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*J Immunol* published online 10 October 2011

http://www.jimmunol.org/content/early/2011/10/09/jimmunol.1101315

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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Vγ4 γδ T Cell-Derived IL-17A Negatively Regulates NKT Cell Function in Con A-Induced Fulminant Hepatitis

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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 bedownregulated 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: 000–000.
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T cell-mediated hepatitis model and survival study

To induce hepatitis, we injected Wt, TCR gd, 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) based on methods recommended by the International Federation of Clinical Chemistry.

Histology

Liver tissues were harvested after treatment of Con A, fixed in 10% formalin, and embedded in paraffin. Five-micrometer sections were affixed to slides, stained with H&E, and images were acquired on a Leica DM3500 microscope using 10x objective. The liver damage extent was quantified by the necrosis area as a percentage of total area using Image-Pro Plus (IPP) 6.0 software (Media Cybernetics, Silver Spring, MD). Three 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 500 x 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 x g. MNCs were collected from the interface and washed twice in PBS.

Adoptive transfer experiments

For expansion of 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 x 10^6 cells/mouse) were i.v. transferred into B6 TCR gd 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 AAT AGA CTC-3′; reverse: 5′-CCT CAA CTT GGA GAA ATT AGA AGA-3′; IFN-γ forward: 5′-ATG AAC GCT ACA CAC TGC ATG-3′; reverse: 5′-CCA TCT TTC TGT CAG TTC CTG CTC-3′; IL-4 forward: 5′-GAA AAC TTC ATG CTT GAA GAA-3′; reverse: 5′-TCT TTC ATG GAT GTG TTT GTC-3′; TNF-α forward: 5′-CTA CTT ATG AAC TGG GTG ATC-3′; reverse: 5′-CAG CTC GTG TAC TCG AAT T-3′; T-bet forward: 5′-TTT CAT TTG GGA AGC TAA AGG-3′; reverse: 5′-GGC TGG TAC TTG TGG AGA GA-3′; GATA-3 forward: 5′-AGA GGT GCG AGT CTT TAT C-3′; reverse: 5′-AGA GAT CGT GCC TGC AGA G-3′; Fas forward: 5′-AGC CTT CAC ATG CAA GTG AGG-3′; reverse: 5′-CAA GGA CAG AAC TCT GAC GCT GAC-3′; RPLP0 forward: 5′-GAA ACT GCT GCC TCA CCG-3′; reverse: 5′-CTG GCA TGA TCA CCT CAC AGC-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 gd 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 gd 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 gd 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 gd 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 gd mice in comparison with Wt mice, as indicated by the serum ALT level (Fig. 1B). This was further evidenced by liver histopathology at 18 h after treatment of Con A (Fig. 1C, 1D). Significantly bigger areas of necrosis in liver tissues were observed in TCR gd mice.

To further define the role of γδ T cells in Con A-induced hepatitis, we adopted an another approach. B6 TCR δ−/− mice were reconstituted with in vitro-activated Wt γδ T cells as described in Materials and Methods. Adoptive transfer of γδ T cells into TCR δ−/− mice significantly reduced hepatitis (Fig. 1E), further confirming the protective role of γδ T cells. These results collectively demonstrated a protective role of γδ T cells in Con A-induced hepatitis model.

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-
IL-17A played important roles in protection against 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 × 10⁶ 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).
**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 for each strain of mice is shown. Scale bars, 200 μm. C, Percentage of necrosis in each strain of mice is shown. Scale bars, 200 μm. D, B6 IL-17A−/− mice (n = 5) were treated with i.p. 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 < 0.01, ***p < 0.001). 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 following 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.
T cells, or left them untreated. These mice were then treated with Con A and analyzed by real-time PCR (Fig. 6B) to determine the cytokine profiles of these T cells. NKT cells were isolated at 2 h after Con A treatment for gene expression analysis, and liver at 2 h after Con A treatment, and the cytokine profiles of these NKT cells and NK cells were analyzed by real-time PCR. Interestingly, NKT cells were then analyzed by real-time PCR. 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, 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 regulating IFN-γ production by NKT cells.
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-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-17 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. 5G). 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 NKT cells and negatively regulated IFN-γ production by NKT 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 gratefully acknowledge technical support from 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.

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