Vγ4 γδ T Cell-Derived IL-17A Negatively Regulates NKT Cell Function in Con A-Induced Fulminant Hepatitis

Na Zhao, Jianlei Hao, Yuanyuan Ni, Wei Luo, Ruifang Liang, Guangchao Cao, Yaping Zhao, Puyue Wang, Liqing Zhao, Zhigang Tian, Richard Flavell, Zhangyong Hong, Jihong Han, Zhi Yao, Zhenzhou Wu and Zhanan Yin

J Immunol 2011; 187:5007-5014; Prepublished online 10 October 2011;
doi: 10.4049/jimmunol.1101315
http://www.jimmunol.org/content/187/10/5007

References
This article cites 44 articles, 25 of which you can access for free at: http://www.jimmunol.org/content/187/10/5007.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Vγ4 γδ T Cell-Derived IL-17A Negatively Regulates NKT Cell Function in Con A-Induced Fulminant Hepatitis

Na Zhao,*†‡,1 Jianlei Hao,*† Yuanyuan Ni,*† Wei Luo,* Ruifang Liang,* Guangchao Cao,* Yapu Zhao,* Puyue Wang,* Liqing Zhao,* Zhigang Tian,* Richard Flavell,*‡ Zhangyong Hong,* Jihong Han,* Zhi Yao,*† Zhenzhou Wu,* and Zhinan Yin*

Con A-induced fulminant hepatitis is a well-known animal model for acute liver failure. However, the role of γδ T cells in this model is undefined. In this report, using TCR δ−/− mice, we demonstrated a protective role of γδ T cells in Con A-induced hepatitis model. TCR δ−/− mice showed significantly decreased levels of IL-17A and IL-17F in the Con A-treated liver tissue, and reconstitution of TCR δ−/− mice with wild-type (Wt), but not IL-17A−/−, γδ T cells significantly reduced hepatitis, strongly suggesting a critical role of IL-17A in mediating the protective effect of γδ T cells. Interestingly, only Vγ4, but not Vγ1, γδ T cells exerted such a protective effect. Furthermore, depletion of NKT cells in TCR δ−/− mice completely abolished hepatitis, and NKT cells from Con A-challenged liver tissues of TCR δ−/− mice expressed significantly higher amounts of proinflammatory cytokine IFN-γ than those from Wt mice, indicating that γδ T cells protected hepatitis through targeting NKT cells. Finally, abnormal capacity of IFN-γ production by NKT cells of TCR δ−/− mice could only be downregulated by transferring Wt, but not IL-17A−/−, Vγ4 γδ T cells, confirming an essential role of Vγ4-derived IL-17A in regulating the function of NKT cells. In summary, our report thus demonstrated a novel function of Vγ4 γδ T cells in mediating a protective effect against Con A-induced fulminant hepatitis through negatively regulating function of NKT cells in an IL-17A-dependent manner, and transferring Vγ4 γδ T cells may provide a novel therapeutic approach for this devastating liver disease. The Journal of Immunology, 2011, 187: 5007–5014.
FTC-conjugated anti-mouse TCR β, allophycocyanin-conjugated anti-mouse CD4 (clone GK1.5), anti-mouse NK1.1 (clone PK136), purified anti-mouse IL-17A (clone 17F3), hamster anti-mouse TCR Vγ1 mAb 2.11, and hamster anti-mouse TCR Vγ4 mAb UC3 were from Sungenne (Tianjin, China); Alexa Fluor 647-conjugated anti-mouse γδ TCR (clone XMG1.2) were purchased from BioLegend (San Diego, CA); PE-conjugated anti-mouse IL-17A clone TC11-18H10 was purchased from BD Biosciences (San Jose, CA).

T cell-mediated hepatitis model and survival study

To induce hepatitis, we injected Wt, TCR-γδ−/−, and IL-17A−/− mice i.v. with Con A (10 mg/kg body weight). For survival study, the dose of Con A was increased to 20 mg/kg body weight. Mice were closely monitored and euthanized before the end point was reached.

Assay for serum transaminase activity

Mice serum samples were obtained at different time points after Con A injection. Serum alanine aminotransferase (ALT) activities were measured using commercial available test kit (Rong Sheng Biotech, Shanghai, China); Alexa Fluor 647-conjugated anti-mouse IFN-γ (clone XMG1.2) were purchased from BioLegend (San Diego, CA) for 4.5 h. Cells were stained first with Abs against surface molecules and then fixed and permeabilized as described previously (15). For IFN-γ staining, liver lymphocytes from different groups were collected, suspended in PBS, and different fields were randomly chosen in every slide, and average necrosis percentage was calculated.

Liver mononuclear cell preparation

Mouse livers were removed and pressed through a 200-gauge stainless-steel mesh. The liver cell suspension was collected, suspended in PBS, and centrifuged at 50 × g for 5 min. Supernatants containing mononuclear cells (MNCs) were collected and resuspended in 40% Percoll (GE Healthcare). The cell suspension was gently overlaid onto 70% Percoll and centrifuged for 30 min at 1260 × g. MNCs were collected from the interphase and washed twice in PBS.

Adoptive transfer experiments

For expansion of γδ, Vγ1, and Vγ4 γδ T cells in vitro, γδ T cells were sorted and expanded from splenocytes either from Wt or IL-17A−/− mice as described previously (19). Expanded cells (1 × 10⁶ cells/mouse) were i.v. transferred into B6 TCR-γδ−/− mice 24 h before Con A administration.

Intracellular cellular cytokine staining

For IL-17A staining, liver lymphocytes were isolated 2 h after Con A treatment and stimulated with PMA (50 ng/ml; Sigma, St. Louis, MO) and ionomycin (1 μg/ml; Sigma) in the presence of GolgiPlug (BD Biosciences, San Jose, CA) for 4.5 h. Cells were stained first with Abs against surface molecules and then fixed and permeabilized as described previously (15). For IFN-γ staining, liver lymphocytes from different groups of mice were isolated 12 h after Con A treatment, and stimulated with plate-coated anti-CD3 and soluble anti-CD28 and IL-2 for 6 h in the presence of GolgiPlug. Cells were then stained with FITC–anti-mouse TCR β and PE–anti-mouse NK1.1 followed by fixation, permeabilization, and intracellular staining as described previously (15).

Cell depletion

Mice were administered with an i.v. injection of 50 μl anti-asialo GM1 (ASGM1; Wako Pure Chemical Industries, Osaka, Japan) diluted in 200 μl pyrogen-free PBS to deplete NK cells. To deplete both NK and NKT cells, we injected mice i.v. with 200 μg anti-NK1.1 mAb (PK136; American Type Culture Collection) diluted in 200 μl pyrogen-free PBS. Depletion was confirmed by flow cytometry. For neutralizing effect of IL-17A in vivo, anti-mouse IL-17A Ab (100 μg/mouse) was injected i.v. 1 h before Con A treatment. Mice with cell depletion were then treated with Con A as described earlier.

Real-time PCR for gene transcription

Total RNA was extracted from liver MNCs by using TRizol Reagent (Invitrogen, Carlsbad, CA) and reverse-transcribed by Quantscript RT Kit (Tiangen, Beijing, China). mRNA expression was quantified by SYBR Premix HotMaster Taq (Tiangen, Beijing, China), and ribosomal protein large P0 (RPLP0) (30) was used as an internal normalizing gene. The primer sequences used were as follows: IL-17A forward: 5′-TGA AGG CAG CAG CGA TCA-3′, reverse: 5′-GGA AGT CCT TGG CCT CAG TGT-3′; IL-17F forward: 5′-CCG CAT TCA GCA AAG AAT CC-3′, reverse: 5′-CTC CAA CCT GAA GAA ATT AGA ACA G-3′; FASL forward: 5′-CTC CAG ATG CAA GTG ATG AT-3′, reverse: 5′-CTT GAG CTC CTC GCA AAC GAC GG-3′; IFN-γ forward: 5′-CTC CAG TGT CAC TGG AAT T-3′; bet-forward: 5′-TTT CAT TTG GGA AGC TAA AG-3′, reverse: 5′-GTC TGG TAC TCT TGG TGG AGA GA-3′; GATA-3 forward: 5′-AGA GGT CGG CTT ACT TTT TAA C-3′, reverse: 5′-AGA GAT CGT CGC GAC GAG T-3′; FASL-forward: 5′-CAG CTT CAG ATG CGA GTG GG-3′, reverse: 5′-CAG AAC GAG AAT CGT GCC TCA CAG CCG-3′, reverse: 5′-CTG GCA CAG TGA CCT CAG ACG-3′.

Statistics

Data are presented as mean values ± SD. Statistical significance between two groups was evaluated by two-tailed unpaired Student t test using Instat version 3.06 software for Windows (GraphPad, San Diego, CA). For comparing values obtained in three or more groups, we used one-factor ANOVA, followed by S-N-K’s post hoc test. The difference of survival was compared and analyzed using the log-rank test, performed by GraphPad Prism 4 for Windows (GraphPad). Throughout the text, figures, and figure legends, the following terminology is used to denote statistical significance: *p < 0.05, **p < 0.01.

Results

γδ T cells play a protective role in Con A-induced fulminant hepatitis

To define the role of γδ T cells in Con A-induced hepatitis, sex- and age-matched B6 Wt and TCR-γδ−/− mice were treated with high dose of Con A (20 mg/kg body weight), and the survival rate of mice was observed and recorded. In comparison with B6 Wt mice, TCR-γδ−/− mice died much earlier and no mice survived after 20 h (Fig. 1A), indicating an essential role of γδ T cells in protection against Con A-induced liver damage. To further dissect the protective role of γδ T cells in this model, we administered different doses of Con A to B6 Wt or TCR-γδ−/− mice, and a lower dose of Con A (10 mg/kg body weight) was found to be the best to demonstrate significant differences between Wt and TCR-γδ−/− mice (data not shown). Thereafter, all the following experiments were performed using this lower dose. On treatment with Con A, hepatitis appeared significantly earlier and lasted much longer in TCR-γδ−/− mice in comparison with Wt mice, as indicated by the serum ALT level at different time points. In the absence of IL-17 cytokine family has multiple members and it has been shown to be involved in many inflammatory diseases. To define the role of IL-17A in Con A-induced hepatitis, we treated sex- and age-matched B6 Wt and IL-17A−/− mice with Con A and measured serum level of ALT at different time points. In the absence of IL-17A, the survival rate of IL-17A−/− mice was compared and analyzed using the log-rank test, performed by GraphPad Prism 4 for Windows. Throughout the text, figures, and figure legends, the following terminology is used to denote statistical significance: *p < 0.05, **p < 0.01.

IL-17A is a critical cytokine for protection against Con A-induced hepatitis

IL-17 cytokine family has multiple members and it has been shown to be involved in many inflammatory diseases. To define the role of IL-17A in Con A-induced hepatitis, we treated sex- and age-matched B6 Wt and IL-17A−/− mice with Con A and measured serum level of ALT at different time points. In the absence of IL-
FIGURE 1. γδ T cells play a protective role in Con A-induced hepatitis. A, Sex- and age-matched B6 Wt and TCR δ−/− mice (n = 15 for each group) were i.v. injected with Con A (20 mg/kg body weight), and the rates of death were observed every 30 min and recorded. One representative experiment of three repeated experiments is shown (*p < 0.05). B, Sex- and age-matched B6 Wt and TCR δ−/− mice (n = 12 for each group) were i.v. injected with Con A (10 mg/kg body weight), and at different time points after Con A treatment, serum samples were obtained for measuring the level of ALT. One representative experiment is shown (**p < 0.01). C, Liver tissues at 18 h after Con A treatment were fixed for H&E staining, and one representative tissue staining is shown. N, necrosis area. Scale bars, 200 μm. D, Percentage of necrosis is calculated and shown. n = 3 mice/group. **p < 0.01. E, B6 TCR δ−/− mice (n = 6) were reconstituted with in vitro-activated total γδ T cells (1 × 10^6 cells/mouse) as described in Materials and Methods, followed with Con A treatment. Serum samples were obtained at different time points after Con A treatment, and the levels of ALT were measured. One of three repeated experiments is shown (*p < 0.05, **p < 0.01).

17A, significantly worse inflammation was observed on Con A treatment, as indicated by the significantly increased level of ALT (Fig. 2A) and bigger necrosis areas shown by liver histopathology (Fig. 2B, 2C), suggesting a protective role of IL-17A in this hepatitis model. B6 IL-17A−/− mice were administered with rmIL-17 and serum ALT level was monitored to further confirm the protective effect of IL-17A. Reconstitution of IL-17A−/− mice with IL-17 resulted in protection against Con A-induced hepatitis, evidenced by serum ALT level (Fig. 2D) and pathology (Fig. 2E, 2F), supporting the protective effect of IL-17 in this fulminant hepatitis model. Consistently, treatment of B6 WT mice with an anti-IL-17A Ab rendered these mice more susceptible to Con A treatment (Fig. 2G). Therefore, our results collectively demonstrate a protective role of IL-17A in this hepatitis model.

γδ T cells are the primary source of IL-17A on Con A treatment

Based on our findings as described earlier that both γδ T cells and IL-17A played important roles in protection against Con A-induced hepatitis, we hypothesized that γδ T cells might be the primary source of IL-17A. To test our hypothesis, we treated sex- and age-matched B6 WT or TCR δ−/− mice with Con A (10 mg/kg), and at 2 h after treatment, liver lymphocytes were collected for RNA extraction and an array of cytokines were analyzed by real-time PCR. In comparison with WT mice, liver lymphocytes of TCR δ−/− mice expressed significantly lower level of IL-17A and IL-17F (Fig. 3A). To further confirm the primary source of IL-17A, we isolated liver lymphocytes from WT mice at 2 h after Con A treatment and activated them, followed by intracellular staining as described in Materials and Methods. Interestingly, >50% of liver γδ T cells expressed IL-17A, and few CD4+ T and TCR αβ+ T cells showed IL-17A+ (Fig. 3B), supporting the notion that γδ T cells, but not Th17 or other αβ+ T cells (such as NKT cells), were the predominant source of IL-17A in Con A-induced hepatitis.

In contrast, no significant changes were observed between these two groups of mice for IFN-γ, IL-4, TNF-α, T-bet, GATA-3, and FASL (Fig. 3C), suggesting a critical role of γδ T cells in providing the primary source of IL-17A in Con A-induced hepatitis model.

γδ T cell-derived IL-17A is critical for protective immune response against Con A-induced hepatitis

To test the hypothesis that the protective effect of γδ T cells is through providing IL-17A, we administered B6 TCR δ−/− mice with rmIL-17 as described earlier and monitored serum ALT level and liver histopathology. Indeed, injection of IL-17A significantly reversed Con A-induced hepatitis in TCR δ−/− mice (Fig. 4A), supporting a critical role of IL-17A in γδ T cell-mediated protective responses on Con A challenge.

To further test the critical role of IL-17A derived from γδ T cells, we adopted a similar approach as described in Fig. 1E. Splenic γδ T cells were isolated either from WT or IL-17A−/− mice, activated in vitro, and then transferred into TCR δ−/− mice (1 × 10^6 cells/mouse). At 24 h after transfer, these recipients were then treated with low dose of Con A as described earlier. Serum samples were collected at different time points for monitoring the ALT level, and liver tissues were collected at 18 h of Con A treatment for histopathology staining. Interestingly, TCR δ−/− mice reconstituted with WT γδ T cells showed significant protection. In sharp contrast, reconstitution of TCR δ−/− mice with IL-17A−/− γδ T cells did not offer any protection (Fig. 4B); the severity of liver damage in these reconstituted mice was almost the same as untreated TCR δ−/− mice, strongly supporting a critical role of γδ T cell-derived IL-17A in mediating the protective effect of γδ T cells in Con A-induced hepatitis model.

Only Vγ4, but not Vγ1, γδ T cells offer protection against Con A-induced hepatitis

Peripheral γδ T cells are mainly composed of two subsets: Vγ1 and Vγ4. These two subsets of γδ T cells have divergent functions in many different disease models (9). Based on the results
FIGURE 2. IL-17A is required for protection against Con A-induced liver damage. A, Sex- and age-matched B6 Wt and IL-17A−/− mice (n = 15 for each group) were i.v. injected with Con A (10 mg/kg body weight), and at different time points after Con A treatment, serum samples were obtained for measuring the level of ALT. One of three repeated experiments is shown (**p < 0.01). B, Liver tissues of B6 Wt or IL-17A−/− mice at 18 h after Con A treatment were fixed and stained with H&E. One representative staining is shown. Scale bars, 200 µm. C, Percentage of necrosis is shown. n = 3 mice/group. **p < 0.01. D, B6 IL-17A−/− mice (n = 5) were treated with i.v. injection of rmIL-17 (1 µg/mouse) or PBS vehicle 30 min before and 1 h after Con A treatment, and serum ALT levels were measured from serum samples 18 h after Con A treatment. One representative experiment is shown (**p < 0.01). E, Liver tissues of B6 IL-17A−/− mice that received PBS or rmIL-17 at 18 h after Con A administration were fixed and stained with H&E. One representative staining is shown. Scale bars, 200 µm. F, Percentage of necrosis is shown. n = 3 mice/group. **p < 0.01. G, For IL-17A blocking, B6 Wt mice (n = 5) were treated with i.v. injection of either anti-mouse IL-17A Ab, 100 µg/mouse, or control Ab 1 h before Con A treatment. Serum samples were collected at different time points after Con A administration for analysis of the ALT level. One representative experiment is shown (*p < 0.05, **p < 0.01). N, necrosis area.

obtained earlier that IL-17A was a critical mediator for the protective effect of γδ T cells, and studies by others (27, 28, 31) and our own unpublished work that Vγ4 γδ T cells are the main producer of IL-17, we hypothesized that Vγ4 γδ T cells might be the critical one to play the protective effect. Vγ1 and Vγ4 γδ T cells were sorted from B6 Wt mice, cultured as described earlier, and used for IL-17 family cytokine analysis by real-time PCR to test this hypothesis. Only Vγ4, but not Vγ1, γδ T cells expressed high levels of IL-17A and IL-17F (Fig. 5A). Consistently, we found the main source of IL-17A in Con A-treated liver was the Vγ4+ subset (Fig. 5B). To directly compare the protective effect of Vγ1 and Vγ4 γδ T cells in vivo, we reconstituted TCR δ−/− mice with either Vγ1 or Vγ4 γδ T cells isolated from B6 Wt mice followed by Con A administration as described earlier. Only Vγ4 γδ T cells offered significant protection against Con A-induced hepatitis. In contrast, reconstitution with Vγ1 γδ T cells did not show any protective effect (Fig. 5C). To test whether the protective effect of Vγ4 γδ T cells was dose dependent, we transferred different amounts of Vγ4 γδ T cells to TCR δ−/− mice followed by Con A treatment. We showed that at least 1 million Vγ4 γδ T cells were required for the protective effect (Fig. 5D). The differential effects of these two subsets of γδ T cells were further confirmed by liver histopathology sections (Fig. 5E, 5F). To further prove the critical role of IL-17A in mediating the protective effect of Vγ4 γδ T cells, we reconstituted TCR δ−/− mice with either Wt or IL-17A−/− Vγ4 γδ T cells as described earlier, and monitored serum ALT level and liver histopathology. Consistently, only Wt, but not IL-17A−/−, Vγ4 γδ T cells showed significant protection. Reconstitution of Vγ4 γδ T cells significantly reduced serum ALT level (Fig. 5G). Therefore, our results determined a critical role of Vγ4 γδ T cells in protection against Con A-induced hepatitis through IL-17A production.

Vγ4 γδ T cell-derived IL-17A regulates IFN-γ secretion by NKT cells

NKT cells play an essential role in the pathogenesis of Con A-induced hepatitis through producing inflammatory cytokines, especially IFN-γ (2). We hypothesized that the protective effect of γδ T cells might be through targeting NKT cells. To test our hypothesis, we treated TCR δ−/− mice with an anti-NK1.1 (clone PK136) or ASGM1 Ab to deplete NK1.1+ (include NK and NKT cells) or NK cells, respectively (32). These mice were then treated with Con A and the serum ALT level was measured at 18 h
after treatment. Depletion of NK1.1+, but not NK, cells in TCR δ−/− mice significantly decreased Con A-induced liver damage, indicating an essential role of NKT cells in the Con A-induced hepatitis model, even in the absence of γδ T cells (Fig. 6A). Based on the fact that NKT cell-derived IFN-γ is critical for the pathogenesis of Con A-induced liver damage, we hypothesized that the protective effect of γδ T cell-derived IL-17A might negatively regulate IFN-γ production by NKT cells. To test our hypothesis, we treated sex- and age-matched B6 WT and TCR δ−/− mice with Con A as described earlier. NKT and NK cells were sorted from liver at 2 h after Con A treatment, and the cytokine profiles of these NKT cells were then analyzed by real-time PCR. Interestingly, NKT cells from TCR δ−/− mice reconstituted with WT Vγ4 γδ T cells expressed a significantly lower level of IFN-γ, indicating a protective role of Vγ4 γδ T cells through altering the proinflammatory cytokine production by NKT cells (Fig. 6C). In contrast, NKT cells from mice reconstituted with IL-17A−/−/Vγ4 γδ T cells showed a similar level of IFN-γ as those in TCR δ−/− mice (Fig. 6C). To further confirm the regulatory effect of Vγ4 γδ T cells on the ability of NKT cells to produce IFN-γ, we sorted NKT cells at 12 h after Con A treatment and activated them for intracellular cytokine staining. In our preliminary studies, at this time point, the biggest differences were observed between WT and TCR δ−/− mice in their IFN-γ production by NKT cells (data not shown). In the absence of γδ T cells, NKT cells produce a significantly higher level of IFN-γ, which was inhibited by transferring WT, but not IL-17A−/−, Vγ4 γδ T cells (Fig. 6D), suggesting a critical role of IL-17A in mediating the protective effect of Vγ4 γδ T cells through targeting IFN-γ production by NKT cells.

**Discussion**

Con A-induced fulminant hepatitis is a well-known animal model for studying the pathophysiological mechanisms of acute liver failure, a devastating liver disease with significant mortality worldwide and without effective therapeutic approaches. Activated T cells, especially NKT cells, have been defined to play a critical role in promoting liver damage through producing cytokines, such as IFN-γ. However, the controlling mechanisms for NKT cells, especially for their cytokine production, have been elusive. In this report, we demonstrated for the first time, to our knowledge, that γδ T cells, especially Vγ4 γδ T cells, played a critical role
in protective immune response against Con A-induced hepatitis through providing IL-17A, which, in turn, regulates NKT cell function, especially the production of IFN-γ.

One of the key findings in this study is establishing for the first time, to our knowledge, a protective role of γδ T cells in Con A-induced hepatitis. γδ T cells have many unique features and functions in comparison with conventional αβ T cells. It has been well documented that γδ T cells play an important role in many aspects of immune responses, including protective immunity against pathogens, tumor immune surveillance, and inflammatory diseases (9, 33). However, the role of γδ T cells in liver immune responses is unclear. Using either TCR δ−/− mice or transferring activated peripheral γδ T cells into TCR δ−/− mice, we collectively demonstrated that γδ T cells played a critical role in protective immune response against Con A treatment (Figs. 1, 5).

Furthermore, reconstitution of TCR δ−/− mice only with Vγ4, but not Vγ1, γδ T cells rendered these mice more protective against Con A-induced liver inflammation (Fig. 5). Our results added another example of divergent functions of different subsets of γδ T cells determined by their TCR usage. Interestingly, Vγ4 and Vγ1 γδ T cells are primarily in peripheral lymphoid organs (spleen and lymph nodes) (8); therefore, our study provided novel evidence that peripheral γδ T cells were involved in liver immune responses on Con A challenge. It is currently unclear whether these Vγ4 γδ T cells are newly recruited from periphery on Con A injection or prestored in the liver as “effector memory-like” γδ T cells. Given the fact that Vγ1 and Vγ4 γδ T cells have divergent functions in many other disease models, it remains to be determined whether Vγ4 γδ T cells play any similar roles in other LPS- or polyinosinic-polycytidylic acid acid-induced hepatitis models, and whether Vγ1 γδ T cells have any regulatory functions in these processes.

NKT cells are enriched in the liver and play an essential role in the pathogenesis of liver inflammation and damage, especially in the Con A-induced fulminant hepatitis model. To answer the question whether γδ T and NKT cells really “talked” to each other, we next provided evidence that the protective effect of γδ T cells indeed was through targeting NKT cells. We demonstrated that depletion of NKT cells completely abolished the severe liver damage in TCR δ−/− mice (Fig. 6A). Furthermore, the hallmark of proinflammatory cytokines IFN-γ (Fig. 6B, 6D), IL-4, and TNF-α (data not shown) expressed by NKT cells was significantly increased in TCR δ−/− mice in comparison with WT mice on Con A injection, indicating a negative regulatory role of γδ T cells in the cytokine production by NKT cells. This effect was not NK1.1 dependent, because no significance was observed for cytokine expression level by NK1.1+CD3− NK cells between these two groups of mice (Fig. 6B). Both γδ T and NKT cells are innate-like lymphocytes, with an activated phenotype (34–36) and the ability to bridge the innate immunity and adaptive immune responses (23–25, 37, 38). Therefore, defining for the first time, to

At 18 h after Con A treatment, serum samples were collected for determination of ALT level. One representative experiment is shown (**p < 0.01). E, Liver tissues of Con A-treated B6 TCR δ−/− mice at 18 h after Vγ1 or Vγ4 γδ T cell (1 × 106 cells/mouse) transfer as described previously were fixed and stained with H&E. One representative staining for each group of mice is shown. N, necrosis area. Scale bars, 200 μm. F, Percentage of necrosis is also shown (n = 3 mice/group, **p < 0.01). G, B6 TCR δ−/− mice were reconstituted with cultured Vγ4 γδ T cells from either WT or IL-17A−/− mice, or with PBS, and then followed by Con A administration. The levels of ALT from serum samples at 18 h after treatment were measured. One representative experiment is shown (**p < 0.01).
our knowledge, the interaction between these two cell types from this study, especially in the liver, which is a special organ with enriched immune cells, may have significant impact on understanding the immune pathology of other liver diseases. Further studies are granted to study the role of γδ T cells in other cell type-mediated liver damage and viral hepatitis.

What could be the critical mediator between γδ T and NKT cells? We hypothesized that it would be a cytokine. Indeed, our study firmly established that it was IL-17A deriving from Vγ4 γδ T cells that played a critical role in mediating the protective immune responses against Con A-induced liver damage. Our conclusion was supported by results obtained from multiple approaches. First, rIL-17A reconstituted the resistance against liver damage on Con A treatment in TCR δ−/− mice (Fig. 4A). Second, in comparison with those of Wt mice, liver tissues of TCR δ−/− mice showed a significantly decreased level of IL-17A (Fig. 3A), and Vγ4 γδ T cells expressed a high level of IL-17A (Fig. 5A, 5B). Third, only transferring Wt, but not IL-17A−/−, Vγ4 γδ T cells into TCR δ−/− mice offered protection against Con A-induced liver damage (Fig. 5B). Finally, consistent with findings described earlier, the regulation of the function of NKT cells by γδ T cells was IL-17A dependent (Fig. 6). Therefore, our results firmly established that Vγ4 γδ T cells provided the primary source of IL-17A and played a protective role in Con A-induced liver damage through targeting NKT cells. These results were also consistent with previous findings that Vγ4 γδ T cells were the main source of IL-17A on pathogen infection (27, 28). Interestingly, there were few, if any, CD4+ IL-17A+ cells, indicating a minor role of Th17 cells in this model. Given the critical role of NKT cells in Con A-induced hepatitis, and based on the fact that IL-17A–producing NKT cells express TCR αβ, but not NK1.1 (39), we also analyzed IL-17A production by αβ T cells. Similarly, a very low percentage of TCR αβ+ IL-17A+ T cells was detected in the Con A-treated liver (Fig. 3B), indicating that the critical role of NKT cells in this model was not through providing IL-17A. The protective role of IL-17A demonstrated in this article was also supported by a previous report that IL-17A protected against α-galactosylceramide-induced hepatitis in mice (40). However, in several other previous reports, there was no significant role for IL-17A in the Con A-induced hepatitis model using IL-17A−/− mice (41). The reason for such a discrepancy is unclear at the present time. We speculated that the main reason for such a discrepancy was due to the environment of the animal facility, which, in turn, resulted in the different intestine microbes, and consequently led to the differential phenotypes. It has been reported that intestine microbes can influence IL-17A production by CD4+ T cells and experimental autoimmune encephalomyelitis (EAE) phenotype (42, 43). Further studies are needed to determine whether treatment of these mice with antibiotic will alter the phenotype.

Differing from those IFN-γ–producing Vγ4 γδ T cells as described in our previous studies (15–17), we speculated that these IL-17A–producing Vγ4 γδ T cells were “Ag-naive” γδ T cells. Further studies are needed to further define the functional differences between these two subtypes of Vγ4 γδ T cells and whether the interactions between these subtypes do exist.

Which function of NKT cells was regulated by γδ T cells? Because it has been well established that IFN-γ is a critical cytokine in the pathogenesis of Con A-induced hepatitis (3, 5), and it was reported that IL-17A can downregulate T-bet and IFN-γ in CD4+ T cells (44), we hypothesized that Vγ4-derived IL-17A might downregulate IFN-γ production by NKT cells. Indeed, in the absence of γδ T cells, NKT cells expressed a significantly higher level of IFN-γ (Fig. 6B, 6D), and transferring Wt, but not IL-17A−/−, Vγ4 γδ T cells into TCR δ−/− mice completely decreased IFN-γ expression from NKT cells (Fig. 6C, 6D). We speculated that Vγ4-derived IL-17A might act on NKT cells to alter IFN-γ–producing programs. Further studies are needed to give us a better clue about the detail of molecular mechanisms.
In summary, our study has demonstrated a critical role of γδ T cells, especially Vγ4 γδ T cells, in protection against Con A-induced hepatitis. This protective effect was mediated by Vγ4 γδ T cell-derived IL-17A, which, in turn, targeted NK cells and negatively regulated IFN-γ production by NK T cells. Thus, for the first time, to our knowledge, our results define a critical role of γδ T cells in Con A-induced hepatitis and show that transferring γδ T cells may provide a novel therapeutic approach for this devastating liver disease.

Acknowledgments
We are grateful to Dr. Mark Bartlam and Marie-Louise Hjareesen for editing the manuscript. We also greatly appreciate technique support by Xinglong Zhou and Qiang Zhao from the core facility of College of Life Sciences, Nankai University.

Disclosures
The authors have no financial conflicts of interest.

References
15. Zhou and Qiang Zhao from the core facility of College of Life Sciences, Nankai University.