Published in final edited form as:

J Hepatol. 2017 October ; 67(4): 791–800. doi:10.1016/j.jhep.2017.05.024.

Crosstalk between type II NKT cells and T cells leads to spontaneous chronic inflammatory liver disease

Xiufang Weng^{1,2}, Ying He¹, Lavanya Visvabharathy¹, Chia-Min Liao^{1,3}, Xiaosheng Tan², Arjun Balakumar¹, and Chyung-Ru Wang¹

¹Department of Microbiology and Immunology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611

²Department of Immunology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Abstract

Background & AIM—NKT cells are CD1d-restricted innate-like T cells that modulate innate and adaptive immune responses. Unlike the well-characterized invariant/type I NKT cells, type II NKT cells with a diverse TCR repertoire are poorly understood. This study defines the pathogenic role of type II NKT cells in the etiology of chronic liver inflammation.

Methods—Transgenic mice with the *Lck* promoter directing CD1d overexpression on T cells in *Ja18* wild-type (*Lck-CD1dTgJa18⁺*; type I NKT cell sufficient) and *Ja18*-deficient (*Lck-CD1dTgJa18⁰*; type I NKT cell deficient) mice were analyzed for liver pathology and crosstalk between type II NKT cells and conventional T cells. CD1d expression on T cells in peripheral blood samples and liver sections from AIH patients and healthy individuals were also examined.

Results—*Lck-CD1dTgJa18⁰* and *Lck-CD1dTgJa18⁺* mice developed similar degrees of liver pathology resembling chronic autoimmune hepatitis in humans. Increased CD1d expression on T cells promoted the activation of type II NKT cells and other T cells. This resulted in T_H1 -skewing and impaired T_H2 cytokine production in type II NKT cells. Dysfunction of type II NKT cells was accompanied by conventional T cell activation and pro-inflammatory cytokine production, leading to a hepatic T/B lymphocyte infiltration, elevated autoantibodies and hepatic injury in *Lck-CD1dTg* mice. A similar mechanism could be extended to humans as CD1d expression is

Corresponding authors: Dr. Chyung-Ru Wang, Department of Microbiology and Immunology, Northwestern University, 320 E. Superior Street, Searle 3-401, Chicago, IL 60611, Phone: (312) 503-9748; Fax: (312) 503-9594, chyung-ru-wang@northwestern.edu. Dr. Xiufang Weng, Department of Immunology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, 13# Hangkong Rd, Wuhan, Hubei, China. Phone: 86 (27) 83692611; Fax: 86 (27) 83693500, wengxiufang@hust.edu.cn.

³Present address: Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia, Philadelphia, PA 19104

Conflict of interest

The authors have declared that no competing interests exist with respect to this manuscript

Authors contributions

X.W. and C.-R. W. conceived and designed the experiments. X.W., Y.H., L.V., C.L., X.T. and A.B. performed the experiments. X.W., Y.H., L.V., C.L., X. T. and C.-R.W. analyzed the data. X.W., L.V. and C.-R.W. wrote the manuscript.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

upregulated on activated human T cells and increased presence of CD1d-expressing T cells was observed in AIH patients.

Conclusions—Our data reveals enhanced crosstalk between type II NKT cells and conventional T cells leads to a Th1-skewed inflammatory milieu, leading to the development of chronic autoimmune liver disease.

Graphical abstract

Enhanced crosstalk between type II NKT cells and T cells triggers a polarized Th1 response, which lead to spontaneous chronic inflammatory liver disease.

Keywords

CD1d; NKT cells; Autoimmune hepatitis; Liver inflammation; Transgenic/knockout mice

Introduction

The liver is constantly exposed to large varieties of antigens derived from the gastrointestinal tract as well as from systemic circulation, a possible explanation for why liver tissue is subject to a greater degree of immune tolerance than some other organs [1]. Despite this, disruption of tolerance leads to inflammatory liver injuries during chronic diseases such as persistent viral infection [2], drug toxicity [3] and autoimmune hepatitis (AIH) [4]. As one of the predominant lymphocyte populations in the liver, CD1d-restricted NKT cells contribute to the maintenance of immune tolerance but also direct adverse immune responses in the liver [5, 6]. NKT cells are divided into two subsets based on TCR usage: type I NKT cells expressing a semi-invariant TCR (V α 14J α 18 in mice and V α 24J α 18 in human), and type II NKT cells having a diverse TCR repertoire. While type I NKT cells are the dominant NKT cell subset in mice, type II NKT cells appear to be more prevalent in humans [7]. Unlike type I NKT cells which can be unambiguously detected by α -galactosylceramide-loaded CD1d tetramer, type II NKT cells are more difficult to study because of the lack of specific markers that can uniquely define them. Although NK1.1 and CD161 are expressed in most NKT cells in mice and humans, NK1.1/CD161 positive T cells also comprise several other innate-like T cell subsets including the newly discovered mucosal associated invariant T (MAIT) cells [8, 9]. Therefore, we have utilized a variety of markers such as NK1.1⁺ CD8⁻ and functional phenotypes to define type II NKT cells more accurately in order to determine the contributions of type II NKT cells to inflammatory liver disease.

CD1d is a non-polymorphic MHC class I-like molecule which presents self- and foreign lipid antigens to NKT cells. CD1d is expressed on a variety of hematopoietic and non-

hematopoietic cells and its altered expression levels are correlated with several disease conditions. While certain viral infections such as human immunodeficiency virus and herpes simplex virus type-1 infection down-regulate CD1d expression as a viral immune evasion strategy [10, 11], CD1d expression is enhanced during chronic inflammatory and autoimmune diseases. In experimental autoimmune encephalomyelitis, CD1d is upregulated on macrophages and T cells invading inflamed CNS tissue [12]. Furthermore, CD1d is strongly upregulated in the liver during hepatitis C infection, which might contribute to liver injury during chronic infection [7, 13]. This raises the possibility that functional differences in NKT cells may be partially mediated by differential interactions between NKT cells and different types of antigen presenting cells (APC) expressing elevated levels of CD1d.

Several transgenic mouse models have been used to examine the effect of altered CD1d expression patterns on NKT cell development and function. By manipulating CD1d expression under the control of a *MHC I*, *MHC II* or proximal *Lck* promoter in the CD1d-deficient background [14–17], we and others have shown that only mice with transgenic *CD1d* driven by *Lck* promoter (*Lck-CD1dTg* mice) are sufficient to support NKT cell development [15, 16]. Interestingly, the *Lck-CD1dTg* mouse model we generated in which both thymocytes and peripheral T cells express high levels of CD1d develops liver pathology in the absence of any exogenous manipulation [16]. Although peripheral T cells express low levels of CD1d in both humans and mice, CD1d could be upregulated on T cells by activation *in vivo* or *in vitro* [18]. Apart from the critical role of thymocytes in NKT cell selection, it is not yet clear whether T cells can function as CD1d-restricted APCs.

Our previous study showed that type I NKT cells in *Lck-CD1dTg* mice are hypo-responsive to α -GalCer stimulation [16]. However, it is unclear whether altered CD1d expression also affects the function of type II NKT cells and by extension whether type II NKT cells contribute to the development of liver pathology. We utilized *Lck-CD1d* transgenic mice to determine whether enhanced crosstalk between type II NKT cells and conventional T cells in the liver affects the development of chronic hepatic inflammation.

Materials and Methods

Mice

Ja18^o, *K^b-CD1dTg*, *Lck-CD1dTg*, *IL-4 GFP* enhanced transcript (4get), and *24a β Tg* mice have been described elsewhere [16, 19–23]. *Lck-CD1dTg* mice were crossed with *Ja18^o* and *24a β Tg* mice to obtain *Lck-CD1dTgJa18^o* and *Lck-CD1dTg24a β Tg*, respectively and then further crossed with 4get mice to track IL-4-expressing cells. Both male and female mice were used in this study. All animal studies and procedures were approved by the Northwestern University Institutional Animal Care and Use Committee.

Human samples

Blood samples from healthy donors and AIH patients were collected from Tongji Hospital, Wuhan, China. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. Paraffin-embedded human normal and AIH liver sections were obtained from tissue bank of Tongji Hospital. Individuals with viral hepatitis, HIV infection,

or alcoholic liver disease were excluded. The characteristics of AIH patients included in this study were detailed in Supplementary Table 2. The project and protocols involving human samples were approved by the ethics committee of Tongji Medical College. Informed consent was obtained from all subjects.

Cell preparations and analysis

Mouse thymocytes, splenocytes, and hepatic leukocytes were prepared by mechanical dispersion as described previously [24]. Type II NKT, conventional T, 24 α β T and B cell populations were purified by FACS or magnetic-activated cell sorting (MACS) using specific antibodies. Bone marrow-derived DCs (BMDCs) were prepared as previously described [19]. Cell surface staining, T cell activation and intracellular cytokine staining were performed as described [25]. Flow cytometry, proliferation assay, cytokine release detection, and RT-qPCR were performed to determine the phenotype and function of various cell types.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Data were analyzed using unpaired Student's *t* test for 2 group comparisons or one way ANOVA for more than two group comparisons, followed by Bonferroni post-hoc test. Values are mean + SEM. Statistical significance is indicated by the following annotation: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Additional descriptions of methodology and reagents are provided in the Supplementary Materials and Methods section.

Results

Lck-CD1dTgJa 18⁰ mice develop chronic inflammatory liver disease spontaneously

We have previously shown that *Lck-CD1dTg* mice develop liver hypertrophy spontaneously [16], regardless of whether the endogenous CD1d is present or not. The liver hypertrophy and the elevated liver-to-body weight ratio found in *Lck-CD1dTgJa 18⁺* mice were also observed in *Lck-CD1dTgJa 18⁰* mice (Figure 1A, B). Additionally, the *Lck-CD1dTgJa 18⁰* mice exhibited splenomegaly, which is often caused by shunting of blood from the liver to the spleen as a result of portal hypertension during chronic liver disease [26]. In contrast, *Lck-CD1dTgJa 18⁰* kidneys were normal in size (Figure 1A). Both *Lck-CD1dTgJa 18⁺* and *Lck-CD1dTgJa 18⁰* mice had elevated hepatic leukocyte numbers and ALT levels when compared to wild-type (WT) and *Ja 18⁰* mice (Figure 1B). Furthermore, H&E stained liver sections showed that hepatocytes from *Lck-CD1dTgJa 18⁰* mice exhibited cytomegaly similar to *Lck-CD1dTg* mice (Figure 1C). In fact, we observed no significant difference in liver-to-body weight ratio, the degree of leukocyte infiltration and changes in liver histology (Figure 1C) between *Lck-CD1dTgJa 18⁺* and *Lck-CD1dTgJa 18⁰* mice. These data suggest that type I NKT cells are dispensable for the development of liver pathology in this model.

To gain a deeper insight into the liver pathology in *Lck-CD1dTgJa 18⁰* mice, immunohistochemical analysis of liver sections was performed. Staining with oval cell-

specific antibody and TUNEL staining unveiled significant oval cell hyperplasia and cell death in *Lck-CD1dTgJa18⁰* livers (Figure 1D, E). Anti-CD45 staining demonstrated leukocyte infiltration and formation of inflammatory foci in livers (Figure 1F). Flow cytometry revealed T and B cells to be two major infiltrating leukocyte populations in *Lck-CD1dTgJa18⁰* livers, though myeloid cells were also slightly increased in number (Figure 1G). Additionally, the severity of liver disease in *Lck-CD1dTgJa18⁰* mice progressed with age and was more profound in female mice. At 11 months of age, most of the female *Lck-CD1dTgJa18⁰* mice had macroscopically visible liver nodules (Figure 1H) while this was largely absent in male mice (data not shown). H&E staining of liver sections from these mice revealed portal inflammation, hepatocyte injury, hepatocyte steatosis (Figure 1I, left panel), and the presence of regenerative nodules surrounded by fibrous connective tissue (Figure 1I, right panel), which bear resemblance to AIH in humans (Supplementary Figure 1).

CD1d-restricted type II NKT cells are reduced in number and dysfunctional in *Lck-CD1dTgJa 18⁰* mice

Type II NKT cells are the major CD1d-restricted T cell population in *Lck-CD1dTgJa18⁰* mice. Because NK1.1, CD122 and IL-4 transcript are expressed in most type II NKT cells in WT mice [23], we sought to evaluate type II NKT cells using NK1.1, CD122 and IL-4-eGFP (4get-GFP) as surrogate markers. Because most CD1d-restricted NKT cells in mice exhibit a CD4⁺ or CD4⁻CD8⁻ (DN) phenotype, we focused our analysis on CD8 negative (CD8⁻) T cell population. We observed a significant decrease in the percentage and number of TCRβ⁺NK1.1⁺CD8⁻, TCRβ⁺CD122⁺CD8⁻ and TCRβ⁺4get-GFP⁺CD8⁻ cells in hepatic lymphocytes from *Lck-CD1dTgJa18⁰* mice compared to those from *Ja18⁰* mice (Figure 2A–C). The percentage and number of type II NKT cells in the thymus of *Lck-CD1dTgJa18⁰* and *Ja18⁰* mice were comparable (Figure 2D–F), indicating the reduction of type II NKT cells in *Lck-CD1dTgJa18⁰* mice was mainly a peripheral event.

FACS sorted type II NKT cells (defined as TCRβ⁺NK1.1⁺CD8⁻) from *Ja18⁰* and *Lck-CD1dTgJa18⁰* livers exhibited CD1d-dependent IFN-γ production upon stimulation with CD1d-overexpressing BMDCs which express CD1d under the control of the MHC-Ia promoter [19] (Figure 3A). However, these type II NKT cells produced lower levels of IFN-γ than those from *Ja18⁰* mice. Further, type II NKT cells from *Lck-CD1dTgJa18⁰* mice were completely defective in IL-4 and IL-13 production upon stimulation with K^b-CD1dTg BMDCs (Figure 3B). Analysis of TCR Vα and Vβ usage by type II NKT cells showed only subtle differences between *Ja18⁰* and *Lck-CD1dTgJa18⁰* mice (Supplementary Figure 2), indicating that the functional impairments observed were unlikely to be attributed to changes in the TCR repertoire of type II NKT cells.

We have previously shown that type II NKT cells have an activated phenotype as CD69^{hi}CD44^{hi}CD62L^{lo}CD122⁺ [23]. Although most type II NKT cells in *Lck-CD1dTgJa18⁰* mice expressed similar levels of CD122 and CD62L as those from *Ja18⁰* mice, they expressed higher levels of CD69 and CD44, suggesting their hyper-activated status *in vivo* (Figure 3C). Hepatic type II NKT cells in *Lck-CD1dTgJa18⁰* mice also had a higher percentage of cell death compared with *Ja18⁰* mice (Figure 3D). This suggested that

activation induced cell death may account for the decreased number of type II NKT cells in *Lck-CD1dTgJa18^o* mice. In contrast, conventional T cells in *Lck-CD1dTgJa18^o* mice did not show increased cell death when compared to that of *Ja18^o* mice. Collectively, these data suggested the type II NKT cells in *Lck-CD1dTgJa18^o* mice are chronically activated and thus hyporesponsive to antigen stimulation, resulting in impaired cytokine responses.

CD1d overexpression on conventional T cells is sufficient to drive type II NKT cell activation and proliferation

To further determine the effects of enhanced T cell-specific CD1d expression on the functionality of type II NKT cells, we crossed monoclonal type II NKT cell TCR transgenic mice (*24αβTg*) [22] into the *Lck-CD1dTg* background (*Lck-CD1dTg/24αβTg*). Although total liver leukocytes increased, the percentage and number of Vα3.2⁺Vβ9⁺ 24αβ T cells significantly decreased in the liver of *Lck-CD1dTg/24αβ Tg* mice compared to *24αβ Tg* mice (Figure 4A, B). This is consistent with the decrease in polyclonal type II NKT cells observed in the *Lck-CD1dTgJa18^o* liver (Figure 2A–C). Moreover, 24αβ T cells similarly lost the capacity to produce IL-4, as shown by the decreased 4get-GFP expression (Figure 4C). The elevated CD69 and CD44 expression in 24αβ T cells in *Lck-CD1dTg/24αβ Tg* mice suggested that dysfunctional monoclonal type II NKT cells were also hyperactivated *in vivo* (Figure 4C).

To determine which APC is primarily responsible for activating type II NKT cells in *Lck-CD1dTg* mice, we compared the stimulatory capacity of T cells, B cells, and BMDCs from *Lck-CD1dTg* mice on 24αβ T cells. 24αβ T cells upregulated CD69 expression and underwent extensive cell proliferation following stimulation with *Lck-CD1dTg*-expressing T (Tg⁺ T) cells, but not WT T (Tg⁻ T) cells (Figure 4D). This effect was abolished in the presence of anti-CD1d mAb, confirming these responses to be CD1d-dependent. In contrast, neither BMDCs nor B cells from *Lck-CD1dTg* mice, which expressed WT levels of CD1d (Supplementary Figure 3), had comparable stimulatory capacities (Figure 4D). This suggests that increased CD1d expression on T cells alone is sufficient to induce type II NKT cell activation.

Enhanced interaction between type II NKT cells and conventional T cells leads to conventional T cell activation in *Lck-CD1dTgJa18^o* mice

NKT cells are known to modulate T cell responses [23, 27]. In order to study the reciprocal effects between type II NKT cells and *Lck-CD1dTg*-expressing T cells *in vivo*, CFSE-labeled 24αβ T cells were adoptively transferred into 1-month-old *Ja18^o* and *Lck-CD1dTgJa18^o* mice. The proliferative capacity of 24αβ T cells and the percentage of CD44^{high}CD62L^{low} effector T (T_{eff}) cells in recipient mice were compared 7 days and 1-month post-transfer. We chose one-month-old mice as recipients because liver pathology in *Lck-CD1dTgJa18^o* mice becomes apparent at 2 months of age. We consistently found that naïve 24αβ T cells exhibited a higher degree of proliferation when adoptively transferred to *Lck-CD1dTgJa18^o* mice, compared to that of *Ja18^o* mice (Figure 4E). Moreover, there was a significant increase in T_{eff} cells in the liver of *Lck-CD1dTgJa18^o* mice that received 24αβ T cells as compared to the liver of *Lck-CD1dTgJa18^o* mice that had not received 24αβ T cells (Figure 4F).

To address whether polyclonal type II NKT cells had similar effects on conventional T cells, we evaluated CD4⁺ and CD8⁺ T cells in *Lck-CD1dTgJa18^o* mice. CD4⁺ and CD8⁺ T cell numbers increased in the liver of *Lck-CD1dTgJa18^o* mice compared to *Ja18^o* controls (Figure 5A). Foxp3⁺ regulatory CD4⁺ T cells (T_{reg}) also increased in *Lck-CD1dTgJa18^o* mice compared to controls (Figure 5B), suggesting that inflammation in *Lck-CD1dTgJa18^o* livers was not due to impaired T_{reg} cell differentiation. However, *Lck-CD1dTgJa18^o* mice had a higher frequency of T_{eff} cells in both the CD4⁺ and CD8⁺ T cell compartments compared to age and sex-matched controls (Figure 5C, D).

Functional analysis of conventional T cells in *Lck-CD1dTgJa18^o* mice showed that IFN- γ producing cells, but not IL-4 producing cells, increased significantly in *Lck-CD1dTgJa18^o* mice compared with *Ja18^o* controls (Figure 5E). Multiplex cytokine assays showed that CD8⁺ T cells from *Lck-CD1dTgJa18^o* livers produced more T_h1-like cytokines upon TCR stimulation (Figure 5F), and conventional CD4⁺ (NK1.1⁻) T cells produced more IL-17 and less IL-4, when compared with their counterparts from *Ja18^o* livers (Figure 5G). Imbalanced T_h1/T_h2 responses and/or increases in T_h17 proportions in the liver have long been thought to be associated with persistent inflammation and chronic liver pathology [28]. Thus, increased pro-inflammatory cytokine production by conventional T cells could partially account for T-cell mediated liver injury in *Lck-CD1dTgJa18^o* mice.

B-cell activation and autoantibodies are increased in *Lck-CD1dTgJa18^o* mice

It has been suggested that hyperactive T cells promote autoreactive B cell responses through increasing costimulatory molecule signaling, cytokines, and/or other stimulatory factors [29]. We found CD4⁺ T cells from *Lck-CD1dTgJa18^o* liver expressed higher mRNA levels of the B-cell activating factors BAFF and IFN- α (Figure 6A). IFN- α upregulates BAFF, and BAFF has been shown to promote autoreactive B cell activation [30]. Indeed, B cells were greatly increased in *Lck-CD1dTgJa18^o* livers (Figure 1G) and were mainly mature B-2 cells (Supplementary Figure 4). They upregulated CD69 to a higher degree and produced more pro-inflammatory IL-6 upon LPS stimulation as compared to B cells from *Ja18^o* livers (Figure 6B–C). Serum IgM levels (but not IgG) were also increased in *Lck-CD1dTgJa18^o* mice that were older than 4 months (Figure 6D and data not shown). Additionally, elevated anti-dsDNA antibodies were detected in *Lck-CD1dTgJa18^o* mice, with IgM and IgG2a/c being notably elevated (Figure 6E). Collectively, our data suggests that the increased inflammatory T cell response in *Lck-CD1dTgJa18^o* mice promotes B cell activation and autoantibody production.

Dysregulation of type II NKT and T cells is also observed in *Lck-CD1dTgJa18⁺* mice

A recent study described a perturbed TCR- α repertoire with lower TCR diversity in *Ja18^o* mice [31]. To assess whether the dysfunction of type II NKT, T and B cells was influenced by changes in TCR repertoire in *Lck-CD1dTgJa18^o* mice, we evaluated the functional status of these cell subsets in *Lck-CD1dTgJa18⁺* mice. Type II NKT cells, defined as CD1d- α GalCer tetramer⁻CD8⁻TCR β ⁺NK1.1⁺, were significantly reduced in *Lck-CD1dTgJa18⁺* mice compared to *Ja18⁺* WT mice (Figure 7A). Moreover, impaired IL-4 production and over-activation of type II NKT cells in *Lck-CD1dTgJa18⁺* mice were also observed (Figure

7B). These results suggested similar dysregulation of type II NKT cells in *Lck-CD1dTgJa18⁺* mice as seen in *Lck-CD1dTgJa18⁰* mice.

In further support of the congruence of these two models, conventional T and B cells remained the two major populations of infiltrating leukocytes in *Lck-CD1dTgJa18⁺* mice (Figure 7C). Enhanced activation and T_H1-biased cytokine production in conventional T cells were also observed in *Lck-CD1dTgJa18⁺* mice, along with elevated levels of circulating autoantibodies (Figure 7D–F). Collectively, these results showed that the dysfunctional phenotypes of type II NKT, conventional T and B cells observed in *Lck-CD1dTgJa18⁰* mice were also found in *Lck-CD1dTgJa18⁺* mice.

Overexpression of CD1d on T cells are found in AIH patients

Since the human liver contains a high frequency of type II NKT cells, it will be of great interest to investigate whether elevated CD1d expression can be detected in AIH patients. Consistent with a previous report that activated T cells upregulate CD1d expression [18], we found in vitro activation of human T cells with anti-CD3 or IFN- γ treatment resulted in CD1d upregulation (Figure 8A). In addition, ex vivo analysis of CD1d expression on peripheral blood T cells revealed higher CD1d expression on pre-activated CD69⁺ T cells than that on CD69⁻ T cells (Figure 8B). Interestingly, PBMC from human AIH patients have increased frequency of activated CD69⁺ T cells, suggesting more circulating CD1d-expressing T cells in AIH patients (Figure 8C). Furthermore, in situ immunofluorescence co-staining of CD3 and CD1d unveiled a proportion of infiltrated T cells expressed high level of CD1d in AIH liver (Figure 8D). These data suggest that upregulation of CD1d on T cells and possibly aberrant activation of NKT cells may contribute to the development of clinical AIH.

Discussion

NKT cells have been shown to play either a pathogenic or protective role in inflammatory liver disease [32, 33]. However, most of these studies used a model of ConA-induced hepatitis which causes acute liver inflammation but does not reflect chronic disease seen in human AIH. Here, we showed that aberrant CD1d expression on T cells resulted in hyperactivation of type II NKT cells and conventional T cells, leading to the development of chronic inflammatory liver disease in the absence of any exogenous antigenic or pharmacological stimuli. Liver pathology observed in *Lck-CD1dTgJa18⁰* mice showed some features reminiscent of human AIH (e.g. portal inflammation, hepatocyte injury, hepatic steatosis and fibrosis), while no signs of pathology were observed in the kidney, stomach, small intestine and colon of these mice. In addition, the disease severity is more profound in female *Lck-CD1dTgJa18⁰* mice as female *Lck-CD1dTgJa18⁰* mice tended to have higher liver to body weight ratios and more severe histopathology than male *Lck-CD1dTgJa18⁰* mice. This is consistent with the finding that AIH is more common in female patients. Further, hypergammaglobulinemia and autoantibody production commonly associated with AIH were also found in *Lck-CD1dTgJa18⁰* mice. Our study thus suggests a potential role for type II NKT cells in the pathogenesis of AIH.

Our finding that *Lck-CD1dTgJa18⁺* and *Lck-CD1dTgJa18^o* mice had similar degrees of liver pathology suggests either that type I and type II NKT cells play a redundant role or that type II NKT cells play a dominant role in liver pathogenesis in this mouse model. The *Ja18^o* mice used in this study have been shown to have lower TCR diversity due to suppressed transcription of *TRAJ* gene segments upstream of *Traj18(Ja18)* [31]. However, the influence of this change on liver inflammation in *Lck-CD1dTgJa18^o* mice is limited because similar liver pathology, immune cell infiltration/activation, and B cell autoantibody production were found in *Lck-CD1dTgJa18⁺* and *Lck-CD1dTgJa18^o* mice. Additionally, we have shown that type II NKT cells in *Ja18^o* mice closely resemble those from wild-type mice in terms of their surface phenotype and cytokine-producing capacity [23], despite having lower TCR diversity.

Previous studies have shown the importance of conventional T cell activation in the etiology and pathogenesis of AIH [34, 35]. Several studies have suggested that impaired T_{reg} function is responsible for over-activation of CD4⁺ and CD8⁺ T cells, which can lead to a breakdown in tolerance to liver antigens and a progression towards liver disease [36, 37]. Here, we showed that type II NKT cells may also play a role in the establishment of AIH. NKT cells from *Lck-CD1dTgJa18^o* mice were able to produce IFN- γ but not IL-4/IL-13 in a CD1d-dependent manner (Figure 3A, B). Though total IFN- γ production by type II NKT cells in *Lck-CD1dTgJa18^o* mice was lower than that in *Ja18^o* mice, the complete lack of IL-4 and IL-13 production suggests that T_H2-mediated inhibition of the T_H1 response in these mice is impaired. This could lead to chronic, low-level production of IFN- γ which can, over time, activate conventional T cells to produce pro-inflammatory cytokines leading to liver pathology. It is unknown why type II NKT cells were unable to produce T_H2 cytokines in *Lck-CD1dTgJa18^o* mice, but we speculate that overexpression of CD1d on T cells induces a chronic, sub-optimal stimulation of type II NKT cells, leading to their aberrant activation and preferential T_H1 polarization.

Finally, increased CD1d expression has been reported in several disease settings and in response to cytokine treatments [7, 12, 38, 39]. Here, we provide evidence that elevated CD1d expression on T cells promotes enhanced interaction between type II NKT and T cells, which leads to T_H1-biased inflammatory liver disease in *Lck-CD1dTgJa18^o* mice. We also present data showing that increased CD1d expression on T cells can be seen in AIH patients. The findings in this study are the first to show the effect of altered CD1d expression on the function of type II NKT cells at the polyclonal level and demonstrate their role in the pathogenesis of spontaneous inflammatory liver disease. Taken together, these results suggest that type II NKT cells, which are enriched in the human liver, could be an important clinical target in the treatment of AIH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support

This work was supported by the National Institutes of Health [NIH grant R01AI43407 to C.-R.W.] and National Nature Science Foundation of China [NSFC grant 31570913 to X. W.]

We thank the Northwestern University flow cytometry core facility for cell sorting; mouse histology & phenotyping laboratory for histology services; the NIH tetramer facility for CD1d tetramer; Sreya Bagchi for critical reading of the manuscript, Dr. Guang-Yu Yang for advice on histological assessment of liver pathology and Dr. Susanna Cardell for providing *24aβ Tg* mice.

References

1. Crispe IN. Immune tolerance in liver disease. *Hepatology*. 2014; 60:2109–2117. [PubMed: 24913836]
2. Koike K. Steatosis, liver injury, and hepatocarcinogenesis in hepatitis C viral infection. *Journal of gastroenterology*. 2009; 44(Suppl 19):82–88. [PubMed: 19148799]
3. Woolbright BL, Jaeschke H. Xenobiotic and Endobiotic Mediated Interactions Between the Cytochrome P450 System and the Inflammatory Response in the Liver. *Advances in pharmacology*. 2015; 74:131–161. [PubMed: 26233906]
4. Liaskou E, Hirschfield GM, Gershwin ME. Mechanisms of tissue injury in autoimmune liver diseases. *Seminars in immunopathology*. 2014; 36:553–568. [PubMed: 25082647]
5. Crispe IN. Hepatic T cells and liver tolerance. *Nature reviews Immunology*. 2003; 3:51–62.
6. Gao B, Jeong WI, Tian Z. Liver: An organ with predominant innate immunity. *Hepatology*. 2008; 47:729–736. [PubMed: 18167066]
7. Durante-Mangoni E, Wang R, Shaulov A, He Q, Nasser I, Afdhal N, et al. Hepatic CD1d expression in hepatitis C virus infection and recognition by resident proinflammatory CD1d-reactive T cells. *Journal of immunology*. 2004; 173:2159–2166.
8. Fergusson JR, Fleming VM, Klenerman P. CD161-expressing human T cells. *Frontiers in immunology*. 2011; 2:36. [PubMed: 22566826]
9. Tilloy F, Treiner E, Park SH, Garcia C, Lemonnier F, de la Salle H, et al. An invariant T cell receptor alpha chain defines a novel TAP-independent major histocompatibility complex class Ib-restricted alpha/beta T cell subpopulation in mammals. *The Journal of experimental medicine*. 1999; 189:1907–1921. [PubMed: 10377186]
10. Chen N, McCarthy C, Drakesmith H, Li D, Cerundolo V, McMichael AJ, et al. HIV-1 down-regulates the expression of CD1d via Nef. *European journal of immunology*. 2006; 36:278–286. [PubMed: 16385629]
11. Yuan W, Dasgupta A, Cresswell P. Herpes simplex virus evades natural killer T cell recognition by suppressing CD1d recycling. *Nature immunology*. 2006; 7:835–842. [PubMed: 16845396]
12. Busshoff U, Hein A, Iglesias A, Dorries R, Regnier-Vigouroux A. CD1 expression is differentially regulated by microglia, macrophages and T cells in the central nervous system upon inflammation and demyelination. *Journal of neuroimmunology*. 2001; 113:220–230. [PubMed: 11164905]
13. Exley MA, He Q, Cheng O, Wang RJ, Cheney CP, Balk SP, et al. Cutting edge: Compartmentalization of Th1-like noninvariant CD1d-reactive T cells in hepatitis C virus-infected liver. *Journal of immunology*. 2002; 168:1519–1523.
14. Xu H, Chun T, Colmone A, Nguyen H, Wang CR. Expression of CD1d under the control of a MHC class Ia promoter skews the development of NKT cells, but not CD8+ T cells. *Journal of immunology*. 2003; 171:4105–4112.
15. Wei DG, Lee H, Park SH, Beaudoin L, Teyton L, Lehuen A, et al. Expansion and long-range differentiation of the NKT cell lineage in mice expressing CD1d exclusively on cortical thymocytes. *The Journal of experimental medicine*. 2005; 202:239–248. [PubMed: 16027237]
16. Zimmer MI, Colmone A, Felio K, Xu H, Ma A, Wang CR. A cell-type specific CD1d expression program modulates invariant NKT cell development and function. *Journal of immunology*. 2006; 176:1421–1430.
17. Forestier C, Park SH, Wei D, Benlagha K, Teyton L, Bendelac A. T cell development in mice expressing CD1d directed by a classical MHC class II promoter. *Journal of immunology*. 2003; 171:4096–4104.

18. Salamone MC, Rabinovich GA, Mendiguren AK, Salamone GV, Fainboim L. Activation-induced expression of CD1d antigen on mature T cells. *Journal of leukocyte biology*. 2001; 69:207–214. [PubMed: 11272270]
19. Chun T, Page MJ, Gapin L, Matsuda JL, Xu H, Nguyen H, et al. CD1d-expressing dendritic cells but not thymic epithelial cells can mediate negative selection of NKT cells. *The Journal of experimental medicine*. 2003; 197:907–918. [PubMed: 12682110]
20. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science*. 1997; 278:1626–1629. [PubMed: 9374463]
21. Mohrs M, Shinkai K, Mohrs K, Locksley RM. Analysis of type 2 immunity in vivo with a bicistronic IL-4 reporter. *Immunity*. 2001; 15:303–311. [PubMed: 11520464]
22. Skold M, Faizunnessa NN, Wang CR, Cardell S. CD1d-specific NK1. 1+ T cells with a transgenic variant TCR. *Journal of immunology*. 2000; 165:168–174.
23. Zhao J, Weng X, Bagchi S, Wang CR. Polyclonal type II natural killer T cells require PLZF and SAP for their development and contribute to CpG-mediated antitumor response. *Proceedings of the National Academy of Sciences of the United States of America*. 2014; 111:2674–2679. [PubMed: 24550295]
24. Felio K, Nguyen H, Dascher CC, Choi HJ, Li S, Zimmer MI, et al. CD1-restricted adaptive immune responses to Mycobacteria in human group 1 CD1 transgenic mice. *The Journal of experimental medicine*. 2009; 206:2497–2509. [PubMed: 19808251]
25. Li S, Choi HJ, Felio K, Wang CR. Autoreactive CD1b-restricted T cells: a new innate-like T-cell population that contributes to immunity against infection. *Blood*. 2011; 118:3870–3878. [PubMed: 21860021]
26. Theodorakis N, Maluccio M, Skill N. Murine study of portal hypertension associated endothelin-1 hypo-response. *World J Gastroenterol*. 2015; 21:4817–4828. [PubMed: 25944995]
27. Terabe M, Matsui S, Park JM, Mamura M, Noben-Trauth N, Donaldson DD, et al. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *The Journal of experimental medicine*. 2003; 198:1741–1752. [PubMed: 14657224]
28. Wick G, Grundtman C, Mayerl C, Wimpissinger TF, Feichtinger J, Zelger B, et al. The immunology of fibrosis. *Annual review of immunology*. 2013; 31:107–135.
29. Ando DG, Sercarz EE, Hahn BH. Mechanisms of T and B cell collaboration in the in vitro production of anti-DNA antibodies in the NZB/NZW F1 murine SLE model. *Journal of immunology*. 1987; 138:3185–3190.
30. Jacob N, Guo S, Mathian A, Koss MN, Gindea S, Putterman C, et al. B Cell and BAFF dependence of IFN-alpha-exaggerated disease in systemic lupus erythematosus-prone NZM 2328 mice. *Journal of immunology*. 2011; 186:4984–4993.
31. Bedel R, Matsuda JL, Brigl M, White J, Kappler J, Marrack P, et al. Lower TCR repertoire diversity in Traj18-deficient mice. *Nature immunology*. 2012; 13:705–706. [PubMed: 22814339]
32. Halder RC, Aguilera C, Maricic I, Kumar V. Type II NKT cell-mediated anergy induction in type I NKT cells prevents inflammatory liver disease. *The Journal of clinical investigation*. 2007; 117:2302–2312. [PubMed: 17641782]
33. Maricic I, Girardi E, Zajonc DM, Kumar V. Recognition of lysophosphatidylcholine by type II NKT cells and protection from an inflammatory liver disease. *Journal of immunology*. 2014; 193:4580–4589.
34. Than NN, Jeffery HC, Oo YH. Autoimmune Hepatitis: Progress from Global Immunosuppression to Personalised Regulatory T Cell Therapy. *Canadian journal of gastroenterology & hepatology*. 2016; 2016:7181685. [PubMed: 27446862]
35. Czaja AJ. Autoimmune hepatitis. Part A: pathogenesis. *Expert review of gastroenterology & hepatology*. 2007; 1:113–128. [PubMed: 19072440]
36. Liberal R, Grant CR, Holder BS, Cardone J, Martinez-Llordella M, Ma Y, et al. In autoimmune hepatitis type 1 or the autoimmune hepatitis-sclerosing cholangitis variant defective regulatory T-

- cell responsiveness to IL-2 results in low IL-10 production and impaired suppression. *Hepatology*. 2015; 62:863–875. [PubMed: 25953611]
37. An Haack I, Derkow K, Riehn M, Rentinck MN, Kuhl AA, Lehnardt S, et al. The Role of Regulatory CD4 T Cells in Maintaining Tolerance in a Mouse Model of Autoimmune Hepatitis. *PLoS One*. 2015; 10:e0143715. [PubMed: 26599014]
38. Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS, Bhan AK. Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. *Immunity*. 2002; 16:219–230. [PubMed: 11869683]
39. Raghuraman G, Geng Y, Wang CR. IFN-beta-mediated up-regulation of CD1d in bacteria-infected APCs. *Journal of immunology*. 2006; 177:7841–7848.

Highlights

1. CD1d overexpression alters type II NKT cell activation/polarization polyclonally.
2. Dysfunctional type II NKT cells trigger an inflammatory loop with T and B cells.
3. Enhanced cross-talk among type II NKT/T/B cells leads to autoantibody production.
4. A pathogenic role of type II NKT cells in autoimmune liver diseases is proposed.

Figure 2. The frequency and number of type II NKT cells are reduced in the liver of *Lck-CD1dTgJa 18⁰* mice

Hepatic lymphocytes and thymocytes were isolated from *Ja18⁰* (n=23), *Lck-CD1dTgJa18⁰* (n=17), *Ja18⁰4get* (n=7), and *Tg⁺Ja18⁰4get* (n=5) mice. CD8⁻ cells were gated and analyzed for TCRβ, NK1.1, CD122, and 4get-GFP expression. (A and D) Dot plots: percentages of TCRβ⁺NK1.1⁺, TCRβ⁺CD122⁺ or TCRβ⁺4get⁺ in CD8⁻ hepatic lymphocytes (A) or CD8⁻thymocytes (D). Quantification of TCRβ⁺NK1.1⁺CD8⁻, TCRβ⁺CD122⁺CD8⁻, or TCRβ⁺4get⁺CD8⁻ T cells in the liver (B and C) and thymus (E and F). Values are mean + SEM. Statistical analysis was performed using Student's t-test. ****P* < 0.001; ***P* < 0.01; **P* < 0.05.

Figure 3. Type II NKT cells in *Lck-CD1dTgJa 18⁰* mice have increased cell death and impaired IL-4 producing capacity

Hepatic TCRβ⁺NK1.1⁺CD8⁻ T cells were FACS sorted and stimulated with *K^b-CD1Tg* BMDCs. (A) Levels of IFN-γ production ± anti-CD1d mAb (n=6). (B) Levels of IL-4, IL-13, TNF-α, IL-6, and IL-17 production (n=4). (C) Activation marker expression on TCRβ⁺NK1.1⁺CD8⁻ T cells from indicated mice. (D) Percentages of dead (7AAD⁺) cells in type II NKT and conventional T cells (n=5). Data shown are from 3 independent experiments. Statistical analysis was performed using Student's t-test. ****P* < 0.001; ***P* < 0.01.

Figure 4. Enhanced interaction between type II NKT cells and *Lck-CD1dTg*⁺ T cells leads to bidirectional activation of type II NKT and conventional T cells
 (A–C) Numbers in representative dot plots show percentage of 24αβ (Vα3.2⁺Vβ9⁺) T cells (A). Percentage and absolute number of 24αβ T cells in indicated mice (n=6) (B). 4get-GFP, CD69, and CD44 expression on 24αβ T cells (n=4) (C). (D) CD69 upregulation and proliferation of 24αβ T cells were determined in 24αβ T cells stimulated with the indicated APCs (n=4). (E and F) CFSE-labeled 24αβ T cells were adoptively transferred to *Ja18*⁰ or *Lck-CD1dTgJa18*⁰ (Tg⁺Ja18⁰) mice. 7 days post-transfer, proliferation of donor 24αβ T cells was determined (E). One month post-transfer, T cells in *Lck-CD1dTgJa18*⁰ mice that received 24αβ T cells (AT) or PBS (No AT) were analyzed for T_{eff} (CD44^{hi}CD62L^{lo}) cells (F) (n=4). Statistical analysis was performed using Student's t-test. ***P < 0.001; *P < 0.05.

Figure 5. Conventional T cells are more activated and produce more pro-inflammatory cytokines in *Lck-CD1dTgJa 18⁰* mice

(A) The total number of CD4⁺ and CD8⁺ conventional T cells in the liver (n=8). (B) The percentage of CD4⁺FoxP3⁺ Treg cells in indicated mice (n=4). (C and D) Representative dot plots (C) and bar graphs (D) depict the percentage of T_{eff} (CD44^{hi}CD62L^{lo}) cells in CD4⁺ and CD8⁺ T cells (n=6). (E) The percentage of IFN- γ producing (left panel) and IL-4 producing (right panel) T cells. (F–G) FACS sorted CD8⁺ (F) and CD4⁺ (G) T cells were stimulated with anti-CD3/CD28 for 48h. Cytokine levels in supernatants were determined by CBA (n=5). Statistical analysis was performed using Student's t-test. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Figure 6. Increased B-cell activation and autoantibody production in *Lck-CD1dTgJa 18°* mice
(A) Expression of various B-cell activation factors in sorted hepatic CD4⁺ T cells. All mRNA levels relative to mRNA expression in CD4⁺ T cells from *Ja18°* mice (n=7). **(B)** CD69 expression on LPS-activated B cells (n=6). **(C)** Cytokine production in supernatants from FACS sorted hepatic B cells stimulated with LPS for 48h, determined by CBA (n=5). **(D)** Levels of serum IgM in indicated mice at different ages (n=7–16 per group). **(E)** Levels of dsDNA-specific IgG1, IgG2a/c, IgG2b, IgG3 and IgM (n=8). Statistical analysis was performed using Student's t-test. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Figure 7. *Lck-CD1dTgJa 18⁺* mice exhibited similar dysregulation of type II NKT, T and B cells as *Lck-CD1dTgJa 18⁰* mice

Hepatic leukocytes and serum were prepared from *Ja18⁺* (n=13), *Lck-CD1dTgJa18⁺* (*Tg⁺Ja18⁺*, n=12), *Ja18⁺4get* (n=4), and *Tg⁺Ja18⁺4get* (n=4) mice. **(A)** Representative dot plots show percentages of TCRβ⁺NK1.1⁺ type II NKT cells in CD1d Tet⁺CD8⁺ hepatic lymphocytes. **(B)** 4get-GFP (left panel) and CD69 expression (right panel) on type II NKT cells. **(C)** Absolute number of various leukocyte subsets (n=4). **(D)** Levels of dsDNA-specific IgM (n=8–10/group). **(E)** Percentage of T_{eff} (CD44^{hi}CD62L^{lo}) cells in conventional CD4⁺ and CD8⁺ T cells (n=6). **(F)** The percentage of IFN-γ producing (left panel) and IL-4 producing (right panel) T cells (n=6). Statistical analysis was performed using Student's t-test. ****P* < 0.001; **P* < 0.05.

Figure 8. Increased CD1d expression on activated T cells that were more prevalent in AIH patients

(A) CD1d expression on unstimulated, anti-CD3 stimulated, and IFN- γ -treated T cells from human PBMCs (n=6/group). (B) CD1d expression on CD69⁺ and CD69⁻ human T cells. (C) Percentage of CD69⁺ T cells from healthy donors (HD, n=8) and AIH patients (AIH, n=8). (D) Representative immunofluorescent staining of normal liver sections from hepatic hemangioma patients (n=2) and inflammatory liver sections from AIH patients (n=3) with anti-CD3 (green) and anti-CD1d (red). Statistical analysis was performed using Student's t-test. ** $P < 0.01$.