ab; panels *c* (original magnification $\times 200$) and *d* (original magnification $\times 400$) presented the FasL expression in liver of mild CHB patients; panels *e* (original magnification $\times 200$) and *f* (original magnification $\times 400$) presented the FasL expression in liver of ACLF patients. Arrows pointed the FasL expression in brown staining. **B**, A representative FACS analysis of the expression of NCRs (NKp30 and NKp46) and FasL on the peripheral NK cells from patients with HBV-ACLF and mild CHB. Patient characteristics are noted in Table II. PBMC from patients with HBV-ACLF and mild CHB were prepared and stained with FITC-conjugated anti-CD3, APC-Cy7-conjugated anti-CD16, APC-conjugated anti-CD56, PE-conjugated anti-NKp30, anti-NKp46, and anti-FasL mAbs. Lymphocytes were first gated according to forward scatter and side scatter; then the NK cells ($CD3^-CD16^+CD56^+$ and $CD3^-CD16^-CD56^+$) were gated and the percentages of NK cells expressing NKp46 $^+$, NKp30 $^+$, or FasL $^+$ were determined individually. **C**, A statistic analyze of the percentages of NKp46 $^+$, NKp30 $^+$, or FasL $^+$ peripheral NK cells from patients with HBV-ACLF ($n = 14$) and mild CHB ($n = 15$). **D**, A statistic analyze of the mean fluorescence intensity of NKp46 $^+$, NKp30 $^+$, or FasL $^+$ peripheral NK cells from patients in (C). Results are the mean \pm SD. * $p < 0.05$ as comparison with mild CHB group.

Recent studies have highlighted important roles of NK cells and their activating receptors in HCV or HIV infection (30, 31). Significant downregulated expression of the NK cells activating receptors (NKG2D, NKp30, and NKp46) and markedly impaired NK cytolytic function were found in patients with HIV infection (30). Nattermann et al. (31) also reported a significantly reduced expression of NKp46 and NKp30 on NK cells in patients with HCV infection. They also found that patients who cleared HCV under antiviral therapy showed normal expression of NKp44, NKp30 (31). In contrast, De Maria et al. (32) reported that NK cells from HCV-infected patients had selective increased expression of NKp30 and NKp46. The controversy on the expression of NCRs on NK cells of patients with HCV infection may derive from the complexity of the disease, such as differences in virus genotyping, patients selection and genetic background. The above data provided evidence for the existence of an association between the abnormal expression of NK cells activating receptors and the impaired innate host response in HIV and HCV patients. In animal studies, NKG2D/NKG2DL pathway triggered by NK T cells has been shown to play a crucial role in inducing acute hepatitis in the HBV-transgenic mouse model (33). Our results, however, showed that the NKG2D/NKG2DL pathway triggered by NK cells partially contribute to the hepatocyte injury. Moreover, interestingly, both Fas/FasL and NKG2D/NKG2DL pathways were found to contribute to liver NK cell-mediated hepatocyte toxicity in MHV-3-induced FHF.

Emerging evidence indicates that NK cells participate in the pathogenesis of fulminant hepatitis B. An increased number of CD16⁺ NK cells has been reported in patients with fulminant hepatitis (34). In addition, NK cells may also participate in the development of HBV-related acute liver failure in HIV-positive patients with severe immunodeficiency (35). In our study, patients with HBV-ACLF had more CD3⁺CD57⁺ NK cells and enhanced expression of FasL in liver and significantly increased expression of NK cell NCRs (NKp30 and NKp46) peripherally, compared with patients with mild CHB. These results provide new insights into the role of NK cells in the pathogenesis of HBV-ACLF.

Disclosures

The authors have no financial conflicts of interests.

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