Intrahepatic Infiltrating NK and CD8 T Cells Cause Liver Cell Death in Different Phases of Dengue Virus Infection

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Abstract

Elevated liver enzyme levels is an outstanding feature in patients with dengue. However, the pathogenic mechanism of liver injury has not been clearly demonstrated. In this study, employing a mouse model we aimed to investigate the immunopathogenic mechanism of dengue liver injury. Immunocompetent C57BL/6 mice were infected intravenously with dengue virus strain 16681. Infected mice had transient viremia, detectable viral capsid gene and cleaved caspase 3 in the liver. In the mean time, NK cell and T cell infiltrations peaked at days 1 and 5, respectively. Neutralizing CXCL10 or depletion of Asialo GM1+ cells reduced cleaved caspase 3 and TUNEL+ cells in the liver at day 1 after infection. CD8+ T cells infiltrated into the liver at later time point and at which time intrahepatic leukocytes (IHL) exhibited cytotoxicity against DENV-infected targets. Cleaved caspase 3 and TUNEL+ cells were diminished in mice with TCRb deficiency and in those depleted of CD8+ T cells, respectively. At day 5 after infection. Moreover, intrahepatic CD8+ T cells were like their splenic counterparts recognized DENV NS4B99–107 peptide. Together, these results show that infiltrating NK and CD8+ T cells cause liver cell death. While NK cells were responsible for cell death at early time point of infection, CD8+ T cells were for later. CD8+ T cells that recognize NS4B99–107 constitute at least one of the major intrahepatic cytotoxic CD8+ T cell populations.

Introduction

Dengue virus (DENV) infection causes dengue fever (DF) which may progress to become life-threatening dengue hemorrhagic fever (DHF) and/or dengue shock syndrome (DSS). About 50 million cases of dengue infection are reported worldwide each year and dengue has become the most important tropical disease second to malaria [1].

The involvement of liver in DENV infection has been well documented. An early report showed that among 270 dengue cases studied, as high as 93% of them had elevated liver enzyme levels [2]. In most cases, the elevation is mild to moderate, indicating liver damage is not severe [2,3]. Nevertheless, the level of aminotransferases correlates with the severity of vascular leakage and bleeding manifestations [4,5]. DENV infects primary human hepatocytes and Kupffer cells as well as hepatoma cell lines [6–9]. Hepatocytes and Kupffer cells as DENV targets are confirmed in biopsies and autopsies of fatal cases [3,10–12]. Both apoptotic and necrotic cell death are detected in infected liver [11]. Even though DENV is demonstrated to cause HepG2 and Huh7 apoptosis [8], whether liver pathology and cell death is directly caused by viral infection remains unclear.

Immune activation has been proposed as a cause for severe dengue illness. The expression of CD69 on CD8+ T and NK cells is high in both DF and DHF, but higher in DHF patients [13]. Serum levels of soluble IL-2 receptor (sIL-2R), sCD4, IL-2 and IFNγ are higher in DF and DHF patients than in healthy donors, and sCD8 is high in only DHF but not DF patients, showing that CD8+ T cell activation correlates with severe disease [14]. Moreover, inflammatory cell infiltration is evident in liver biopsy of patients with high aminotransferase levels [10]. These studies suggest that immune cell activation and possibly intrapathelial infiltration may be involved in liver pathology.

Small animal models were developed to study DENV infection. Severe combined immunodeficient (SCID) mice xenografted with human cell lines showed viremia [15–17]. RAG2−/−γc−/− mice engrafted with human CD34+ hematopoietic stem cells exhibited viremia and detectable anti-dengue immunoglobulins [18]. Injection of AG129 mice with low dose of non-mouse adapted DENV resulted in spleen damage, liver dysfunction and increased vascular permeability and eventual death [19]. BALB/c and C57BL/6 mice infected with DENV showed elevated liver enzymes and intrahepatic cellular infiltration [20–22]. The elevation of liver enzymes coincided with the kinetics of CD4+CD8+ mononuclear cell infiltration [21]. Together, these reports show that intrahepatic infiltration of activated immune cells positively correlates with DENV-induced liver damage. However, the direct proof of the relationship between them needs further investigation.

In this study, we employed immunocompetent mouse model to study the immunopathogenesis of DENV-induced liver injury. Intravenously infected mice experienced transient viremia and viral capsid gene was detected in the liver. Intrahepatic NK cell infiltration peaked at day 1 when TUNEL+ cells and cleaved caspase 3 appeared. Blocking or depleting NK cells diminished liver cell death. Cytotoxicity assay demonstrated that intrahepatic leukocytes were cytotoxic against dengue virus-infected Hepa 1–6...
recognized NS4B99–107. Taken together, we demonstrated for the first time in mouse model that intrahepatic infiltrating NK and CD8+ T cells cause liver cell death at different phases of infection respectively and that intrahepatic cytotoxic CD8+ T cells recognized NS4B99–107.

Materials and Methods

Mice

Wild type and TCRβ knockout (KO) mice in C56BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in the Laboratory Animal Center, National Taiwan University College of Medicine. STAT1 KO [23] were maintained in LEVEL Biotechnology (Taipei, Taiwan). This study was carried out in strict accordance with the recommendations in the Guidebook for the Care and Use of Laboratory Animals, The Third Edition, 2007, published by The Chinese-Taipei Society of Laboratory Animal Sciences. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of the National Taiwan University College of Medicine (Permit Number: 20080169).

Virus and Infection

DENV-2 strain 16681 was propagated in C6/36 cells (ATCC CRL-1660, gift from Dr. Chuan-Liang Kao, College of Medicine National Taiwan University, Taipei, Taiwan). Viral titers were determined by plaque-forming assay on BHK cells. To obtain UV-inactivated virus, viral stocks were treated with UV at 50 mJ/cm² in 30 sec by UVlink crosslinker (UVItec, UK). Mice were inoculated intravenously with 1x10⁸ PFU of viable virus or the equivalent UV-inactivated virus. To obtain higher numbers of intrahepatic leukocytes for cytotoxicity assay, a second injection of virus at day 7 after primary infection was given. STAT1 KO mice were inoculated intravenously with 1x10⁷ PFU of viable virus or the equivalent UV-inactivated virus. At indicated time points, mouse livers were perfused with PBS through the portal vein before harvest.

RT-PCR and Real-time PCR

Serum RNAs were extracted by QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). RNAs in the liver and C6/36 cells cultured in medium containing mouse sera (at 1:2 dilution) were extracted by Trizol (Invitrogen, Grand Island, NY). Viruses were reversely transcribed by C14A (5’-AACGATGCAGGAAACGGAGAACCCGAGAAGGAAACGACG-3’) and C69B (5’-CCCATCTTGCTTGCTTTGGACGACGTG-3’) [24] for viral capsid gene expression and by random primers (Promega, Tokyo, Taiwan) for β-actin, GAPDH and chemokine gene expression. Viral capsid gene in serum and liver was then determined by PCR using primer pairs mouse GAPDH: forward 5’- ACCACAATGCTGAGACACGACCACGAC-3’ and reverse 5’-TCCACCACCCTTGCTTTGGACGACGTG-3’ [25] C6/36 β-actin: forward 5’-CCAATGCTGAGACACGACCACGACGAC-3’ and reverse 5’-CAGCTTTGCTAGCCCTTGGAGCGATAT-3’ [26]. Viral capsid gene in serum was determined by real-time PCR by MyiQ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA)

ELISA

CXCL10 and CCL5 levels in serum were determined using ELISA antibody pairs purchased from Bender MedSystems (La Jolla, CA) for CXCL10 and R&D systems (Minneapolis, MN) for CCL5.

Western Blotting

Protein was extracted from perfused liver tissues by RIPA lysis buffer containing 1% protease inhibitor mixture (Sigma-Aldrich), and then boiled in SDS sampling buffer. The released proteins were separated by 12% SDS-polyacrylamide gels, transferred to nitrocellulose membrane (GE Healthcare Bio-Sciences, Sweden) and blotted with anti-caspase 3 or anti-actin antibody (each at 1:1000, Cell Signaling, Danvers, MA) followed by HRP-conjugated goat anti-rabbit IgG antibody (1:2000, Jackson ImmunResearch Laboratories, West Grove, PA). The blots were visualized using the ECL detection system (Thermo, Rockford, IL). Actin was used as loading control.

Isolation of Intrahepatic Leukocytes (IHLs)

After perfusion, the liver was incubated in digestion buffer (0.1% collagenase IV and 0.01% DNase I (Sigma-Aldrich) at 37°C for 40 min. The digested tissues were washed through 70 μm mesh (BD Falcon, Franklin Lakes, NJ) and the passed-through cells were collected and separated in Ficoll-Paque PLUS (GE Healthcare Bio-Sciences) by centrifugation at 2000 rpm (760 g) for 20 min. The intrahepatic leukocytes at the interface were collected.

Immunohistochemistry and TUNEL Staining

Liver tissues were embedded in OCT embedding medium (Shandon, CRYOTOME SME, Pittsburgh, PA), snap frozen in liquid nitrogen. Frozen tissues were cryosectioned at 5 μm-thicknesses. After fixation in acetone, rat anti-mouse CD49b (DX5) antibody (BD Pharmingen, San Diego, CA) was added and left at 4°C overnight. HRP-conjugated goat anti-rat Ig antibody (Jackson ImmunResearch Laboratories) was added and incubated at 37°C for 5 h. DAB (2,4-Diaminobenzylic Acid, Sigma-Aldrich) was used as substrate for color development and hematoxylin was used as counterstain.

To detect dead cells, cryosectioned tissues were fixed in 4% paraformaldehyde before treatment with 0.1% Triton X-100 on ice. TUNEL reaction mixture (Roche Applied Science, Indianapolis, IN) was added and left at room temperature for 60 min. Converter-POD (anti-FITC, Roche Applied Science) was then added and the slides were incubated at room temperature for another 30 min. DNase1 (1 μg/ml) treated sections were used as positive control. DAB substrate was added for color development. The slide was counterstained with methyl green.

Antibody Preparation and Treatment

Hybridomas that produce anti-CD4 (GK1.5, ATCC TIB-207), anti-CD8 (2.43, ATCC TIB-210) and anti-CXCL10 (kindly provided by Dr. Thomas E. Lane, Molecular Biology and Biochemistry Research, University of California, Irvine) antibodies were cultured in DMEM (Gibco BRL, Gaithersburg, MD)
Liver Cell Death in DENV Infection

Results

Animal Model to Study DENV-induced Liver Injury

Wild type mice intravenously infected with DENV strain 16681 have elevated serum levels of ALT and AST [21]. Here, we employed this model to investigate the mechanism of liver injury in DENV infection. RT-real-time PCR (Fig. 1A) and PCR (Fig. 1B) results show that dengue virus capsid gene was detectable in sera of infected mice at days 0.6 and 1 and viable virus detectable at days 0.6, 1, 3, 5 after infection. Capsid gene expression was also detected in liver tissues at the early phase after infection (Fig. 1C). While UV-inactivated DENV did not have any effect, transient DENV infection induced caspase 3 cleavage in the liver at days 1, 3 and 5 (Fig. 1D and E), showing that liver cells undergo apoptosis after infection. These results together demonstrated that accompanied elevated serum levels of ALT and AST [21], transient DENV infection induced liver cell apoptosis.

Upregulation of CXCL10 and CCL5 after DENV Infection

Examining chemokine expression, we found that the protein and mRNA levels of CXCL10 were significantly elevated in the serum and liver of infected mice at days 0.6 and 1 after infection. The CCL5 levels were the highest at day 0.6 and remained significantly higher than controls until day 5 (Fig. 2A and B). In addition, DENV infection of mouse hepatoma cell line, Hepa1–6, induced the expressions of CXCL10 and CCL5 mRNA (Fig. 2C). These results indicate that CXCL10 and CCL5 are upregulated in the liver after DENV infection and hepatocytes are likely one of their source.

Analysis of intrahepatic leukocytes (IHL) revealed two waves of cellular infiltration into the liver after infection. While NK cells peaked at day 1, CD4+ and CD8+ T cells did at day 5 (Fig. 2D). Notably, intrahepatic CD4+/CD8+ T cell ratios changed from 1.4 in uninfected controls to 0.6 in infected mice at day 5. These results demonstrate that correlating to hepatic CXCL10 and CCL5 expressions, there is a transient intrahepatic infiltration of NK at day 1 and T cells at day 5 after DENV infection.

NK Cell Infiltration Results in Liver Cell Death in Early Phase of Infection

Figure 3A shows that neutralization of CXCL10 abrogated NK cell recruitment, which established that CXCL10 mediates intrahepatic NK cell infiltration. Additionally, while infection of wild type mice resulted in caspase 3 cleavage at day 1, anti-AGM1 antibody treatment diminished the expressions of cleaved caspase 3 and TUNEL+ cells in the liver (Fig. 3B and C). Together, these results established a causal relationship between NK cell infiltration and liver cell death during early phase of DENV infection.

Intrahepatic CD8+ T Cells are Cytotoxic against Infected Targets

Since caspase 3 cleavage was observed in liver not only in day 1 but also days 3 and 5 after infection [Fig. 1C] and T cell infiltration peaked at day 5 (Fig. 2D), we employed TCRβ KO mice to investigate whether T cells mediate liver cell death at later time points. While caspase 3 cleavage was detectable at days 1 and 3, it was almost completely diminished in TCRβ KO mice at day 5 after infection (Fig. 4A). These results strongly indicate that T cells are the cause of liver cell death at day 5 after infection. Results of in vitro assay showed that intrahepatic leukocytes from infected wild type mice were cytotoxic against DENV-infected Hepa 1–6 targets. At an effector-to-target ratio of 10:1, the cytotoxicity was

Detection of Viral Antigen, Cell Surface Marker and Intracellular Cytokine

C6/36 cells were cultured in sera (at 1:2 dilution) for 3 days before harvest and viral protein expression was determined by staining with rabbit anti-DENV antiserum (gift from Dr. Wen Chang, Academia Sinica, Taipei, Taiwan) [21] followed by PE-goat anti-rabbit antibody. Splenocytes and IHLs were collected at different time points after infection. Cells were stained with anti-CD4 (GK1.5), anti-CD8 (53-6.7) (eBioscience, La Jolla, CA) and anti-CD49b (DX5) (BD Pharmingen) antibodies. For intracellular staining, cells were fixed and then stained with anti-INFγ antibody (eBioscience) in Perm/Wash buffer (containing 0.5% saponin). Cells were acquired by FACSCalibur and data were analyzed by CellQuest (BD Biosciences, San Jose, CA).

Peptide Stimulation

Splenocytes or IHLs were harvested from infected wild type or STAT1 KO mice at different time points after infection. Peptides C51–19: VAFLRFLTI; E451–458: VSWTMKIL; NS2A 8–15: STNTDVT; NS4B59–66: SSVNVSLT; NS4B 99–107: YSAVN–GK1.5 and 2.43 (250 μg/ml) in the presence of monensin (2 μM, Sigma-Aldrich) and soluble anti-CD28 antibody (1 μg/ml) for 6 h. IFN-γ-producing CD8+ T cells were determined by intracellular cytokine staining as described above.

Statistical Analysis

Two-tailed Student t test was used to determine statistical significance.
about 15% (Fig. 4B). Depletion of CD8$^+$ but not CD4$^+$ T cells completely eliminated TUNEL$^+$ cells in the liver (Fig. 4C). Thus, intrahepatic CD8$^+$ T cells and their cytotoxic activity against DENV-infected hepatocytes is the cause of liver cell death.

Both Intrahepatic and Splenic CD8$^+$ T Cells Recognize NS4B_{99-107} Epitope

Core$_{31-39}$, NS2A$_{11-15}$, NS4B$_{99-107}$, and NS5$_{237-245}$ were identified to be DENV-specific epitopes that are recognized by splenic CD8$^+$ T cells in DENV clone S221-infected mice [21]. Since DENV 16681 share the same peptide sequence with the parental D2S10 virus in these positions, we asked whether the intrahepatic CD8$^+$ T cells recognize any of these epitopes. Interestingly, infection of both wild type mice and STAT1 KO mice resulted in intrahepatic caspase 3 cleavage at days 3 and 5 though cleaved caspase 3 was not observed in STAT1 KO mice at day 1 due to their lack of functional NK cells [30] (Fig. 5A). Figure 5B shows that splenic CD8$^+$ T cells in both DENV 16681-infected wild type and STAT1 KO mice recognized only NS4B$_{99-107}$ but not other peptides. NS4B$_{99-107}$-reactive CD8$^+$ cells expanded in both wild
type and KO mice from days 3 to 7 after infection. Injection of UV-inactivated DENV did not elicit CD8\(^+\) T cell response to any of the peptides tested (Fig. 5B). Taking advantage of the greater magnitude of CD8\(^+\) T cell response in STAT1 KO mice, we stimulated intrahepatic cell from infected STAT1 KO mice with different peptides. The results show that intrahepatic CD8\(^+\) T cells, like their splenic counterparts, recognized NS4B99–107 but not other peptides (Fig. 5C), indicating that intrahepatic CD8\(^+\) T cells that recognize NS4B99–107 constitute at least one of the major cytotoxic T cell populations that kill infected hepatic cells.

**Discussion**

We have observed the correlations between T cell activation, hepatic cellular infiltration and elevated levels of AST and ALT in DENV-infected immunocompetent animals [21]. In this study, employing the same model we demonstrated that NK and T cells are recruited to the liver at different phases of infection. We provided direct evidence to show that while recruited NK cells are responsible for liver cell death at an early time point, intrahepatic infiltrating CD8\(^+\) T cells are for later time point. Infiltrating CD8\(^+\) T cells are cytotoxic against DENV-infected hepatocytes and NS4B99–107-reactive cells constitute at least one the major intrahepatic CD8\(^+\) T cell populations.

It is reported earlier that recombinant CXCL10 is able to inhibit the binding of DENV to heparan sulfate on hepatoma cell surface [31]. Here we showed that anti-CXCL10 antibody abolishes NK cell infiltration and that NK cell infiltration is critical to liver cell death at early phase of DENV infection. Thus, it appears that DENV-induced CXCL10 production is beneficial as well as harmful to the infected host. Through competition for heparan sulfate, CXCL10 inhibits DENV infection, but in the mean time, CXCL10-mediated recruitment of NK cells causes liver cell death.

*In vitro* studies show that DENV infection causes liver cell death. DENV infection of Hepa 1–6 induces cell death through a mitochondria-mediated pathway [32], while infection of HepG2 causes cell death through the upregulations of CD95 [33], CD137 [34] and TRAIL [35]. Studies showed that poly I:C-induced intrahepatic infiltrating NK cells express high levels of TRAIL and are responsible for liver damage [36]. Blocking TRAIL in EMCV-
infected mice diminishes NK cell cytotoxicity, which demonstrated that NK cell anti-EMCV response is TRAIL-mediated [37]. We showed in this study that DENV was transiently detected in the liver in the early phase of infection (Fig. 1C) during which time NK cells cause liver cell death (Fig. 3). It is our speculation that DENV infection of the liver, although very transient, up-regulates death receptors and renders hepatocytes susceptible to NK cell killing at early time point.

STAT1 KO mice are known to have impaired NK cell cytotoxic function. The mice are unable to reject NK sensitive tumor [30]. Our data showed that caspase 3 cleavage was induced only in the liver of infected-wild type but not -STAT1 KO mice at day 1 after infection. These results together with the anti-Asialo GM1 depletion experiment confirm the contribution of NK cells in DENV-induced liver injury during early phase of infection.

Yauch et al. observed specific anti-DENV CD8 T cell response in the spleen of mice infected by DENV S221 [29]. Although the sequences of capsid51–59 (Kb), NS2A8–15 (Kb), NS4B99–107 (Db) and NS5237–245 (Db) peptides in DENV 16681 are identical to that in the parental D2S10, our study showed that splenic as well as intrahepatic CD8+ T cells from DENV 16681-infected mice recognize only NS4B99–107 epitope, and intrahepatic infiltrating CD8+ T cells are antigen-specific and not by-stander cells. While splenic CD8+ T cells are shown to be protective [29], our results showed that intrahepatic CD8+ T cells are cytotoxic and cause liver cell death. Therefore, CD8+ T cell immune response induced by DENV infection is a double-edge sword, on one hand it clears the virus yet when recruited to the liver they cause injury.

Elevated liver enzymes are reported in other hemorrhagic virus infections, such as Ebola and Crimean-Congo hemorrhagic fever [39,40]. Apoptotic cells are found in the liver of yellow fever virus-infected patients [41]. Interferon-signaling deficient mice infected with Sindbis virus or Crimean-Congo hemorrhagic fever virus exhibit high liver enzymes and hepatomegaly [42,43]. Infection of SCID mice with mouse-adapted Marburg hemorrhagic fever virus induces lethality and significantly elevated liver enzymes [44,45]. Therefore, it appears that liver injury is common to infection by most hemorrhagic viruses. Although mouse differs from human in the composition of immune cells in the blood, the results of our study suggest that the involvement of NK and CD8+ T cells can not be ignored.

In summary, DENV can be detected in the serum and liver transiently after intravenous infection. DENV-triggered expression...
Figure 5. Intrahepatic as well as splenic CD8+ T cells recognize NS4B99–107 epitope. (A) Liver lysates were collected from uninfected and infected wild type and STAT1 KO mice. Arrow points to cleaved caspase 3. Data presented are representative of three independent experiment (n = 3 per time point in each experiment). (B) Splenocytes were isolated from wild type and STAT1 KO mice at day 3 (empty, 5 (darkened), and 7 (hatched) after infection). (C) Liver lysates were collected from wild type and STAT1 KO mice at days 5 after DENV infection. Splenocytes and IHLs were stimulated with or without (control) indicated peptides. The percentages of IFN-γ-producing CD8+ T cell within the total CD8+ T population were analyzed by flow cytometry. Data presented are representative of four independent experiments (n = 3 per time point in each experiment). ** P value <0.01 compared with the control group.

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of CXCL10 recruits NK cells to the liver which cause liver cell death early after infection. Intrahepatic leukocytes recruited to the liver at later time points are cytotoxic against DENV-infected hepatocytes. The intrahepatic infiltrating CD8+ T cells are responsible for liver cell death and they recognize NS4B99–107. Our study indicate that NK and CD8+ T cells are both critical to dengue liver injury and that intrahepatic NS4B99–107-specific CD8+ T cells are the major cytotoxic T cell population that kill infected hepatic cells.

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