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γδ T Cells Contribute to the Outcome of Murine Fulminant Viral Hepatitis via Effector Cytokines TNF-α and IFN-γ*

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Summary: The mechanisms involved in virus-induced severe hepatitis have not been fully elucidated. In this study, we investigated the role of gamma delta T cell receptors (γδ) T cells in the pathogenesis of fulminant viral hepatitis (FVH) induced by murine hepatitis virus strain 3 (MHV-3). The model of FVH was established by intraperitoneal injection of MHV-3 into Balb/cJ mice. The survival days of mice, and the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were examined. The proportions of γδ T cells in blood, spleen and liver, and cytokines secreted by hepatic γδ T cells were analyzed by flow cytometry. The function of hepatic γδ T cells was examined by cytotoxicity assay. Balb/cJ mice died in 3 to 6 days post MHV-3 infection, with severe hepatic necrosis and significant augmentation of serum ALT and AST levels. The proportions of γδ T cells in blood, spleen and liver were significantly increased post MHV-3 infection, while those of the early activating molecule CD69-expressing γδ T cells and productions of cytokines tumor necrosis factor-alpha (TNF-α) and interferon-γ (IFN-γ) increased remarkably in the liver. These highly activated liver γδ T cells were cytotoxic to MHV-3-infected hepatocytes *in vitro* and this effect of liver γδ T cells against hepatocytes might involve the TNF-α and IFN-γ pathway. These results demonstrated that γδ T cells might contribute to the pathogenesis of MHV-3-induced FVH through the effector cytokines TNF-α and IFN-γ.
Key words: fulminant viral hepatitis; murine hepatitis virus strain 3; gamma delta T cell receptors T cells; tumor necrosis factor-α; interferon-γ

Hepatitis B virus (HBV) infection is one of the most common causes of fulminant viral hepatitis (FVH) especially in Asian countries[1–4]. It has been reported that the incidence of FVH is about 1% in patients with acute viral infection and the mortality rate approaches almost 80%[5]. Virus-induced liver damage generally results from a complicated and sustained interaction between virus replication and host defense. However, the underlying immunological mechanisms of FVH are not well defined. Many studies found that some cytotoxic cells, especially viral specific cytotoxic lymphocyte (CTL) and natural killer (NK) cells, play an important role in the pathogenesis of FVH[3, 6, 7], whereas the contribution of other immune cells, especially gamma delta T cell receptors (γδ) T cells in FVH is still elusive.

The γδ T cells are a diverse population of lymphocytes that have been found to play a critical role in immune regulation[8]. Early researches have shown that γδ T cells have many properties of cells of the innate immune system. γδ T cells appear early

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during immune responses and efficiently produce inflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-alpha (TNF-α)[9]. Previous studies have shown that γδ T cells play a major role in linking innate and adaptive immunity[10]. γδ T cells are the early infiltrating cells of inflammation caused by infection[11–14] and autoimmune diseases[15, 16].

To date, the role of hepatic γδ T cells in murine hepatitis virus type 3 (MHV-3)-induced FVH has not been investigated. In the present study we examined the characteristics and role of hepatic γδ T cells during MHV-3-induced FVH in Balb/cJ mouse. We found a predominant increase in proportions of γδ T cells post MHV-3 infection. The hepatic γδ T cells produced inflammatory cytokines including TNF-α and IFN-γ post MHV-3 infection. Then further study demonstrated that both TNF-α and IFN-γ, produced by hepatic γδ T cells contribute to the cytotoxic effect against infected hepatocytes *in vitro*. Thus, our data suggest that γδ T cells may contribute, through the effector cytokines TNF-α and IFN-γ, to the pathogenesis of MHV-3 -induced FVH.

1 MATERIALS AND METHODS

1.1 Mice

All animal experiments were carried out according to the guidelines of the Chinese Council on Animal Care and approved by Tongji Hospital Committees on Animal Experimentation (Tongji Medical College, Huazhong University of Science and Technology, China), and the research protocol was reviewed and approved by the hospital institutional review board.

Female BALB/cJ mice, 6–8 weeks old and weighing 20–22 g, were purchased from Hubei Laboratory Animal Center (Wuhan, China) and were maintained in the animal facility at the Tongji Hospital using approved protocols and procedures.

1.2 Virus and Animal Model

MHV-3 was obtained from the American Type Culture Collection (ATCC, USA), plaque purified on monolayers of delayed brain tumor (DBT) cells and titrated on L2 cells according to a standard plaque assay. MHV-3 was reconstituted in sterile PBS at a concentration of 500 PFU/mL. Mice were injected intraperitoneally (i.p.) with MHV-3 (100 PFU per mouse) in a total volume of 200 μL.

1.3 Antibodies and Reagents

γδ T cells were characterized by fluorescence-conjugated mAbs and respective isotype controls that specifically recognize the CD3 (clone 145-2C11), γδ-TCR (clone eBioGL3), CD25 (clone PC61.5), CD28 (clone 37.51), αβ-TCR (clone B20.1), γδ-TCR (clone eBioGL3), IL-2 (clone JES6-5H4), IL-4 (clone 11B11), IL-10 (clone JES5-16E3), IFN-γ (clone XMG1.2), TNF-α (clone MP6-XT22), FasL (clone MFL3), granzyme B (clone 16G6), and perforin (clone eBioOMAK-D) (all from eBioscience, USA). Data were acquired and analyzed on a FACS Aria flow cytometer (BD, USA).

1.4 Isolation of γδ T Cells

Murine hepatic γδ T cells were purified by magnetic cell sorting. In brief, mononuclear cells (MNCs) from the livers of normal animals or MHV-3 48-h-infection BALB/cJ mice were treated with γ/δ T Cell Isolation Kit (MiltenyiBiotec, Germany) to purify γδ T cells. Viability and purity of the cells were monitored by flow cytometry, and purity of CD3+ γδ-TCR+ T cells was >95%.

1.5 Isolation and Culture of Hepatocytes

Hepatocytes were isolated from MHV-3-infected and uninfected BALB/cJ mice by a two-step hepatic portal vein perfusion technique. Briefly, after the induction of anesthesia with pentobarbital sodium i.p. (400 mg/kg), the peritoneal cavity was exposed and the liver was perfused *in situ* via the portal vein first for 5 min at 37°C with calcium-free HEPES buffer and then for 10 min in HEPES buffer containing 50 mg/100 mL collagenase D and 70 mg/100 mL $CaCl_2$. The perfusion rate was set at 5 mL/min for both solutions. The viability of isolated hepatocytes was 90% by the Trypan blue exclusion test.

Isolated hepatocytes were cultured in the hepatocyte growth medium: PRIM 1640 medium (GIBCO, USA), supplemented with 10% FBS and 100 mg/L insulin-transferrin-selenium (Life Technologies, USA), 100 U/mL penicillin/streptomycin, 20 mg/L hepatocyte growth-promoting factors and 5 mg/L dexamethasone.

1.6 Preparation of Blood, Spleen and Liver Samples for Flow Cytometry

MNCs from blood, spleens and livers were collected from MHV-3-infected BALB/cJ mice at 0, 24, 48 and 72 h post-infection for further observation. Samples from each time point were assayed in triplicate. Four-color cytofluorometric analysis was performed on a BD FACS Aria Flow Cytometer. Approximately 10^6 cells/100 μL of the cell suspensions from processed spleens and livers were lysed in RBC-lysing buffer (eBioscience) and then specifically labeled with PerCP-cy5.5-anti-CD3, APC-conjugated anti-γδ-TCR (eBioscience). Blood samples were labeled with antibodies before lysing for 10 min. In addition, 100 μL of each cell type was used for labeling with homotype comparison antibodies to serve as controls.

1.7 Analysis of Cell Surface Markers on γδ T Cells

For phenotyping, hepatic γδ T cells surface markers were determined by flow cytometry analysis. Cell suspensions of processed livers from BALB/cJ mice at 0 h and 48 h post MHV-3 infection were stained with PerCP-cy5.5-anti-CD3, PE-anti-CD25, APC-anti-γδ-TCR, PE-anti-CD28, PE-anti-CD30, PE-

anti-CD44, PE-anti-CD69 monoclonal antibodies or isotype control antibody.

1.8 γδ T Cells Cytotoxicity Assay against MHV-3-infected Hepatocytes

Freshly isolated hepatocytes from BALB/cJ mice that were infected with MHV-3 for 48 h were used as target cells. As effector cells, liver γδ T cells were isolated and purified from BALB/cJ mice at 48 h post-infection. After the hepatocytes (10^4 cells/well) had been cultured with liver γδ T cells at a ratio of 10:1 for 4 h at 37°C in a 5% CO_2 incubator, γδ T cells cytotoxicity assay against MHV-3-infected hepatocytes was measured by CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, USA) according to manufacturer's instructions. In some experiments, the assay was performed in the presence of 10 μg/mL of anti-TNF-α (clone MP6-XT22), 10 μg/mL anti-IFN-γ blocking mAb (clone XMG1.2) or isotype control from Biolegend.

1.9 Intracellular Cytokine Staining

Spleen lymphocytes harvested from control and experimental mice at the 48 h time point were stimulated with PMA/ionomycin/monensin for 4 h. After blocking with anti-CD16/32 mAb (clone 2.4G2), cells were incubated with PerCP-cy5.5-anti-CD3, APC-conjugated anti-γδ-TCR (eBioscience) at 4°C for 30 min, washed twice with PBS, fixed, permeabilized, and then stained with PE-conjugated anti-IFN-γ, PE-conjugated anti-TNF-α (eBioscience).

1.10 Statistical Analysis

Quantitative data were expressed as $\bar{x}\pm s$. Statistical analysis was performed by the Student's *t*-test or one-way ANOVA post hoc test ANOVA. $P<0.05$ is considered significantly different.

2 RESULTS

2.1 Remarkable Increases of Frequencies of γδ T Cells in Blood, Spleen and Liver in MHV-3-infected BALB/cJ Mice

To establish and identify the model of murine FVH, mice were injected i.p. with MHV-3. MNCs from the liver, blood and spleen were stained with PerCP-cy5.5-anti-CD3 and APC-conjugated anti-γδ-TCR mAb. Cells were first gated according to forward scatter and side scatter. Then $CD3^+$ cells were gated, and then γδ T cells were defined (fig. 1A). The proportion of γδ T cells in the liver, blood and spleen were measured by FACS analysis. After MHV-3 infection the proportion of γδ T cells in the blood, spleen and liver were markedly increased and peaked at 48 h post infection compared with normal control from 0.66%±0.20%, 1.86%±0.46% and 4.23%±1.23% to 1.46%±0.25%, 3.76%±0.55% and 17.36%±1.55%, respectively, and remained high in liver (10.63%±3.61%) thereafter (fig. 1B).

2.2 Phenotype of Hepatic γδ T Cells

To further characterize the phenotype of the γδ T cells population, we analyzed the expression of CD25, CD28, CD30, CD44, and CD69 on the γδ T cells from livers of Balb/cJ mice at 0 h and 48 h post MHV-3 infection. γδ T cells were negative for CD25, CD28, CD30, but majority of hepatic γδ T cells expressed CD44 (90.37 ±2.15%) (fig. 2A). Since BALB/cJ mice are naive NK1.1 allelic negative mice[17], we used α-GalCer:mCD1d complex-specific mAb to determine if these γδ T cells express α-Galcer, which is detected in NKT cells in an Ag-specific fashion. The expression of α-Galcer on γδ T cells was negative, demonstrating these cells are different from NKT cells (fig. 2B). Taken together, the phenotype of hepatic γδ T cells is as follows: γδ-TCR+CD3+ CD4– CD8– CD25– CD28– CD30– CD44+.

Furthermore, the activation of the hepatic γδ T cells was determined by measuring the proportions of expression of CD69 at various time points post MHV-3 infection. The proportion of hepatic γδ T cells expressing CD69 increased significantly post MHV-3 infection, from 0.90%±0.07% at 0 h to 27.43%±5.58% at 48 h, peaking at 72 h (38.27%±0.98%) (fig. 2C and 2D). These results suggested that these cells have a rapid activation in liver after MHV-3 infection.

2.3 Significant Increases in Production of TNF-α and IFN-γ of Hepatic γδ T Cells in MHV-3-infected Mice

To further investigate the characteristics of hepatic γδ T cells, liver lymphocytes were isolated from normal and FVH mice at 24 h, 48 h and 72 h post MHV-3 infection, then intracellular cytokines in hepatic γδ T cells were assayed. The productions of TNF-α and IFN-γ by hepatic γδ T cells were increased significantly post MHV-3 infection and peaked at 72 h (14.33%±3.97%, and 13.96%±1.68%, respectively) (fig. 3A and 3B). The results suggested an important involvement of TNF-α and IFN-γ in MHV-3-induced FVH.

2.4 Enhanced Cytotoxicity of Hepatic γδ T Cells Against MHV-3-infected Hepatocytes Via Effector Cytokines TNF-α and IFN-γ

To assess cytotoxicity against MHV-3-infected hepatocytes *in vitro*, hepatic γδ T cells from BALB/cJ mice that were normal or infected with MHV-3 for 48 h purified by magnetic cell sorting of liver were used as effectors and MHV-3-infected hepatocytes at 48 h post-infection were used as targets. Then two neutralizing monoclonal antibodies (anti-TNF-α and anti-IFN-γ mAb) were applied in a hepatocyte cytotoxicity assay to further determine the possible mechanism involved in the γδ T cells-induced hepatocyte toxicity. The E:T ratios were 10:1. The cytotoxicity of hepatic γδ T cells was significantly enhanced at 48 h post-infection when the E:T ratios were 10:1 and 20:1 (fig. 4A). Blockage with TNF-α neutralizing monoclonal antibody inhibited

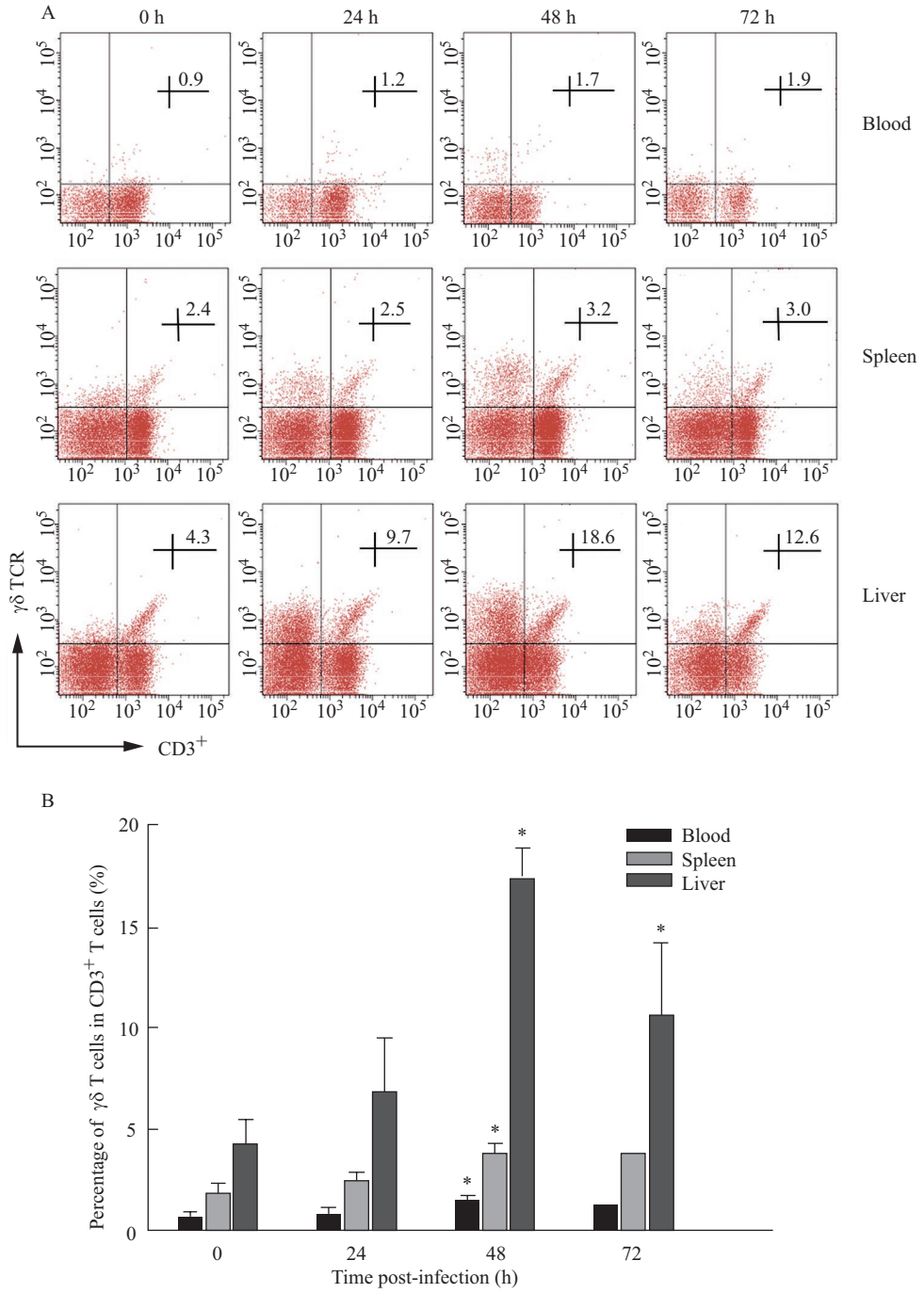

Fig. 1 Remarkable increases of frequencies of γδ T cells in blood, spleen and liver in MHV-3-infected BALB/cJ mice

Mononuclear cells from blood, spleens and livers were collected from MHV-3-infected BALB/cJ mice at 0, 24, 48 and 72 h post-infection and specifically labeled with PerCP-cy5.5-conjugated anti-CD3, APC-conjugated anti-γδ-TCR mAb. Cells were gated according to forward scatter and side scatter. Then $CD3^+$ cells were gated, and $CD3^+$γδ-TCR+ T cells were defined. The percentages of isolated γδ T cells were determined by FACS. A: a representative FACS analysis of different tissues-derived γδ T cells at 0, 24, 48 and 72 h post MHV-3 infection. The results presented represent one of three independent experiments; B time course study of the proportions of different tissues-derived γδ T cells post MHV-3 infection. The results presented are the $\bar{x}\pm s$. For each time point, five mice were analyzed. $^*P<0.05$ *vs*. uninfected groups. TCR: T cell receptors

cytotoxicity directed to hepatocytes, and blockage with IFN-γ neutralizing monoclonal antibody also inhibited cytotoxicity to some extent post MHV-3 infection (fig. 4B). Taken together, these data demonstrate that γδ T cells contribute to the killing of infected hepatocytes via effector cytokines TNF-α and IFN-γ.

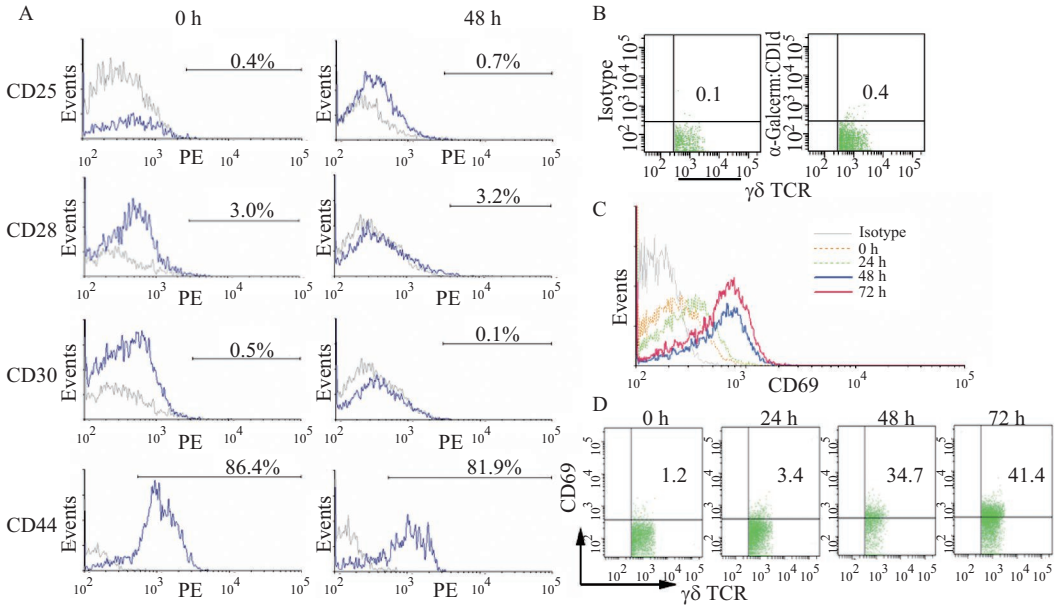

Fig. 2 Phenotype analyses of hepatic γδ T cells

Liver lymphocytes were stained with PerCP-cy5.5-anti-CD3, APC-anti-γδ-TCR, PE-anti-CD25, PE-anti-CD28, PE-anti-CD30, PE-anti-CD44, PE-anti-CD69 monoclonal antibodies or isotype control antibodies. A: a representative FACS analysis of expression of CD25, CD28, CD30, and CD44 on hepatic γδ T cells; B: a representative FACS analysis of the expression of PE-conjugated anti-α-GalCer: mCD1d complex-specific mAbs on hepatic γδ T cells; C and D: a representative FACS analysis of expression of CD69 on hepatic γδ T cells at 0, 24, 48 and 72 h post MHV-3 infection. For each time point, five mice were analyzed. TCR: T cell receptors

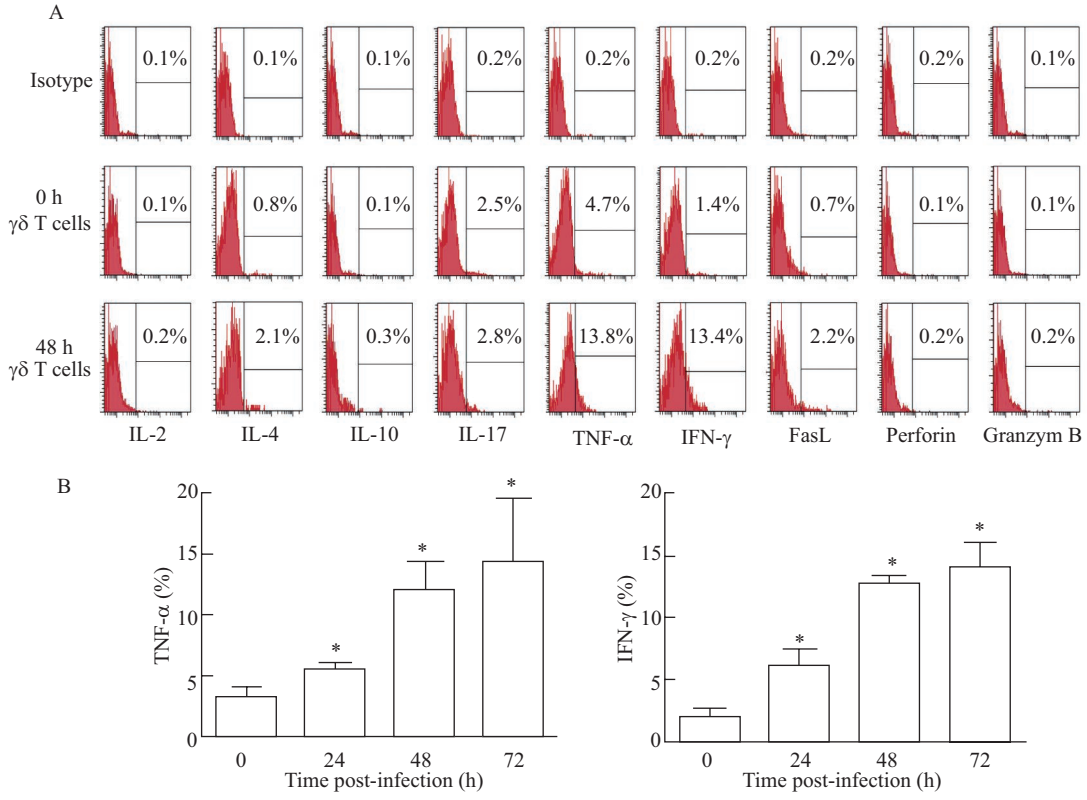

Fig. 3 Significant increases in production of TNF-α and IFN-γ of hepatic γδ T cells in MHV-3 infected mice

Enhanced production of TNF-α and IFN-γ from hepatic γδ T cells post MHV-3 infection. Liver lymphocytes from BALB/cJ mice (uninfected or infected with MHV-3 at 48 h) were collected and analyzed by FACS using PerCP-cy5.5-conjugated anti-CD3, APC-conjugated anti-γδ-TCR, PE-conjugated anti-TNF-α and APC-conjugated anti-IFN-γ mAbs. A: a representative frequency analysis of cytokines secreting hepatic γδ T cells by FACS assay; B: the percentage of liver γδ T cells secreting TNF-α and IFN-γ at 0, 24, 48 and 72 h post-MHV-3 infection. The results presented are $\bar{x}\pm s$ of three independent experiments. $^*P<0.05$ *vs*. uninfected groups

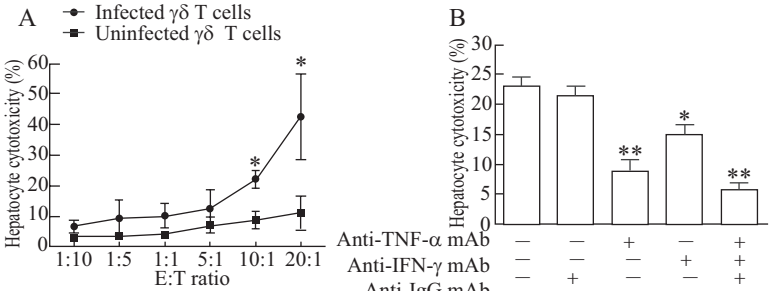

Fig. 4 Enhanced cytotoxicity of hepatic γδ T cells against MHV-3 infected hepatocytes through effector cytokines TNF-α and IFN-γ Hepatic γδ T cells from BALB/cJ mice (0 h and 48 h post MHV-3 infection, n=30) were purified by magnetic cell sorting (purity >95%) and used as effectors. Hepatocytes isolated from BALB/cJ mice (48 h post MHV-3 infection, n=2) were used as target cells. The cytotoxicity assay was conducted at the indicated E:T ratios. Then cytotoxicity assays were performed at an E:T ratio of 10:1 in the presence or absence of anti-TNF-α mAb, anti-IFN-γ mAb, and IgG mAb. A: cytotoxicity of hepatic γδ T cells against hepatocytes isolated from MHV-3 infected BALB/cJ mice; B: Blockage of TNF-α and IFN-γ by neutralizing monoclonal antibodies inhibited the toxicity of hepatic γδ T cells against MHV-3 infected hepatocytes. The results presented are the $\bar{x}\pm s$ of three independent experiments performed in triplicate. $^{*}P<0.05$, $^{**}P<0.01$ *vs*. uninfected groups

3 DISCUSSION

HBV infection is one of the leading causes of severe viral hepatitis[1–4]. The host immune response to HBV plays an important role in the pathogenesis of HBV infection[18, 19], thus uncovering the underlying mechanisms of FVH may help us to understand the immune system and is essential for the future success of cellular immunotherapy. Previous works have shown that some cytotoxic cells, especially the virus-specific CTL and NK cells, are critical determinants of viral clearance[20, 21] and hepatocyte injury in the pathogenesis of FVH[3, 6, 7]. However, the contribution of γδ T cells in FVH is still elusive.

The γδ T cells are a diverse population of lymphocytes that have been found to play a critical role in immune regulation[8]. Previous works have shown that γδ T cells invade inflamed organs[22] during the early phase of viral infection and the inflammation which is involved in autoimmune diseases of central nervous system (encephalomyelitis)[23] and intestines (colitis)[24]. Recently it has been reported that γδ T cells exert antiviral activity against several viruses[25]. Some works suggest that the livers of patients with chronic hepatitis C virus (HCV) infection contain increased numbers of γδ T cells and these γδ T cells have enhanced cytotoxic activity against different target cells, thus activation of circulating γδ T cells from HCV infected patients induces the inhibition of HCV replication[26–28].

In this present study, to investigate the contribution of liver γδ T cells to the pathogenesis of hepatocyte injury and FVH, we measured the numbers of γδ T cells in different organs post MHV-3 infection and found that the proportions of γδ T cells in blood, spleen and liver increased significantly, peaked at 48 h post-MHV-3 infection, The marked increases of γδ T cells were closely correlated with the severity of hepatocytes injury post-infection as displayed by increased levels of serum ALT and AST, and pathological liver damage, suggesting an important involvement of γδ T cells in MHV-3-induced FVH. The hepatic γδ T cells had a phenotype of γδ-TCR+ CD3+ CD4– CD8– CD25– CD28– CD30– CD44+, and we found that these hepatic γδ T cells were activated dramatically post MHV-3 infection.

Furthermore, measurement of inflammatory cytokines TNF-α and IFN-γ produced by hepatic γδ T cells from MHV-3-infected BALB/cJ mice showed that the expression levels of TNF-α and IFN-γ were highly upregulated post-infection. TNF-α, known as a common inflammatory cytokine, has been found to participate in lymphocyte-mediated cell death and inhibition of viral replication as well as inflammation[29]. It also has been reported that IFN-γ was able to induce the production of inflammatory chemokines, adhesive molecules and proapoptotic proteins which resulted in liver injury through stimulating the expression of IFN-γ receptor on hepatocytes and nonparenchymal cells[30, 31]. In our work we found that hepatic γδ T cells isolated from MHV-3 infected mice showed enhanced cytotoxic effect against infected hepatocytes, thus this result provides direct evidence that hepatic γδ T cells may participate in lymphocyte-mediated liver damage in MHV-3-induced FVH. To further explore the mechanism involved in the γδ T cells- induced hepatocyte toxicity, we used neutralizing monoclonal antibody of TNF-α and IFN-γ, and found that blocking either TNF-α or IFN-γ inhibited cytotoxicity against hepatocytes. Taken together, these data indicate the hypothesis that the TNF-α and IFN-γ pathways may contribute to γδ T cells-induced hepatocyte injury in MHV-3-induced FVH.

One recent study demonstrates that γδ T cell is an important source of innate IL-17, which provides critical contribution to host immune responses[32, 33], and our previous study found that IL-17 might contribute to

the pathogenesis of FVH[34]. The role of IL-17 produced by γδ T cells and the complex interaction of these cytokines in the pathogenesis of FVH needs further investigation. Furthermore, depletion of the γδ T cells from the circulation will help to better determine the related mechanisms. These cytokines may serve as potential targets for therapeutic intervention in FVH.

In summary, our present work demonstrates that MHV-3 infection can induce remarkably increased proportions and activation of γδ T cells in the liver, and these hepatic γδ T cells may contribute to host susceptibility to MHV-3-induced FVH through the effector cytokines TNF-α and IFN-γ pathways. This work provides new insights into the critical role of γδ T cells in MHV-3-induced murine FVH.

Conflict of Interest Statement

The authors declare no conflicts of interest.

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