Research Article

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CD1d-restricted natural killer T cells contribute to hepatic inflammation and fibrogenesis in mice

Sachiko Ishikawa[1], Kenichi Ikejima[1,*], Hisafumi Yamagata[1], Tomonori Aoyama[1], Kazuyoshi Kon[1], Kumiko Arai[1], Kazuyoshi Takeda[2], Sumio Watanabe[1]

[1]*Department of Gastroenterology, Juntendo University School of Medicine, Tokyo, Japan;* [2]*Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan*

Background & Aims: Several lines of evidence suggest that innate immunity plays a key role in hepatic fibrogenesis. To clarify the role of natural killer (NK) T cells in hepatic inflammation and fibrogenesis, we here investigated xenobiotics-induced liver injury and subsequent fibrogenesis in mice lacking mature NKT cells caused by genetic disruption of the CD1d molecule.

Methods: Male *CD1d*-knockout (KO) and wild-type (WT) mice were given repeated intraperitoneal injections of thioacetamide (TAA, 3 times/week; 0.1–0.2 mg/g BW) for up to 9 weeks, or a single intraperitoneal injection of CCl_4 (1 μl/g). Liver histology was evaluated, and expression levels of cytokines and matrix-related genes in the liver were quantitatively measured by real-time reverse transcription-polymerase chain reaction (RT-PCR).

Results: Mortality following repeated injections of TAA was prevented almost completely in *CD1d*-KO mice. TAA-induced inflammatory responses and hepatocellular damage were markedly ameliorated in *CD1d*-KO mice. TAA-induced expression of smooth muscle α-actin (SMA) and transforming growth factor *(TGF)β1* mRNA in the liver were also prevented largely in *CD1d*-KO mice. In fact, *CD1d*-KO mice developed minimal hepatic fibrosis after 9-weeks of administration of TAA, which caused overt bridging fibrosis in WT mice. Indeed, TAA-induced increases in α1(I)procollagen (*COL1A1*) and tissue inhibitor of matrix metalloproteinase *(TIMP)-1* mRNA were blunted significantly in *CD1d*-KO mice. Similarly, acute CCl_4-induced hepatic injury and subsequent profibrogenic responses were also reduced significantly in *CD1d*-KO mice.

Conclusions: These findings clearly indicated that CD1d-restricted NKT cells contribute to xenobiotics-induced hepatic inflammation, hepatocellular damage, and subsequent profibrogenic responses in the liver.

Keywords: Innate immunity; NKT cells; CD1d; Thioacetamide; Liver injury; Hepatic fibrogenesis.

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* Corresponding author. Address: Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. Tel.: +81 3 3813 3111; fax: +81 3 3813 8862.

E-mail address: ikejima@juntendo.ac.jp (K. Ikejima).

Abbreviations: NASH, nonalcoholic steatohepatitis; NK, natural killer; TCR, T cell receptor; TAA, thioacetamide; WT, wild type; AST, aspartate aminotransferase; ALT, alanine aminotransferase; H–E, hematoxylin–eosin; TdT, terminal deoxynucleotidyl transferase; TUNEL, (TdT)-mediated dUTP nick-end labeling; αSMA, smooth muscle α-actin; HSC, hepatic stellate cell; RT-PCR, reverse transcription-polymerase chain reaction; TNFα, tumor necrosis factor-α; TGFβ1, transforming growth factor-β1; COL1A1, α1(I)procollagen; TIMP-1, tissue inhibitor of metalloproteinase-1; IL-4-6, interleukin-4-6; IFNγ, interferon-γ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance.

Introduction

Increasing lines of evidence indicate the importance of innate immunity in the pathogenesis of various liver diseases including viral hepatitis [1–3], autoimmune liver diseases [4,5], alcoholic liver disease [6–8], and nonalcoholic steatohepatitis (NASH) [9,10]. The basic concept that innate immunity plays a profound role in hepatic inflammation, tissue repair, and fibrogenesis has been proposed [11–14]; however, the molecular and cellular basis of innate immune-regulation in the progression of chronic liver diseases has not been fully elucidated.

The liver contains a variety of innate immune cells, with natural killer (NK) and invariant NKT cells predominating [15,16]. Especially, the proportion of NKT cells in the liver mononuclear cells is considerably higher as compared to blood and other lymphoid organs including spleen and thymus [15]. NKT cells are a heterogeneous subset of lymphocytes expressing both NK and T cell surface markers [17,18]. Classical (Type I) NKT cells express an invariant T cell receptor (TCR) containing Vα14-Jα18 chain in mice, whereas non-classical (Type II) NKT cells express diverse TCR [17]. NKT cells recognize a glycolipid antigen presented by CD1d, one of the major histo-compatibility complex molecules, on antigen presenting cells such as dendritic cells and macrophages [17,19]. Since CD1d is essential for the development of NKT cells [19], genetic disruption of this molecule results in depletion of both type I and II NKT cells in the whole body [20]. Recent lines of evidence suggest the possible role of NKT cells in a wide spectrum of liver diseases including viral hepatitis [21–24], autoimmune hepatitis [25], drug-induced liver injury [26–28], alcoholic liver disease [29], and NASH [11]. Several studies suggested that NKT cells modulate hepatic inflammation and fibrogenesis [30–32], however, the precise role of these cells in liver patho-physiology is still controversial.

Thioacetamide (TAA) is a known hepato-toxicant which is metabolized by microsomal enzyme cytochrome P450 (CYP) 2E1 in hepatocytes. This TAA-metabolizing process elicits reactive oxygen species (ROS) generation, thereby causing oxidative stress-triggered liver injury and subsequent hepatic fibrogenesis. In this study, therefore, we investigated the differences in inflammatory responses and progression of hepatic fibrosis caused by TAA in mice lacking mature NKT cells due to genetic disruption of the CD1d molecule.

Materials and methods

TAA-induced hepatic fibrosis model

CD1d-knockout (KO) mice raised from C57Bl/6 strain (a generous gift from the Department of Immunology, Juntendo University of Medicine, Tokyo, Japan, after backcrossing for 12 generations) were maintained in the animal facility in our institution [20,33]. The wild-type (WT), male C57Bl/6 mice were obtained from CLEA Japan Inc. (Tokyo, Japan) seven weeks after birth. Mice were housed in air-conditioned specific pathogen-free animal quarters with lighting from 08:00 to 21:00 h, and given unrestricted access to a standard lab chow and water for 1 week prior to experiments. All animals received humane care in compliance with the experimental protocol approved by the Committee of Laboratory Animals according to institutional guidelines. Male *CD1d*-KO and wild-type (WT) mice 8 weeks after birth were given repeated intraperitoneal injections of TAA (3 times/week; 0.1 mg/g BW for the initial week, 0.2 mg/g BW for latter weeks) for up to 9 weeks. Mice were euthanized by exsanguinations from the inferior vena cava at 5 and 9 weeks, and serum and liver samples were obtained. For histology and immunohistochemistry, tissue specimens were fixed in buffered formalin. Serum and liver samples were kept frozen at −80 °C until assayed.

Measurement of serum aminotransferases

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured spectrophotometrically by a standard enzymatic method using a commercial kit (KAINOS Laboratories Inc., Tokyo, Japan).

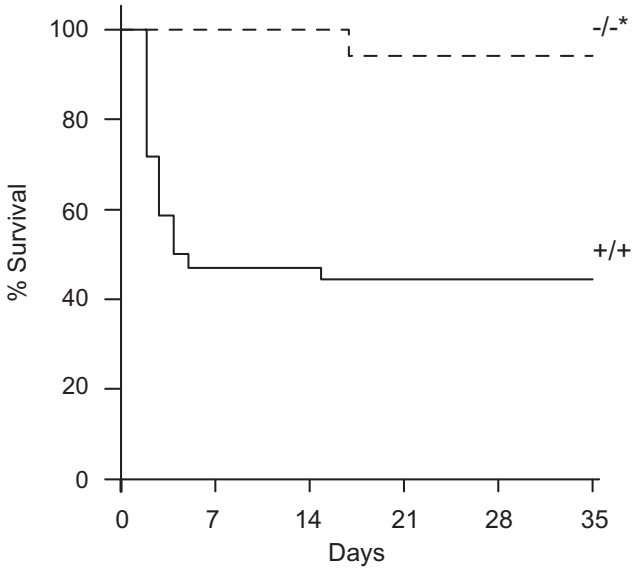

Fig. 1. Survival rate following long-term TAA treatment in CD1d-KO mice. Both wild-type (WT) and *CD1d*-knockout (KO) mice were given repeated injections of thioacetamide (TAA, 3 times per week) for 5 weeks. Percentages of survival in WT controls (+/+, solid line, $n = 36$) and *CD1d*-KO mice (−/−, dashed line, $n = 17$) following TAA treatment are plotted (*$p < 0.001$ vs. WT).

Histopathological evaluation

Formalin-fixed, paraffin-embedded liver sections were deparaffinized, and stained with hematoxylin–eosin (H–E) for regular histopathological observation. To evaluate granulocyte/macrophage infiltration, double staining for a-naphthyl acetate esterase and naphtol AS-D chloroacetate esterase was performed using a kit (Sigma Diagnositics Inc., St. Louis, MO). For visualization of hepatic fibrosis, tissue sections were stained using picro-Sirius Red in combination with Fast Green (Sigma–Aldrich Corp., St. Louis, MO). Specimens were observed and photographed

Table 1. Primer sets for real-time RT-PCR.

Gene (GeneBank accession)	Primer sequences	Product size
TNFα (NM_013693)	forward: 5'-AAGCCTGTAGCCCACGTCGTA-3' reverse: 5'-GGCACCACTAGTTGGTTGTCTTTG-3'	122 bp
TGFβ1 (NM_011577)	forward: 5'-GTGTGGAGCAACATGTGGAACTCTA-3' reverse: 5'-TTGGTTCAGCCACTGCCGTA-3'	143 bp
COL1A1 (NM_007742)	forward: 5'-CCTGGCAAAGACGGACTCAAC-3' reverse: 5'-GCTGAAGTCATAACCGCCACTG-3'	150 bp
TIMP-1 (NM_011593)	forward: 5'-CGAATCAACGAGACCACCTT-3' reverse: 5'-GTAGTCCTCAGAGCCCACGA-3'	399 bp
IL-4 (NM_021283)	forward: 5'-TCTCGAATGTACCAGGAGCCATATC-3' reverse: 5'-AGCACCTTGGAAGCCCTACAGA-3'	183 bp
IFNγ (NM_008337)	forward: 5'-CGGCACAGTCATTGAAAGCCTA-3' reverse: 5'-GTTGCTGATGGCCTGATTGTC-3'	199 bp
IL-6 (NM_031168)	forward: 5'-CCACTTCACAAGTCGGAGGCTTA-3' reverse: 5'-GCAAGTGCATCATCGTTGTTCATAC-3'	112 bp
GAPDH (NM_001001303)	forward: 5'-TGTGTCCGTCGTGGATCTGA-3' reverse: 5'-TTGCTGTTGAAGTCGCAGGAG-3'	150 bp