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CD4–CD8–T cells contribute to the persistence of viral hepatitis by striking a delicate balance in immune modulation

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ABSTRACT

Viral hepatitis remains the most common cause of liver disease and a major public health problem. Here, we focus on the role of CD4 CD8 double negative T (DN T) cells involved in the mechanisms of viral persistence in hepatitis. C3H/HeJ mice infected with murine hepatitis virus strain 3 (MHV-3) were used to display chronic viral hepatitis. DN T cells dramatically increased in MHV-3 infected mice. Adoptive transfer of DN T cells from MHV-3 infected mice led to a significant increase in mice survival. The DN T cells with production of IFN- γ and IL-2 are able to kill virus-specific CD8⁺ T cells via the Fas/FasL dependent pathway. The delicate balance of multiple effects of DN T cells may lead to viral persistence in MHV-3 induced hepatitis. In short, our study identified DN T cells contributing to viral persistence in MHV-3 induced hepatitis in C3H/HeJ mice, which provides a rationale for modulating DN T cells for the management of viral hepatitis.

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1. Introduction

Viral hepatitis remains a major public health problem in the world with considerable morbidity and mortality. It is estimated that there are over 350 million carriers of hepatitis B virus (HBV) and 250 million carriers of hepatitis C virus (HCV) worldwide [1,2]. Persistent infection may progress to chronic liver disease, cirrhosis and primary liver cancer [3].

The viral proteins expressed in hepatocytes may influence the severity and progression of liver disease. However, extensive studies suggest that the mechanisms of liver injury in viral hepatitis are due to the host immune responses, but not to the direct cytopathic effects of viruses. The direct killing of infected cells by virus-specific CD8⁺ cytotoxic T lymphocytes (CTLs) is considered as the central mechanism resulting in both liver damage and virus control. In addition, CD4⁺ T helper response is also found to have a strong association with viral clearance in the acute phase of hepatitis [4].

Viral persistence during hepatitis infection may be the direct result of a weak antiviral immune response to the viral antigens,

Abbreviations: DN T cells, double negative T cells; Tregs, regulatory T cells; MHV-3, murine hepatitis virus strain 3; MCMV, murine cytomegalovirus; FasL, Fas ligand; Foxp3, Forkhead box P3; CTLs, cytotoxic T lymphocytes.

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with corresponding inability to eradicate virus within the infected cells. Accumulating evidence has indicated that regulatory T (Treg) cells play an important role in the suppression of virus specific immune responses [5–7]. Many subsets of Treg cells have been studied including CD4⁺CD25⁺ Tregs [8–13], CD4⁺DX5⁺ T cells [14], Ag-specific CD4⁺ or CD8⁺ T cells that secrete the immuno-regulatory cytokines IL-10 (Tr1 cells) or TGF- β (Th3 cells), TCR $\gamma\delta$ ⁺ T cells [15,16], and TCR $\alpha\beta$ ⁺ CD3⁺CD4[–]CD8[–] double-negative (DN) T cells [17–19].

Among Treg cells, CD4⁺CD25⁺ Tregs are the most extensively studied. A transcription factor Foxp3 is considered as the optimal marker of classic natural Treg [20]. In patients with HBV or HCV infection, CD4⁺CD25⁺ Foxp3⁺ Tregs have increased levels and impair the immune responses directed against hepatitis viruses, leading to persistent infections and chronic liver injury [21–23]. Lately, Shalev et al. reported that adoptive transfer of Tregs from fgl2 +/+ mice into fgl2 –/– mice resulted in increased mortality to MHV-3 infection, demonstrating the critical role for CD4⁺CD25⁺ Tregs in the pathogenesis of MHV-3 induced fulminant hepatitis [24].

DN T cells are a novel subset of Treg cells which is first identified by Zhang and colleagues. In mice, DN Treg cells could kill activated CD8⁺ T cells with the same TCR specificity, and infusion of in vitro-activated DN Treg cells led to significant prolongation of donor-specific skin and heart graft survival [17,25–27]. Recently, increasing attention has been focused on these novel Treg cells. Crispin et al. demonstrated that DN T cells produce IL-17/IFN- γ

and contribute to the pathogenesis of kidney damage in patients with SLE [28]. Another study showed that DN T splenic cells from young NOD mice could provide long-lasting protection against type 1 diabetes [29]. In SIV-induced CD4⁺ T cell depletion in sooty mangabeys which do not present immune dysfunction and clinical AIDS, DN T cells partially compensates for CD4⁺ T cell function in these animals [30]. In cutaneous leishmaniasis infection, TCR $\alpha\beta$ ⁺ DNT cells present an increased bias in their capacity to induce inflammatory immune responses, and TCR $\gamma\delta$ ⁺ DNT cells show a regulatory profile [31].

In the present study, we investigated the characteristics and contribution of splenic DN T cells in a MHV-3 induced chronic viral hepatitis murine model. And our study provides a rationale for modulating DN T cells for the management of viral hepatitis.

2. Materials and methods

All animal studies were carried out according to the guidelines of the Chinese Council on Animal Care, and were approved by the Tongji Hospital of Tongji Medical School Committees on Animal Experimentation (No.2009619).

2.1. Establishment of the chronic viral hepatitis murine model

MHV-3 was obtained from the American Type Culture Collection (ATCC), plaque-purified on a monolayer of DBT cells, and titered on L2 cells using a standard plaque assay. C3H/HeJ mice were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Science (Shanghai, ROC). MHV-3 (10 Pfu/200 μ L) was individually injected into the peritoneal cavity of C3H/HeJ mice as previously described [32]. Peripheral blood and livers were obtained on different time points after MHV-3 infection. C3H/HeJ mice receiving 200 μ L of 0.9% NaCl were used as controls. The survival, serum biochemistry parameters including ALT, AST, TP, ALB, and liver histology were observed. Virus titers were determined in the liver tissue of MHV-3 infected C3H/HeJ mice at various time points by standard plaque assay as described before [32].

2.2. Preparation of blood, spleen, and liver samples for flow cytometry

Blood, spleen, and liver were collected from MHV-3 infected C3H/HeJ mice on indicated days post infections. At each time point, triplicate samples were examined. C3H/HeJ mice treated with 200 μ L NaCl were used as control.

Three-color cytofluorometric analysis was performed on the FACS Aria Flow Cytometer (BD Biosciences, San Jose, CA, USA). In all experiments, approximately 10⁶ cells/100 μ L (from the single cell suspensions of processed PBMC, spleen and liver) were stained by FITC-anti-CD3, PE-anti-CD4 and PerCP-anti-CD8 (eBioscience, San Diego, CA, USA) monoclonal antibodies. Corresponding isotype antibodies were used as controls.

2.3. Adoptive transfer

Five days after MHV-3 infection, three groups (30 mice in each group) of mice were treated respectively with DN T cells, splenocytes or DN T-depleted splenocytes from MHV-3 infected mice via the tail vein. Another 30 C3H/HeJ mice treated with PBS were used as controls. Liver tissue samples of recipient mice from each group (3–5 mice in each group) were harvested and stained with H&E 12 days post the adoptive transfer.

The Knodell hepatitis activity index (HAI) was used to evaluate the severity of the necroinflammation. The Knodell HAI score consists of four separate scores, including periportal necrosis with or without bridging necrosis (0–10), intralobular degeneration and

focal necrosis (0–4), portal inflammation (0–4) and fibrosis (0–4) [33]. Virus titers in the liver tissues of recipient mice from each group were tested by standard plaque assay at indicated time points.

2.4. Analysis of cell surface markers on DN T cells

Expression of DN T cell surface markers were determined by flow cytometry analysis. Cell suspensions of processed spleens from C3H/HeJ mice on days 4, 10, 15, 20, and 30 post MHV-3 infection were stained with PEcy5.5-anti-CD3, FITC-anti-CD4, FITC-anti-CD8, APC-anti-CD25, APC-anti-TCR β , PE-anti-TCR $\gamma\delta$, PE-anti-CD28/PE-anti-CD30, PE-anti-CD44, PE-anti-CD95L, PE-anti-CD95 monoclonal antibodies or isotype control Ab (eBioscience, San Diego, CA, USA).

2.5. Cytokines analysis

Cells were harvested from the spleen of MHV-3 infected C3H/HeJ mice. Flow cytometric detection of intracellular cytokines was performed as previously described. The cells were stimulated with 25 μ M ionomycin and 10 ng/mL PMA in the presence of monensin (10 μ g/mL) for 4 h at 37 °C in 5% CO₂. Cells were washed and fixed in 1 mL 4% ice-cold paraformaldehyde for 20 min. Before the initial staining, 10⁷ cells/tube were washed with saponin/PBS buffer to permeabilize the plasma and intracellular membranes. PE-anti-IL-2, PE-anti-IL-4, PE-anti-IFN- γ , PE-anti-IL-10, PE-anti-TNF α , PE-Anti-perforin, PE-anti-Granzyme B or isotype-matched, irrelevant control Ab (eBioscience, San Diego, CA, USA) was respectively added to the suspension and incubated for 15 min. After two further washes in saponin/PBS buffer, cells were stained with PEcy5.5-anti-CD3, FITC-anti-CD4, FITC-anti-CD8 (eBioscience, San Diego, CA, USA) for 20 min. Then the cells were analyzed by flow cytometry.

2.6. Cell isolation

The spleens from C3H/HeJ mice were made into suspensions and incubated with magnetic bead sorting buffer. Non-T cells, i.e. B cells, NK cells, dendritic cells, and macrophages were magnetically labeled by a cocktail of biotin-conjugated antibodies and anti-biotin microbeads (Miltenyi Biotec, Cologne, Germany). Isolation of highly pure T cells was achieved by depletion of magnetically labeled cells.

Using the CD4⁺ T Cell Isolation kit (Miltenyi Biotec, Cologne, Germany), CD4⁺ T cells were then isolated from T cells obtained previously by depletion of non-CD4⁺ T cells (negative selection). CD8⁺ T cells were isolated from the non-CD4⁺ T cells using the CD8⁺ T Cell Isolation kit (Miltenyi Biotec, Cologne, Germany). The unlabeled non-CD4⁺, non-CD8⁺ T cells were DN T cells.

2.7. Cytotoxic assays

Cytotoxicity of DN T cells was measured using the CytoTox 96 Non-Radioactive Cytotoxicity assay kit (Promega, Madison, WI, USA) following the manufacturer's instruction. This cytotoxicity kit measures the lactate dehydrogenase (LDH) activity released into the culture medium by lysed cells. MHV-3 specific CD8⁺ T cells, normal CD8⁺ T cells, MHV-3 infected hepatocytes, and normal hepatocytes were used as target cells, DN T cells were used as effector T cells.

The experiment was repeated after FasL blockade with anti-mFasL antibodies (R&D Minneapolis, MN, USA) with a 5:1 ratio of DNT cells to CD8⁺ T cells.

Mice infected with the Smith strain of murine cytomegalovirus (MCMV) were taken as an unrelated control. CD8⁺ T cells were iso-

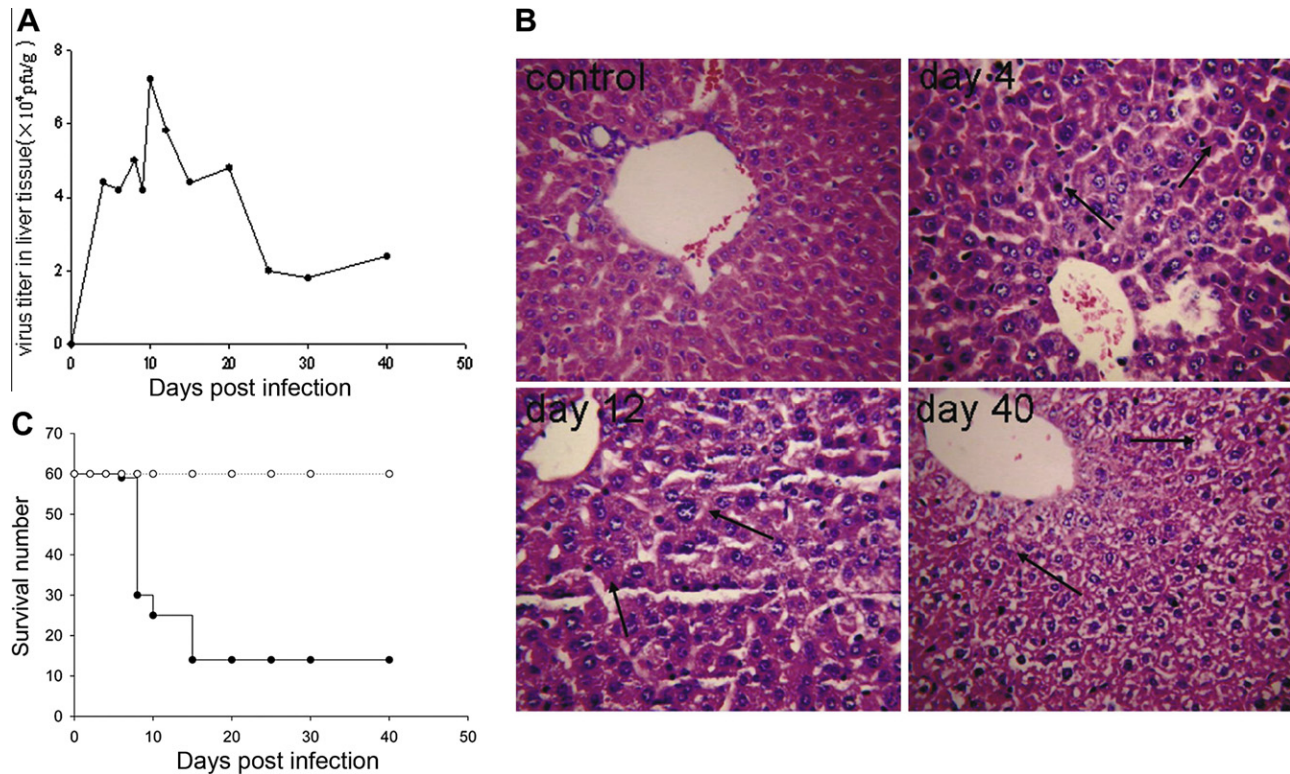


Fig. 1. MHV-3 infected C3H/HeJ mice displayed the characteristics of chronic hepatitis. (A) Virus titer in liver tissue in MHV-3 infected C3H/HeJ mice. The virus titer in livers showed significant increase since Day 2 post MHV-3 infection and last until 40 days of the observation time in C3H/HeJ mice, while no virus titer was detected in control mice. (B) Survival of C3H/HeJ mice post MHV-3 infection. Between day 6–15, approximately 76.7% of MHV-3 infected C3H/HeJ mice died [●], and the survivors gradually recovered after Day 15 post infection. All the control mice survived [○]. (C) Liver histopathology in MHV-3 infected C3H/HeJ mice (HE staining, 400×). Liver histopathology was manifested as hepatocyte swelling, hydropic degeneration, spotty necrosis and infiltration of T cells since 2 days post MHV-3 infection in C3H/HeJ mice. Mild to moderate even severe inflammation of liver were observed till day 12–15 days and the histopathology gradually recovered afterwards. No pathologic change was detected in control mice. The arrows in the figures show hepatocytes swelling, hydropic degeneration, spotty necrosis and infiltration of lymphocytes.

Table 1
MHV-3 infection led to liver dysfunction in C3H/HeJ mice.

Time point/Parameters	ALT (U/L)	AST (U/L)	TP (g/L)	ALB (g/L)
Control	24.67 ± 2.08	54.67 ± 2.52	52.33 ± 1.72	36.03 ± 0.25
Day4	25.00 ± 2.65	54.67 ± 4.51	53.77 ± 1.56	35.33 ± 1.21
Day6	28.67 ± 5.51	61.33 ± 8.50	54.20 ± 0.46	35.50 ± 0.20
Day8	24.33 ± 1.55	72.67 ± 11.15	49.63 ± 0.95*	34.50 ± 0.66
Day9	38.67 ± 1.53	109.33 ± 36.90	47.73 ± 1.66*	33.67 ± 2.32
Day10	530.33 ± 63.32*	954.33 ± 278.10*	43.43 ± 3.20*	28.40 ± 2.36*
Day12	1044.33 ± 508.58*	553.33 ± 113.74*	41.37 ± 1.60*	27.27 ± 4.44*
Day15	40.00 ± 22.61	105.00 ± 9.17	52.50 ± 2.40	36.47 ± 0.50
Day20	37.67 ± 13.42	97.00 ± 21.52	54.33 ± 2.08	35.37 ± 1.12
Day25	24.33 ± 3.51	93.33 ± 8.08	52.40 ± 2.70	35.77 ± 1.70
Day30	26.67 ± 3.06	91.33 ± 14.98	53.83 ± 1.39	35.57 ± 1.10
Day40	26.67 ± 5.51	65.67 ± 6.03	53.43 ± 2.41	35.87 ± 2.84

Serum on indicated days from MHV-3 infected and uninfected C3H/HeJ mice were collected and tested. Compared to uninfected mice, serum levels of ALT, AST in MHV-3 infected C3H/HeJ mice have increased and TP, ALB have decreased. The difference of all the four parameters between MHV-3 infected and uninfected mice was significant on day 10 and day 12 post infection. (Note: * $P < 0.05$ in comparison with the controls) Three independent experiments were performed with 3 mice used in each group.

lated from the spleens of C3H/HeJ mice post MCMV infection. The cytotoxic effect of DN T cells (from the spleens of MHV-3 infected mice) on CD8⁺ T cells (from the spleens of MCMV infected mice) was then examined.

2.8. Transwell experiment

Both DN T cells and CD8⁺ T cells were isolated from the spleen of MHV-3 infected C3H/HeJ mice. Transwell (Corning, Lowell, MA, USA) experiments were conducted in 24 wells in 0.8 mL of complete tissue culture medium. The semipermeable membrane

separating the upper and lower chambers allows diffusion of soluble materials but not cells. To measure the effect of non-contact cytotoxicity of DN T cells on CD8⁺ T cells, CD8⁺ T cells (10^5) were cultured in the lower chamber and DN T cells (5×10^5 or 2.5×10^5) in the upper chamber. CD8⁺ T cells were cultured in the lower chamber alone as control. After an incubation period of 24 h, CD8⁺ T cells were stained with FITC-anti-CD8, washed, and then stained with APC-anti-annexin V (Bender Med system, Vienna, Austria) and PI (Jingmei Biotech, Shanghai, China). At least 5,000 CD8⁺ T events were collected for annexin V/PI analysis. In this approach, the percent of annexin V⁻/PI⁻ events (viable cell

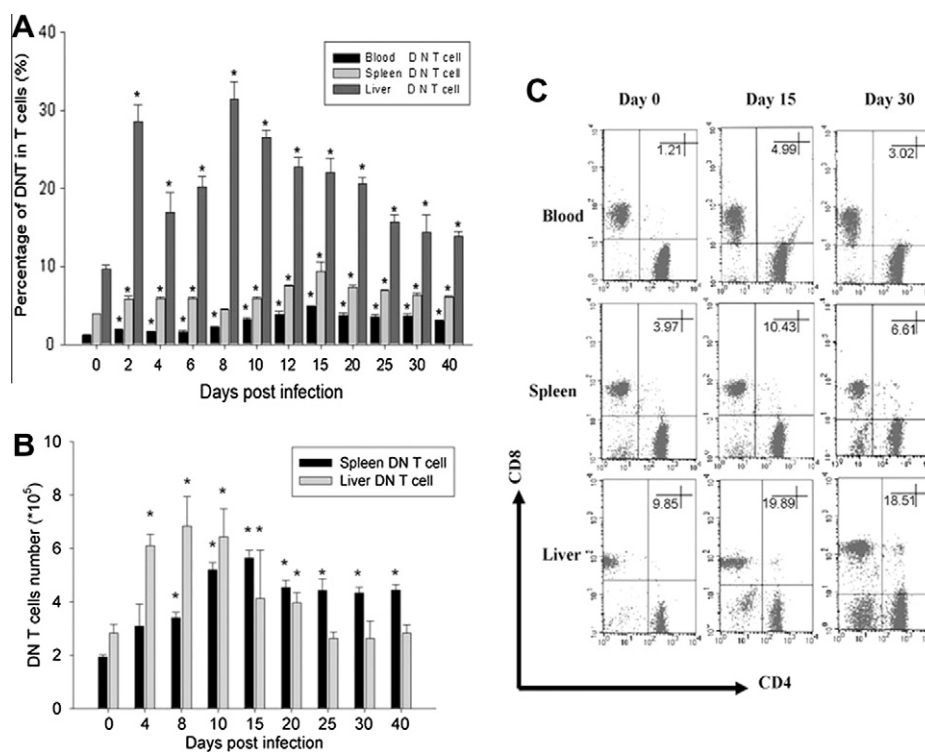


Fig. 2. The DN T cells significantly increased in C3H/HeJ mice with MHV-3 induced chronic viral hepatitis. (A) The proportions of DNT cells increased in total T lymphocytes of blood, spleen and liver in C3H/HeJ mice post MHV-3 infection, peaked between Day 12 and 15 and then decreased gradually. Data represent means \pm SEM from at least 6–9 mice in each group. * $P < 0.05$ versus day 0. (B) The absolute count of DNT cells in either spleen or liver also increased during the disease course post MHV-3 infection (C) Flow cytometry dot-plots displaying proportions of DNT cells in blood, spleen, liver on day 0, 15, 40 post MHV-3 infection in C3H/HeJ mice.

population) were used to correct for spontaneous apoptosis with the following formula:

% Cytotoxicity =

$$\frac{[\% \text{ control} - \text{viable cells}] - [\% \text{ of coincubated} - \text{viable cells}]}{[\% \text{ control} - \text{viable cells}]}$$

The result reflects the rate of cells undergoing apoptosis/death at the time of sampling.

2.9. Statistical analysis

All data are presented as mean \pm SEM. Comparisons of data were performed by using the Student's two-tailed *t*-test or one-way analysis of variance (ANOVA), followed by the Student-Newman Keuls test. Statistical package for the social science (SPSS) was used for data analysis. In all cases, significance was determined at $P < 0.05$.

3. Results

3.1. C3H/HeJ mice develop chronic hepatitis after MHV-3 infection

In order to investigate the role of DN T cells in antiviral responses, we developed a chronic hepatitis model by infecting C3H mice with MHV-3 as formerly reported. For the first time we extensively characterized the disease chronicity, liver function, and pathology of viral hepatitis in this model.

The infected C3H/HeJ mice began to have anorexia and acratia 4–6 days post infection. Between Day 6 and Day 15 post MHV-3 infection, approximately 76.7% mice died, and the survivors became persistently infected afterwards (Fig. 1A). The virus in the liver tissues of infected mice could be detected during the whole

course of observation. The virus replication peaked on day 12, and then decreased and maintained at a low level. (Fig. 1B) Various degrees of hydropic degeneration, hepatocellular necrosis and inflammatory infiltration were observed in the liver of C3H/HeJ mice after 4 days of infection. The most severe liver injury was observed between day 10 and day 12, with dramatically impaired liver function, including increased ALT and AST as well as decreased total protein and albumin (Table. 1). After that, the hepatocellular injury in the survived mice was alleviated, accompanied with less infiltration and improved liver function. But all the inflammatory changes could still be observed in the livers of survivors during the period of observation (Fig. 1C).

3.2. The DN T cells significantly increased in C3H/HeJ mice with MHV-3 induced chronic viral hepatitis

The proportion of DN T cell in the blood, liver, and spleen rose significantly after MHV-3 infection in C3H/HeJ mice (Fig. 2A, C), peaked between Day 12 and 15 and then decreased gradually. The absolute count of DN T cells in either liver or spleen presented a similar change as the proportion did (Fig. 2B).

3.3. Adoptive transfer of DN T cells from MHV-3 infected C3H/HeJ mice increased the survival rate and improved liver histology of recipient mice infected by the same virus strain but had little impact on the virus titer of liver tissue

Fig. 3A showed that no significant difference in virus titer of liver tissue was observed among DN T cells group, splenocytes group, DNT-depleted splenocytes group and PBS control group.

After adoptive transfer of either DN T cells or splenocytes from MHV-3 infected C3H/HeJ mice, the survival rate of the recipient

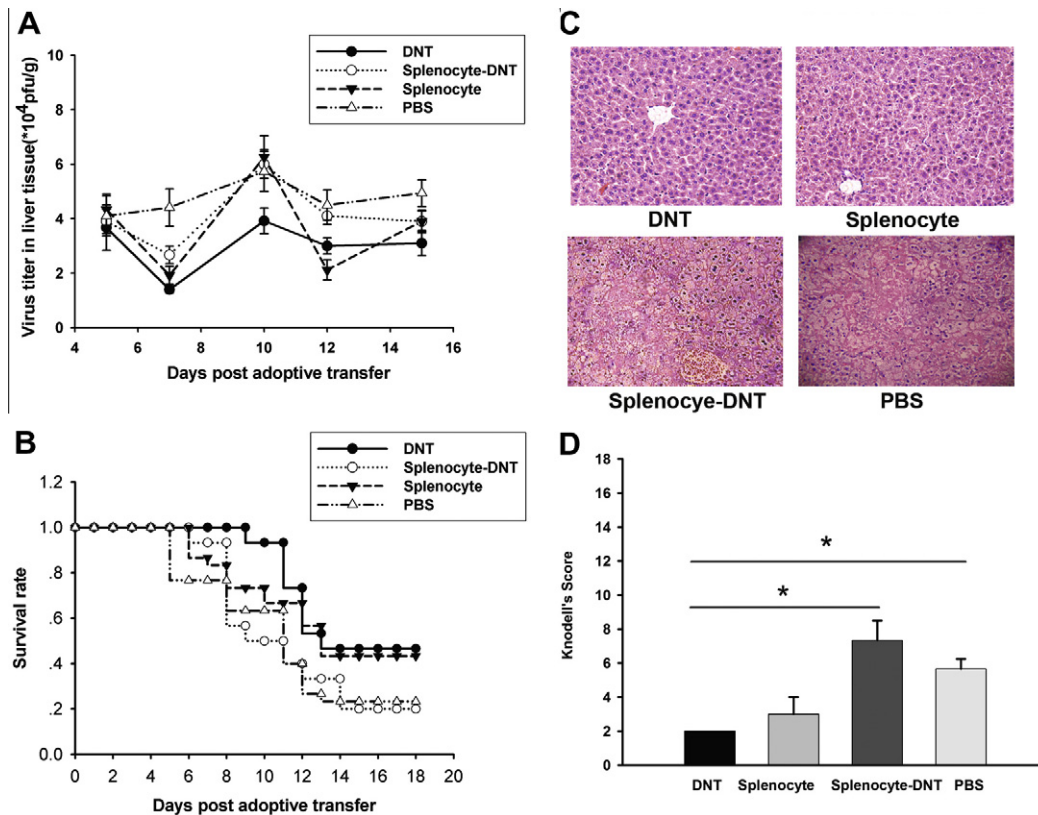


Fig. 3. Adoptive transfer of DN T cells from MHV-3 infected C3H/HeJ increased the survival rate and improved the liver histology of recipient mice infected by the same virus strain but had little impact on the virus titer of liver tissue. (A) Virus titers of liver tissues in each group of the adoptive transfer experiment were tested at indicated time points. No significant difference in virus titers was observed among DNT cells group, splenocytes group, DN T-depleted splenocytes group and PBS control group. Data represent means \pm SEM from three independent experiments performed in each group. $^*P < 0.05$. One-way ANOVA. (B) DN T cells, splenocytes or DN T-depleted splenocytes from C3H/HeJ mice 15 days post MHV-3 infection were injected into C3H/HeJ mice 5 days post MHV-3 infection. PBS was used as control. After adoptive transfer of DN T cells or splenocytes, 46.67% (●) or 43.33% (▼) of the recipient mice survived, while only 23.33% or 25% of C3H/HeJ mice receiving PBS [Δ] or DN T-depleted splenocytes [\square] survived. (C) Liver tissues from each group of mice were obtained on Day 17 post infection and were performed HE staining. Compared with C3H/HeJ mice receiving PBS or DN T-depleted splenocytes, histological improvements including decreases of hydropic degeneration, inflammatory cell infiltration as well as bridge necrosis were evidenced after adoptive transfer of DN T cells or splenocytes from MHV-3 infected C3H/HeJ. (D) Comparison of Knodell score (HAI) among the four groups on day 12 after the adoptive transfer was performed. Significant difference was found when DN T cells group versus DN T-depleted splenocytes group (2.0 ± 0 versus 7.3 ± 1.1 , $^*P < 0.01$), and DN T cells group versus PBS control group (2.0 ± 0 versus 5.7 ± 0.6 , $^*P < 0.01$).

mice raised notably to 46.67% and 43.33%, respectively, while only 23.33% of mice receiving PBS or 20% of mice receiving DN T-depleted splenocytes survived (Fig. 3B).

As shown in Fig. 3C, mice receiving DN T cells or splenocytes had alleviated hydropic degeneration, inflammatory cells infiltration, as well as bridge necrosis in their livers compared with mice receiving PBS or DN T-depleted splenocytes. Compared with splenocytes group, the hepatocytes swelling in the recipient mice in DN T cells group were not as much severe. Comparison of Knodell score (HAI) among the four groups on day 12 after the adoptive transfer was shown in Fig. 3D. Significant difference was found when DN T cells group vs. DN T-depleted splenocytes group (2.0 ± 0 versus 7.3 ± 1.1 , $^*P < 0.01$), and DN T cells group vs. PBS control group (2.0 ± 0 versus 5.7 ± 0.6 , $^*P < 0.01$).

3.4. The phenotypes and cytokine profile of splenic DN T cells

To further characterize the DN T cells, we detected the cell surface markers of DN T cells from spleens of C3H/HeJ mice post MHV-3 infection (Fig. 4A). The majority of DN T cells expressed $TCR\alpha\beta^+$ while only a small part expressed $TCR\gamma\delta^+$. Adhesion molecule CD44 was highly expressed on DN T cells, while activation markers CD25, CD28 or CD30 were rarely detected. Moreover, these DN T cells did not recognize α -GalCer (which NKT cells do

in an Ag-specific fashion), demonstrating that DN T cells are different from NKT cells (data not shown). Intracellular cytokines of DN T cells were detected on Day 0, 6, 10, 15, 20, and 30 after MHV-3 infection. The infected DN T cells either producing IL-2 or IFN- γ have increased since day 7 after MHV-3 infection, peaked at day 15 and maintained a stable level hence. Only marginal levels of IL-4, IL-10, TNF- α , perforin and granzyme B were detected, and there was no significant difference between infected and non-infected mice in the expression of these intracellular cytokines at any time points (Fig. 4B, C, D).

3.5. DN T cells exert a profound cytotoxic effect on virus-specific $CD8^+$ T cells through the Fas-FasL pathway in MHV-3 infected C3H/HeJ mice

The cytotoxic effects of DN T cells from the spleen of C3H/HeJ mice 0, 4, 15, 30, 40 days after MHV-3 infection were examined. DN T cells had significant cytotoxic effects on MHV-3 infected $CD8^+$ T cells, but no apparent effect on infected hepatocytes or non-infected $CD8^+$ T cells and hepatocytes (Fig. 5A).

To further investigate the cytotoxic specificity of DN T cells, a murine cytomegalovirus (MCMV) was used as an unrelated control. After peritoneal MCMV infection, the livers of C3H/HeJ mice were evidently infected by MCMV, and virus replication was observed in the liver (as revealed by a standard plaque assay; data

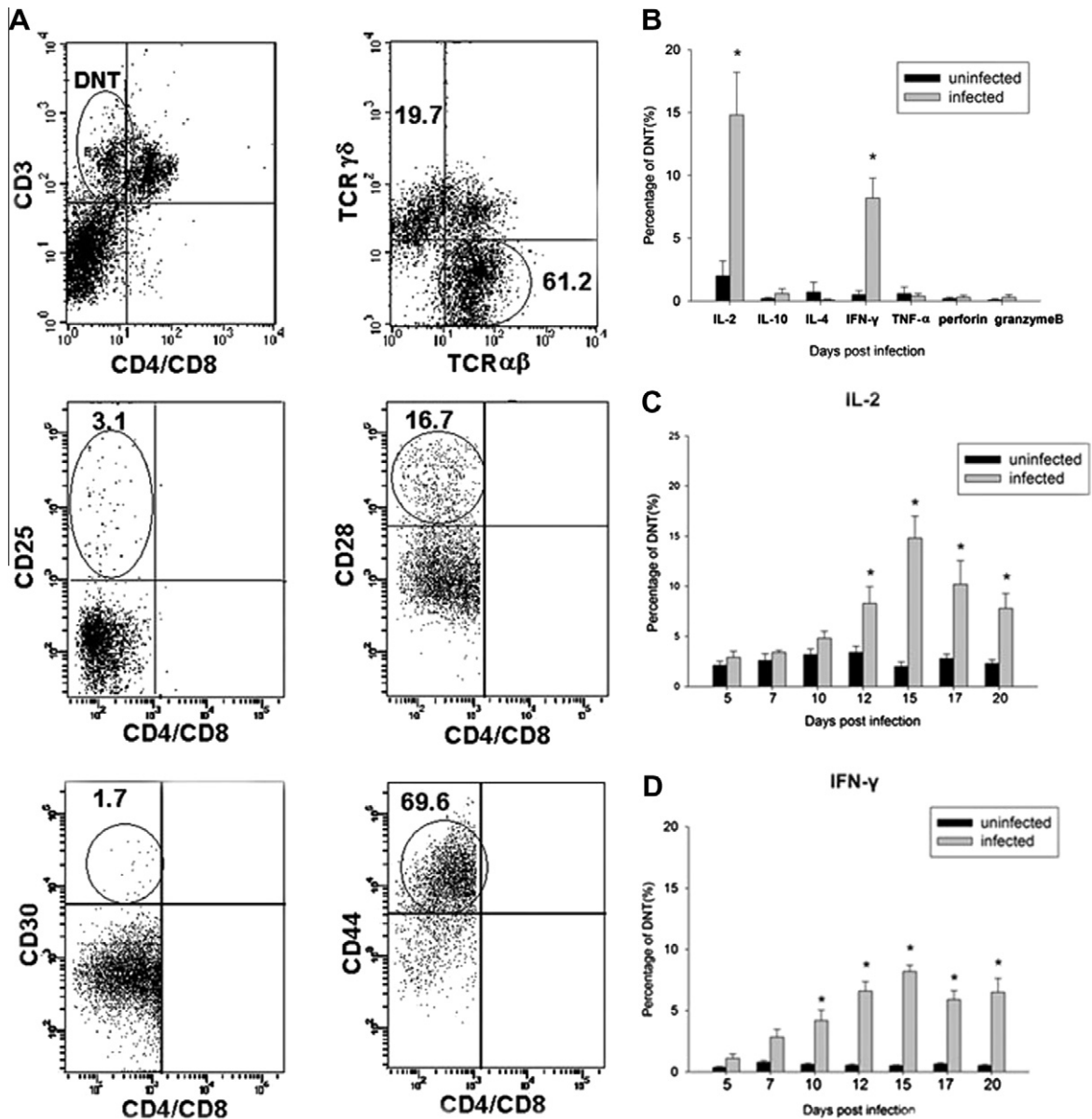


Fig. 4. Splenic DN T cells of MHV-3 infected C3H/HeJ mice displayed a unique set of cell surface markers and had enhanced production of IL-2 and IFN- γ post MHV-3 infection (A) Representative flow cytometry plots display the surface markers of splenic DN T cells of MHV-3 infected C3H/HeJ mice. The DN T cell population was analyzed by gating on CD3⁺ and CD4⁻ CD8⁻ population. The cell surface markers include CD25, CD28, CD30 and CD44. And the majority of DN T cells were TCR $\alpha\beta$ ⁺ CD4⁻ CD8⁻ CD25⁻ CD28⁻ CD30⁻ CD44⁺. (B) shows the intracellular cytokines expression (IL-2, IL-4, IL-10, IFN- γ , TNF- α , perforin, Granzyme B) of splenic cells of C3H/HeJ mice 15 days post MHV-3 infection. (C) and (D) show the IL-2 and IFN- γ expressions of splenic cells of C3H/HeJ mice respectively at different time points post MHV-3 infection. Three independent experiments were performed with 3 mice used in each group. * $P < 0.05$. *t*-test.

not shown). DN T cells from the spleens of MHV-3 infected mice showed no obvious cytotoxic effect on CD8⁺ T cells from the spleens of MCMV-infected mice, strongly suggesting that DN T cells only specifically kill CD8⁺ T cells with same virus specificity, and that this effect is not due to bystander cytotoxicity (Fig. 5B).

A transwell experiment was performed to determine whether the cytotoxic effect is contact-dependent or is mediated by cytokines. Results showed that DN T cells cytotoxicity was remarkably weaker when DN T cells and CD8⁺ T cells were in different chambers as opposed to being in the same chamber (Fig. 5C). To identify the precise mechanism involved in DN T cells cytotoxicity, the expression of FasL, perforin, and granzyme B in DN T cells was studied. Perforin and granzyme B were rarely detected (Fig. 4B), while (47.53 ± 12.83) % of DN T cells express FasL. Moreover,

DN T cells cytotoxicity dramatically decreased when FasL was blocked (effector:target = 5:1) (Fig. 5D).

4. Discussion

Lacking of adequate animal model has long being a major obstacle in studying viral hepatitis. Only chimpanzee and a few other primates are susceptible to hepatitis viruses, but the endangered status and financial considerations limit their widespread use. Researchers have also used surrogate animal models, such as ground squirrel, duck, tamarins and woodchuck. In addition to the wild-type animal models, inbred strains of animals are valuable to investigate the pathogenesis of viral infection. There are

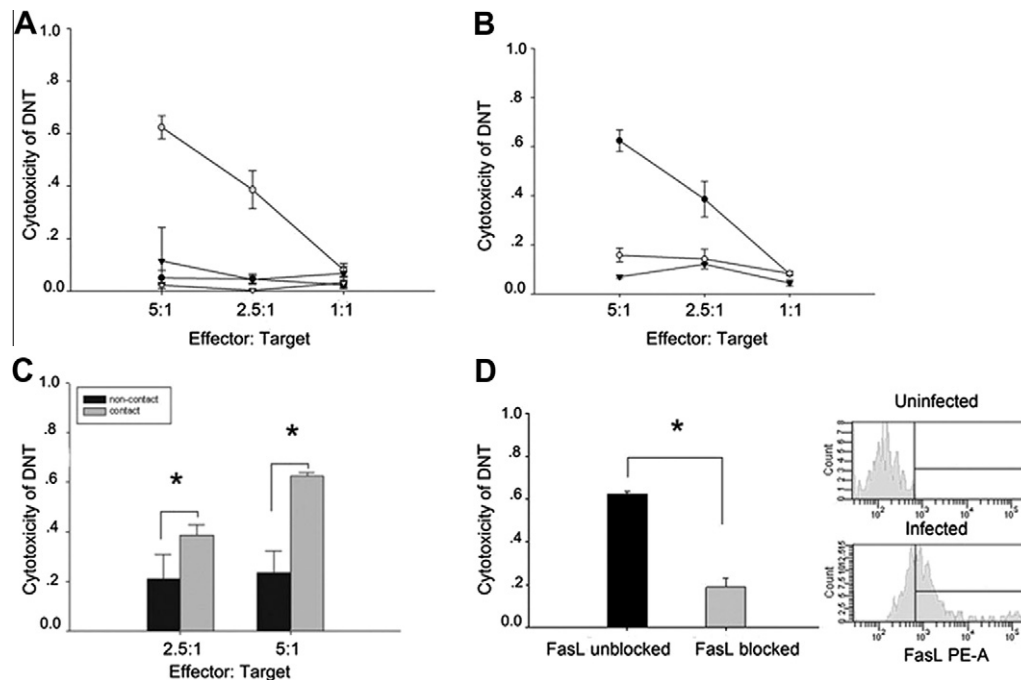


Fig. 5. DNT cells from spleen exerted profound cytotoxic effects on virus-specific CD8⁺ T cells through the Fas–FasL pathway in MHV-3 infected C3H/HeJ mice. (A) Splenic DNT cells demonstrate a remarkable cytotoxic effect on CD8⁺ T cells from MHV-3 infected mice [○] but neither on CD8⁺ T cells [●] and hepatocytes [▼] from naïve mice nor on hepatocytes from MHV-3 infected mice [▽]. The ratio of effector cells (DNT cells) versus target cells (CD8⁺ T cells or hepatocytes) were 5:1, 2.5:1 and 1:1. (B) MHV-3 infected DNT cells demonstrated a significant cytotoxic effect on CD8⁺ T cells from MHV-3 infected mice [●], but not on CD8⁺ T cells from MCMV infected mice [○]. Meanwhile, basically no cytotoxic effect of DNT cells from spleen of the naïve mice was detected on CD8⁺ T cells from MHV-3 infected mice [▼]. (C) DNT cells (5×10^5 or 2.5×10^5) did not kill CD8⁺ T cells (1×10^5) effectively (23.4% or 20.93%) when cultured in separate chambers of the Transwell in comparison with the high killing rate as 63.8% (effector:target = 5:1) or 38.6% (effector:target = 2.5:1) when these two groups of cells cultured in one well. (D) $(47.53 \pm 12.83)\%$ of infected splenic DNT cells expressed FasL on their surfaces while almost no FasL was expressed on uninfected DNT cells. DNT cells cytotoxicity dramatically decreased from 61% to 18.75% when FasL was blocked (effector: target = 5:1). Three independent experiments were performed with 3 mice used in each group. For A and B, comparison between groups was performed using one way ANOVA. For C and D, each point is a mean of triplicate cultures \pm SEM. * $P < 0.05$, *t*-test.

transgenic mouse models as well as immunodeficient mice or tolerized mice or rats, transplanted with human hepatocytes or hepatoma cells. Animal models mentioned above enable us to better understand the pathogenesis of viral hepatitis, but all have limitations [34,35].

Using MHV-3, which produces a strain-dependent viral hepatitis in inbred strains of mice, has brought insights into the pathogenesis of viral hepatitis from our group and others [32,36–40]. MHV-3 belongs to the coronavirus family and has a single-stranded positive-sense RNA genome. As reported, susceptible inbred mouse strains such as Balb/cj or C57BL/6 develop fulminant hepatitis and die within 3–5 days following peritoneal inoculation of the virus. In contrast, A/J mice are resistant and develop no clinical signs of hepatitis, and clear the virus within 10 days of infection [41]. C3H/HeJ mouse, a semisusceptible mouse strain develops acute hepatitis which progresses to mild chronic hepatitis [42]. In our study, the mice have presented acute inflammation from 6 to 15 days after infection, and a proportion of the infected mice died. However, after this phase, the liver injury in the survived mice was alleviated, accompanied with the recovery of the liver function. Only mild inflammation has been observed in the livers of the survivors, with persistent low-level virus replication, suggesting the persistence of viral infection. Since the disease course, histopathology, and serum biochemical changes observed in the mouse model are similar to those observed in patients with chronic viral hepatitis, this mouse model could be useful to understand the pathogenesis of chronic viral hepatitis in humans.

Host immune response exercises a great influence on the pathogenesis and outcome of viral hepatitis. Some viruses can be eliminated by the host immune system in the acute phase of infection, but certain viruses, like HBV and HCV, can evade the host immune

responses, resulting in viral persistence [43]. In order to better understand the mechanisms leading to virus persistence in the model of MHV-3 infected C3H/HeJ mice, we studied the variations of different subgroups of T cells in C3H/HeJ mice after MHV-3 infection. The results showed that the frequency of DN T cells rose significantly in blood, liver, and spleen after MHV-3 infection. We also observed an increase of CD4⁺CD25⁺ Tregs (data not shown) in the liver, but the increase of DN T cells was more pronounced than the increase of CD4⁺CD25⁺ Tregs, indicating that DN T cells might play a critical role in controlling the pathogenesis of the disease.

Based on our observation, the liver injury of infected mice began around 4–5 days after infection. We assumed that the adoptive transfer of DN T cells at this time point might interfere the development of liver injury. Therefore, we chose to transfer DN T cells to recipient mice at 5 days post infection. The results demonstrated that DN T cells could lead to both the dramatic increase in the survival rate of recipient mice and amelioration in liver histology. However, differences in the virus titers of liver tissue had barely been observed between DN T group and control groups. We presume that DN T cells may play a role in inhibiting the host immune responses which cause liver injury. Nevertheless, the virus replication and clearance appear to reach a dynamic balance in the process.

The following cytotoxic assays revealed that after MHV-3 infection, DN T cells showed significant cytotoxic effects on virus-specific CD8⁺ T cells. Since the virus-specific CD8⁺ T cells play a key role in causing liver damage, this result made a sensible explanation to the survival increase and improvement of liver histology in recipient mice in adoptive transfer experiment. Transwell experiments indicated that cell–cell contact is necessary to the

cytotoxicity mediated by DN T cells. Moreover, the Fas/FasL pathway instead of perforin/granzyme pathway plays a vital part in DN T cell killing. Thus we presume that, in MHV-3 infected C3H mice, DN T cells rapidly proliferated following the development of liver inflammation, and contributed to the control of liver injury by killing the virus-specific CD8⁺ T cells. The phenomenon observed here is in agreement with what has been described for TCR $\alpha\beta^+$ CD25⁺CD30⁺ DN T cells, which are able to prevent the rejection of skin and heart allografts by specifically inhibiting CD8⁺ T cells through Fas-FasL interaction [17]. However, Zhang et al. reported that B220⁺CD25⁺ DN T cells can control B and T cell responses in perforin/granzyme-dependent mechanisms [25]. Voelkl also reported that human DN Tregs do not eliminate effector T cells by Fas/FasL-mediated apoptosis, but to suppress by an active cell contact-dependent mechanism [44]. Thus, it seems that the functions of DN Tregs change in accordance with the different phenotypes in specific settings.

We found that the splenic DN T cells of the infected mice bear a distinctive array of phenotypes (TCR $\alpha\beta^+$ CD4⁻CD8⁻CD25⁻CD28⁻CD30⁻CD44⁺) which were completely different from previously described T cells [45,46]. Meanwhile, these DN T cells showed a relatively increased production of IFN- γ and IL-2, but low IL-4, IL-10, TNF- α . It is reported that, in other disease models, DN T cells with different phenotypes present different cytokine profiles. In autoimmune diseases like SLE, TCR $\alpha\beta^+$ TCRV α 24⁻ DN T cells are the major producers of IL-17, one of the key inflammatory cytokine involved in SLE. And the results suggested that these DN T cells contribute to the pathogenesis of kidney damage in patients with SLE [28]. In CD4-low mangabeys, DN T cells have increased expression of IFN- γ (Th1), IL-4(Th2) and IL-17(Th17), suggesting these cells may be capable of performing multiple functions, including CD4 helper-like function and regulatory function. And the latter could potentially contribute to controlling immune activation during nonpathogenic SIV infection [30]. In our study, we assume that the DN T cells may, to some degree, exert the role of Th1 cells, which promote viral elimination. Nevertheless, the limited quantity of these cytokines constrained their power for viral clearance and allowed the low-level viral persistence. We postulate that it was the delicate balance of multiple effects of DN T cells contributing to the viral persistence in this model.

In conclusion, we report herein for the first time that splenic DN T cells with production of IFN- γ /IL-2 have profound immunomodulatory effects by killing viral-specific CD8⁺ T cells via Fas/FasL pathway in MHV-3 induced viral hepatitis, suggesting the contribution of these DN T cells to viral persistence. Our findings may provide a rationale for modulating DN T cells for the management of viral hepatitis.

Disclosures

The authors have no financial conflict of interest.

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