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Early IL-17 Production by Intrahepatic T Cells Is Important for Adaptive Immune Responses in Viral Hepatitis

Lifei Hou,*1 Zuliang Jie,*1 Mayura Desai,* Yuejin Liang,* Lynn Soong,*† and Jiaren Sun*

This study was conducted to examine the interactions among the innate and adaptive immune components of the liver parenchyma during acute viral hepatitis. Mice were i.v. infected with a recombinant adenovirus, and within the first 24 h of infection, we found a transient but significant accumulation of IL-17 and IL-23 in the liver. In vivo neutralization of these interleukins alleviated the liver injury. Further investigations showed that IL-17 neutralization halted the intrahepatic accumulation of CTLs and Th1 cells. A majority of the IL-17−producing cells in the liver were γδ T cells. Additionally, intrahepatic IL-17+ γδ T cells, but not the IFN-γ+ ones, preferentially expressed IL-7Rα (CD127) on their surface, which coincided with an elevation of hepatocyte-derived IL-7 at 12 h postinfection. IL-7Rα blockade in vivo severely impeded the expansion of IL-17−producing cells after viral infection. In vitro, IL-7 synergized with IL-23 and directly stimulated IL-17 production from γδ T cells in response to TCRγδ stimulation. Finally, type I IFN (IFN-γ) signaling was found to be critical for hepatic IL-7 induction. Collectively, these results showed that the IFN-γ/IL-7/IL-17 cascade was important in priming T cell responses in the liver. Moreover, the highly coordinated cross talk among hepatocytes and innate and adaptive immune cells played a critical role in anti-viral immunity in hepatitis. The Journal of Immunology, 2013, 190: 621–629.

Viral hepatitis is one of the most common health problems in the world. Many viruses (e.g., hepatitis B and C viruses, CMV, and adenovirus) can cause liver inflammation, which is often characterized by various degrees of CTL/Th1 responses. Among these viruses, adenovirus (Ad) is a prototypical DNA virus and an important pathogen. It is also one of the preferred vectors for gene therapy, cancer therapy, and experimental vaccines (1). When i.v. injected, the virus preferentially targets the liver and encounters the host defense system. Strikingly, a majority of the viruses have been found to be eliminated by innate immune mechanisms within 24 h (2). However, in subsequent periods, viral elimination reportedly slowed, while disease resolution depended on virus-specific CTL and Th1 responses (3, 4). Additionally, it was reported that overzealous T cell responses can result in necroinflammatory hepatitis, treated similarly by innate immune mechanisms within 24 h (2). However, in subsequent periods, viral elimination reportedly slowed, while disease resolution depended on virus-specific CTL and Th1 responses (3, 4). Additionally, it was reported that overzealous T cell responses can result in necroinflammatory hepatitis, treated similarly by innate immune mechanisms within 24 h (2).

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Abbreviations used in this article: Ad, adenovirus; ALT, alanine aminotransferase; IFNAR, IFN-α receptor; IHL, intrahepatic lymphocyte; qRT-PCR, quantitative reverse transcriptase PCR.

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il-7 plays a critical role in viral hepatitis

interesting questions. Do γδ T cells secrete IL-17 in virus-induced hepatitis, especially during the early stage? If so, what role do these cells play in anti-viral immune responses?

Another important question in this and other types of viral hepatitis is whether hepatocytes, the most abundant and metabolically active cell type in the liver, participate in these crucial immunological dialogues or not. Our previous reports showed that hepatocytes could regulate the anti-viral reaction in the liver through the expression of some costimulatory molecules, including CD40 and CD86 (4, 39). More recently, Sawa et al. (40) reported that hepatocytes could produce IL-7, a potent immune-stimulatory cytokine, in response to IFN-I stimulation, and that hepatocyte-derived IL-7 was pivotal for Th17 cells development in an autoimmune model. Also, in vivo blocking of IL-7Rα could preferentially suppress Th17 development and IL-17-mediated murine autoimmune disease (41). However, the definite role of IL-7 on IL-17-producing γδ T cells awaits further elucidation.

Given that the role of IL-17 in viral hepatitis is still elusive, and early intrahepatic events are extremely crucial to the development of protective immune responses and disease outcome, we decided to examine the effects and regulation of intrahepatic IL-17-producing cells after an adenovirus infection. In this study, we used in vivo Ab neutralization and demonstrated that IL-17 played a critical role in initiating successful anti-viral CTL reactions. In addition, IL-7Rα γδ T cells, but not Th17 cells, were the major IL-17 producers. Finally, we demonstrated that IFN-I was pivotal for inducing IL-7 production from hepatocytes after Ad infection, which might be responsible for the IL-17 production from γδ T cells. This study collectively showed that, in addition to the expression of costimulatory molecules, hepatocytes could also orchestrate the adaptive anti-viral immune response by secreting IFN-I and IL-7.

Materials and Methods

Animals

Female C57BL/6 (B6) mice were purchased from The Jackson Laboratory. Breeding pairs of IFN-α receptor (IFNAR)−/− mice from the 129/Sv background were kindly provided by Dr. Herbert Virgin (Washington University School of Medicine, St. Louis, MO). Wild-type 129/SvEve mice were purchased from Taconic (Germantown, NY). All mice were maintained and bred under specific pathogen-free conditions at the University of Texas Medical Branch animal care facility. Mice were used at 7–10 wk of age according to National Institutes of Health guidelines and with the approval of the University of Texas Medical Branch Institutional Animal Care and Use Committee. Ad carrying the lacZ gene (AdLacZ) was used to induce hepatitis, as described previously (45), and then positively selected through FITC-conjugated anti-TCRγδ (GL-3) and anti-FITC MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of resultant cells was determined by flow cytometry and was consistently higher than 85%. Purified γδ T cells were stimulated with plate-coated anti-TCRγδ (GL-4, 5 µg/ml; BD Biosciences), together with a combination of recombinate mouse IL-1β (5 ng/ml), IL-7 (20 ng/ml), IL-23 (20 ng/ml; all from eBioscience), TNF-α (5 ng/ml; Peprotech), or recombinate human IFN-β (3 ng/ml; Peprotech). These concentrations of cytokines were selected according to our preliminary experiments and in vitro Th17 cell differentiation conditions (data not shown). Culture supernatants were collected at 48 h and stored at −20 °C for ELISA assays. For detecting proliferation, after a 48-h incubation, cells were pulsed with 1 µCi/well of [3H]thymidine and incubated for another 8 h. Cells were then harvested onto glass fiber filters, and incorporated radioactivity was counted by using a β Scintillation Counter (MicroBeta Trilux, PerkinElmer, Waltham, MA).

In vivo neutralization of IL-17 and IL-23 and blocking of IL-7Rα

To block the effects of IL-17 or IL-23, we injected the mice i.p. with 100 µg mAb against IL-17 or IL-23 (clone 50104 and 320229, respectively; R&D Systems) at days −1, 0, and 1 and 3 postinfection. To block the effects of IL-7, we injected mice i.p. with 100 µg anti-mIL-7Rα mAb (clone SB/14; BD Biosciences) at days −1 and 0 and euthanized at day 1 postinfection. Normal rat IgG (Sigma) was administered as an isotype control.

In vivo IFN-β and IFN-α treatment

To examine the effect of IFN-I on intrahepatic immune responses, we directly challenged mice i.v. with IFN-β (10,000 IU; PBL IFNSource) or IFN-α (1000 IU; HyCult, Plymouth Meeting, PA), as described previously (43). Animals were euthanized at 5 h postchallenge. After perfusion with cold PBS to remove the blood, livers were collected for subsequent analyses.

Primary hepatocyte isolation and culture

Primary hepatocytes were isolated from C57BL/6 mice by an adaptation of a two-step collagenase perfusion technique (44). In brief, the mouse liver was perfused with HBSS (pH 7.4, without calcium and magnesium) containing 1 mM EGTA and 10 mM HEPES for 10 min, followed by HBSS with calcium and magnesium plus collagenase D (Roche Applied Science, Indianapolis, IN) for 10 min at 37°C. The digested liver was then excised, rinsed, and disaggregated in a 150-µm polystyrene Petri dish. Subsequently, the disaggregated material was filtered through a 70-µm cell strainer, and the filtrate was gently centrifuged for 3 min at 50 × g. The pellet was resuspended in 45% Percoll in PBS and recentrifuged at 50 × g for 10 min. After enrichment by Percoll isodensity purification, the cells were washed and gently centrifuged, and the pellets were resuspended in the DMEM supplemented with 10 mM HEPEs, 2 mM l-glutamine, in-sulin-transferin-sodium selenite medium (Sigma), and 10% FBS. The viability of the cells was assessed by the trypan blue dye exclusion method. The cells were placed (2.5 × 107 cells/3.0 ml attachment media) in 6-well plates (Corning, Corning, NY) precoated with collagen.

Isolation of intrahepatic lymphocytes

Intrahepatic lymphocytes were isolated according to our previous method with slight modifications (4, 39). Briefly, liver tissues were pressed and distributed and then collected in complete RPMI 1640. After washing (300 × g, 10 min), cell suspensions were resuspended in complete RPMI 1640 containing collagenase IV (0.05%; Roche) at 37°C for 30 min. After digestion, cell suspensions were passed through 70-µm nylon cell strainers to yield single-cell suspensions. Intrahepatic mononuclear cells were isolated by centrifugation (400 × g) at room temperature for 30 min over a 30/70% discontinuous Percoll gradient (Sigma). The cells were collected from the interphase, thoroughly washed, and resuspended in complete RPMI 1640 containing 10% FBS (HyClone, Logan, UT).

Splenic γδ T cell purification and stimulation

Splenocytes were processed through immune-magnetic negative selection to deplete TCRβ T cells, B220+ cells, NK cells, and I-Ab+ APCs, as described previously (45), and then positively selected through FITC-conjugated anti-TCRγδ (GL-3) and anti-FITC MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of resultant cells was determined by flow cytometry and was consistently higher than 85%. Purified γδ T cells were stimulated with plate-coated anti-TCRγδ (GL-4, 5 µg/ml; BD Biosciences), together with a combination of recombinant mouse IL-1β (5 ng/ml), IL-7 (20 ng/ml), IL-23 (20 ng/ml; all from eBioscience), TNF-α (5 ng/ml; Peprotech), or recombinate human IFN-β (3 ng/ml; Peprotech). These concentrations of cytokines were selected according to our preliminary experiments and in vitro Th17 cell differentiation conditions (data not shown). Culture supernatants were collected at 48 h and stored at −20 °C for ELISA assays. For detecting proliferation, after a 48-h incubation, cells were pulsed with 1 µCi/well of [3H]thymidine and incubated for another 8 h. Cells were then harvested onto glass fiber filters, and incorporated radioactivity was counted by using a β Scintillation Counter (MicroBeta Trilux, PerkinElmer, Waltham, MA).

Intracellular staining

Methods for intracellular staining were consistent with previous reports (4, 45). Splenocytes were incubated for 4 h with PMA (50 ng/ml) and ionomycin (750 ng/ml). For the simultaneous detection of surface and intracellular cytokines, cells were stimulated by plate-coated anti-CD3 mAb (145-2C11, 10 µg/ml; eBioscience) for 4 h, in the presence of GolgiStop (BD Biosciences). At the end of incubation, cells were collected and blocked with FcγR blocker before extracellular staining for corresponding fluorochrome-labeled surface mAb. After surface staining, cells were fixed, permeabilized, and stained for cytokines by using a fixation/ permeabilization kit (eBioscience).

Flow cytometry

Murine lymphocytes were blocked with anti-mCD16/CD32 (eBioscience) and stained with fluorochrome-labeled Abs or biotinylated mAbs, followed by fluorochrome-conjugated streptavidin, collected by LSRII FACSFortessa (Becton Dickinson, San Jose, CA), and analyzed by using FlowJo software (Tree Star, Ashland, OR). The following staining panels were used: Re-staining to stain the intracellular IL-17, IFN-γ, or Ki-67; PE-Cy7-anti-mCD3, allophycocyanin-anti-mTCRγδ, Pacific blue–anti-mCD4, PerCP-Cy5.5–anti-mCD8, FITC-anti-mCD127, and PE-anti-mIL-17 or PE-anti-mIFN-γ or PE-anti-mZh-67. We stained IL-17 and IFN-γ simultaneously with PE-Cy7-anti-mCD3, FITC–anti-mIFN-γ, PE-anti-mIL-17, allophycocyanin–anti-mTCRγδ or other fluorochrome-labeled anti-CD4, CD8, and NK1.1. Intracellular IFN-γ and surface CD107a/b were simultaneously stained with PE-Cy7–anti-mCD3, PerCP-Cy5.5–anti-mCD8, PE-Cy5.5–anti-mIFN-γ, Alexa Fluor 488–anti-CD107a, and Alexa Fluor 488–anti-CD107b. For staining the subsets of γδ T cells, cells were blocked with FcγR blockers and then stained with biotin-labeled anti-Vγ1, washed,
which was followed by avidin–PerCP–Cy5.5. After washing, the cells were stained with PE–Cy7–anti-mCD3, allophycocyanin–anti-mTCRγδ, FITC–anti-m-IL-17 and PE–anti-m-IL-17 was applied. For intracellular staining, PE–anti-m-IL-17 was applied. For staining the Vγ5 subset of γδ T cells, cells were blocked with FcR blockers and then were stained with anti-Vγ5 and washed, followed by FITC-labeled goat anti-rat IgG. After washing, the cells were stained with PE–Cy7–anti-mCD3 and allophycocyanin–anti-mTCRγδ.

All fluorochrome-labeled mAbs and their corresponding isotype controls were purchased from BD Pharmingen (San Diego, CA) and eBioscience. The mAb for Vγ1 (clone 2.11) was kindly provided by Dr. Rebecca O’Brien at the National Jewish Medical and Research Center, Denver. The mAb for Vγ5 was clone 17D1, a kind gift from Dr. Robert Tigelaar at Yale School of Medicine. If used alone, it stains Vγ5, but in the presence of anti-TCRγδ (clone GL-3), clone 17D1 stains Vγ6δ1. In all two-step surface staining and intracellular staining, isotype controls were strictly used.

Western blot assay

Proteins were extracted by homogenization with a syringe plunger on ice from frozen liver tissues in a lysis buffer. Subsequently, equal amounts of proteins were loaded onto 10% SDS-polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). Membranes were incubated with goat anti-mouse IL-23p19, IL-17 (clones 320229 and 50104, respectively; R&D Systems), or anti-β-actin (clone AC-15; Sigma), followed by HRP-conjugated secondary Abs for 1 h. Blots were visualized by ECL (ECL-Plus; Amersham Biosciences, Piscataway, NJ).

Real-time PCR

Total RNA was extracted from the liver and other tissues (e.g., the lungs, heart, kidneys, spleen, bone marrow, and thymus) with an RNAqueous kit and digested with DNase I (Ambion, Austin, TX). For relative quantitation of the cytokine and chemokine mRNA levels, cDNA was prepared from 1 μg RNA by using a TaqMan Reverse Transcription Kit (Bio-Rad), and 2 μl of the cDNA was amplified in a 25-μl reaction mixture containing 12.5 μl DNA Master SYBR Green 1 (Roche) and 0.9 μM each of gene-specific forward and reverse primers. The quantitative reverse transcriptase PCR (qRT-PCR) assays were performed in triplicate with the SYBR Green PCR Master Mix (ABI 4364344), as specified by the manufacturer. The PCR assays were denatured for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The PCR was performed with the ABI Prism 7000 Sequence Detection System. Relative quantitation of mRNA expression was calculated as the fold increase in expression by using the ΔΔCt method. PCR products were subjected to melting curve analysis to ensure that a single amplification product was produced. To assess IL-7 expression among different tissues, we used the method described by Sawa et al. (40). To detect the viral DNA copy numbers, DNA was isolated from the livers, and PCR amplification was done according to our previous description (4). The sequences of the forward and reverse gene-specific primers are listed in Supplemental Table I.

ELISA assays

The levels of IL-17A in serum or culture supernatant were assayed by using an ELISA kit (eBioscience) according to the manufacturer’s instructions. The detection limit of IL-17A was 1.6 pg/ml.

Statistical analysis

For statistical analyses, the two-tailed Student t test was used, one-way ANOVA was performed by using ANOVA: *p < 0.05 was considered significant and **p < 0.01 as highly significant.
**Results**

**IL-17 and IL-23 production increased at an early stage in Ad-induced hepatitis**

Intravenous injection of Ad can induce acute hepatitis, which reaches its peak in B6 mice at around days 5–6; the clinical and pathological manifestations of liver inflammation resolve spontaneously ∼3–4 wk thereafter (3). To determine the role of IL-17 and IL-23 in this model, we injected i.v. B6 mice with $2 \times 10^{9}$ PFU of Ad carrying the lacZ gene (AdLacZ), as described previously (4). The animals were sacrificed at 0, 8, 12, and 24 h, and 6 d postinfection. Western blot analysis revealed a significant accumulation of IL-17 in the liver during the first 24 h (Fig. 1A). A parallel elevation of IL-23p19, an important cytokine to induce IL-17 production, was also observed. An ELISA assay confirmed that the intrahepatic surge of IL-17 was accompanied by its increase (3-fold) in the serum at the same time (Fig. 1B).

To examine the source of IL-17 in the liver, we isolated the intrahepatic lymphocytes (IHLs) and analyzed these cells by flow cytometry. As shown in Fig. 1C, most IL-17–producing cells in the liver were CD3+ T lymphocytes, and these cells expanded from 0.5% at 0 h to 1.5% at 24 h postinfection. This surge of IL-17+ T cells was liver-specific, as their frequencies remained low (0.1%) in the spleen, either on day 1 or day 6 (Supplemental Fig. 1A). Whereas IFN-$

Neutralization of IL-17 or IL-23 ameliorated Ad-induced hepatitis

To determine whether this early elevation of IL-17 and IL-23 affects liver injury, we injected the mice i.p. with anti–IL-17 or anti–IL-23 mAb on days −1, 0, 1, and 3 after Ad infection. The mice treated with isotype control Ab developed hepatitis, characterized by inflammatory infiltration, hepatocytes with megakaryoblastic changes, and single-cell necrosis, at 6 d postinfection (Fig. 2A). The animals treated with anti–IL-17 or anti–IL-23 mAb, in contrast, displayed milder liver inflammation and lower pathological scores (Fig. 2B). IL-17 or IL-23 neutralization also greatly reduced the serum alanine aminotransferase (ALT) levels (Fig. 2C). These data demonstrated that IL-17 or IL-23 neutralization could alleviate liver injury.

Because Ad infection induces strong CTL and Th1 responses in the liver, resulting in tissue destruction and serum ALT elevation (3), we then examined the activation and accumulation of CTLs and Th cells in the IL-17–neutralized group. Ad infection resulted in the infiltration of activated CD8+ T cells, with significantly increased CD44 expression and decreased CD62L expression on their surface. In contrast to isotype control mice, IL-17 neutralization significantly suppressed the infiltration of activated CD44hi CD8+ T cells (Fig. 3A, 3B). Functionally, anti–IL-17 mAb administration significantly suppressed the IFN-$

**FIGURE 3.** IL-17 neutralization suppressed the intrahepatic Th1 and CTL responses induced by Ad infection. C57BL/6 mice were treated i.p. with anti–IL-17 or isotype control (rat IgG2a) mAb at days −1, 0, and 3 after Ad infection. Mice were sacrificed at day 6 postinfection. The liver tissues were isolated after the perfusion. (A) Representative flow cytometric examination of CD44 and CD62L expression on CD8+ T cells. (B) Calculated absolute cell numbers according to total IHLs and individual percentages. *$p < 0.05$, **$p < 0.01$. (C) IHLs were isolated and stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop. (D) IHLs were isolated and stimulated with anti-CD3 for 4 h in the presence of GolgiStop. The cells were then stained for surface markers and intracellular cytokines and examined by flow cytometry. Shown are representative flow cytometric results, $n = 6–8$ mice per group; cumulative mean ± SD is shown in quadrant.
of both CD4+ and CD8+ T cells in the liver (11 ± 6% versus 33 ± 13% and 40 ± 14% versus 69 ± 12%, respectively; Fig. 3C). IL-17 neutralization not only reduced IFN-γ/CD8+ frequencies, but also interfered with their effector function in the liver (Fig. 3D). In the control group, considerable IFN-γ+ cells (27 ± 8%) expressed LAMP-1/2 (CD107a/b), indicative of their ability to degranulate cytolytic vesicles. In the anti–IL-17–treated animals, however, a lower percentage of cells (9 ± 4%) expressed surface CD107a/b.

Although neutralization of IL-17 alleviated liver inflammation, hepatic Ad viral copy numbers in IL-17 neutralization mice did not significantly differ from that in control mice at day 6 after AdLacZ infection (p > 0.05). For the control mice and IL-17 neutralization mice, viral copy numbers in liver tissue (means ± SEM; n = 4–6 mice per group) were 4683 ± 698 and 3923 ± 733 per gram, respectively.

IL-17 predominantly produced by γδ T cells in the liver

Both innate and acquired T cells were reported to produce IL-17. We found that in the liver, more than two-thirds of IL-17–producing cells expressed TCRγδ on their surface, as judged by their percentages and absolute numbers (Fig. 4A). Small populations of IL-17+ γδ− intrahepatic T cells were heterogeneous and include CD4+, CD8+, and NKT cells (Fig. 4A, Supplemental Fig. 1B). However, compared with the high percentage of IL-17+ γδ T cells (42%, Fig. 4B), the IL-17+ γδ− T cell subsets were all below 0.9% within their respective populations (Supplemental Fig. 1B). We found that the IL-17+ γδ T cells in the liver expanded more than 2-fold on day 1 and waned during the next several days, whereas the IFN-γ+ γδ T cells did not expand until after day 1. On day 6, most (~35%) of the intrahepatic γδ T cells expressed IFN-γ, but not IL-17 (Fig. 4B). It has been reported that most γδ T cells in the liver of naïve mice belong to the Vγ1 and Vγ4 subsets, and the latter

FIGURE 4. γδ T cells were the major cellular source of IL-17 in the liver. C57BL/6 mice were injected i.v. with 2 × 10^9 PFU of Ad and sacrificed at 0, 1, and 6 d postinfection. After perfusion, IHLs were isolated and stimulated with PMA and ionomycin for 4 h in the presence of GolgiStop. The cells were then collected and stained for surface markers and intracellular cytokines and examined by flow cytometry. (A) Left, Flow cytometric examination of IL-17 expression in TCRγδ+ and TCRγδ− subsets of intrahepatic CD3+ T cells. Right, Cumulative statistical results of absolute cell number. (B) Left, Flow cytometric examination of IL-17 and IFN-γ expression in intrahepatic γδ T cells. Right, Cumulative statistical results of percentage. (C) Representative flow cytometric examination of surface CD3, TCRγδ, Vγ1, Vγ4, and Vγ5 expression. Cumulative mean ± SD is shown in quadrant. n = 3 mice per time point. Experiments were repeated at least three times with similar pattern. *p < 0.05, **p < 0.01. (D and E) Mice were injected i.v. with 2 × 10^9 PFU of Ad and sacrificed at 0, 12, and 24 h postinfection (four mice per time point), and liver cells were collected for FACS staining. Cells were gated on CD3+ TCRγδ+ populations. (D) Intracellular staining of Ki-67. The top panel shows representative dot plots; the bottom panel shows statistical results. *p < 0.05. (E) Surface staining of CD69 and CD44. Cumulative mean ± SD is shown in quadrant.
are the major IL-17 producers in peripheral lymphoid organs (46). In this study, we found that the proportions of intrahepatic γδ T cell subsets (Vγ1, Vγ4, and Vγ5) did not change significantly at day 1 (Fig. 4C). During the initial 24 h of infection, intrahepatic γδ T cells were activated and progressed into the cell cycle, as reflected by their elevated intracellular Ki-67 as well as surface CD69 and CD44 expression (Fig. 4D, 4E). Consistent with an earlier report (22), we found that the Vγ4 subset was the major IL-17 producer in the liver after Ad infection (Supplemental Fig. 2A).

**IL-17-producing γδ T cells regulated by hepatic IL-7**

Because γδ T cells can proliferate and secrete IL-17 merely in the presence of IL-7, IL-1β, and/or IL-23 in sepsis and autoimmune disease (23, 47), we investigated the levels of these cytokines in livers infected with Ad virus. We injected i.v. B6 mice with 2 × 10⁹ PFU AdLacZ and sacrificed the animals at 0, 6, 12, and 24 h, as well as on days 3 and 6 postinfection. We detected a significant increase in TNF-α and IL-23p19 expression at 6 h, followed by a robust elevation of IL-7 mRNA levels at 12 h (Fig. 5A). This increased level of IL-7 expression was only detected in the liver, and not in the lungs, heart, kidneys, bone marrow, spleen, and thymus (Fig. 5B). Along with an accumulation of total IHLs and CD44hiCD62lo CTL infiltrates during the peak of liver injury at day 6 (Fig. 3B), there was another increase in IL-7, IL-1β, and TNF-α messengers in the liver (Fig. 5A). To examine the proliferative potential of γδ T cells in response to these cytokines in vitro, we used purified splenic γδ T cells [because of comparable percentages of γδ T cell subsets in naive mouse liver and spleen (Supplemental Fig. 2B) and technical issues of obtaining sufficient numbers of hepatic γδ T cells]. Similar to a previous report (47), our results showed that IL-7 alone was sufficient to induce γδ T cell proliferation but not IL-17 secretion (Fig. 5C, 5D). IL-7 could synergize with IL-1β and TNF-α for γδ T cell proliferation, as well as act synergistically with IL-23 and IL-1β for IL-17 secretion. In the presence of an anti-TCRγδ mAb, IL-7 dramatically augmented IL-17 production in γδ T cells (Fig. 5E), suggesting that IL-7 could affect the γδ T cells to produce IL-17 with or without TCR engagement. Finally, IL-7 seemed to prolong the survival of γδ T cells ex vivo (Supplemental Fig. 2C).

Flow cytometric analysis demonstrated that the intrahepatic IL-17⁺ γδ T cells preferentially expressed high levels of IL-7Rα (Fig. 6A). In contrast, IFN-γ⁺ γδ T or CD4⁺ T cells did not express IL-7Rα (Fig. 6A). Compared with NK, NKT, and αβ⁺ T cells, γδ T cells in the liver expressed the highest level of IL-7Rα (Fig. 6B). When we treated the mice with an antagonistic mAb against IL-7Rγδ, the blockade of IL-7R signaling halted the expansion of IL-17⁺ cells in the liver (Fig. 6C). These data may mean that hepatocytes regulate the γδ T cell population and function through the IL-7/IL-7R interaction after Ad infection. The early IL-7 and IL-17 production creates a favorable cytokine environment in the liver for effective Ag presentation and T cell development.

**Hepatocyte-derived IL-7 induced by IFN-I signaling**

IL-7 was previously thought to be produced constitutively in the thymus and bone marrow (49, 50). A more recent study reported that liver can produce IL-7 in response to TLR4 signaling in vivo (40). In this study, we found a transient but robust IFN-β expression at 6 h postinfection (Fig. 7A), followed by a similar rise in IL-7 mRNA levels at 12 h (Fig. 5A). We hypothesized that IFN-I signaling was partially responsible for hepatic IL-7 production after Ad infection. By using Ad-infected wild-type and IFNAR−/− mice, we found that in the absence of IFN-I signaling, the animals failed to express IL-7 in the liver despite viral challenge (Fig. 7B). In vitro studies indicated that IFN-β could significantly induce the IL-7 expression only in primary hepatocytes isolated from wild-type mice, but not from IFNAR−/− mice (Fig. 7C). To confirm further the inducible effect of IFN-I signaling on hepatic IL-7 production, we injected naive mice i.v. with murine IFN-α or
IFN-β and examined the expression levels of IL-7, IL-1β, TNF-α, IL-12, and IL-23 in the perfused liver at 5 h posttreatment. Results showed that IFN-β injection resulted in a significant increase in the expression levels of IL-7 and TNF-α (Fig. 7D), but not in those of IL-23 and IL-12 (data not shown). In contrast, both IFN-β and IFN-α significantly suppressed IL-1β mRNA levels in the liver. These results may indicate that the IFN-I signaling pathway is pivotal in inducing IL-7 expression by hepatocytes, which might exert immunomodulatory functions during Ad infection.

Discussion

The primary goal of this study is to understand the complex interactions of the innate and adaptive immune components in the liver parenchyma in Ad-induced hepatitis. We and others have shown previously that serum ALT levels peak around day 6–7 after adenoviral infection in C57BL/6 mice (42). We further demonstrated in this study that hepatic IL-17–producing cells, as well as liver and serum IL-17 levels, peaked at day 1 and subsided at day 6 postinfection (Figs. 1 and 4, respectively), which was critical for regulating the subsequent CTL and Th1 responses (Figs. 1–3).

These results are consistent with previous observations that IL-17 stimulates dendritic cells to promote Th1 responses (14, 16, 35). Importantly, we found that it is γδ T cells that predominantly expanded and produced a bolus of IL-17 early in the infection (Fig. 4). Other minor populations of intrahepatic cells also produced IL-17 at this stage of infection, though at much lower levels (Fig. 4B, Supplemental Fig. 1B). These cells were TCRγδ-negative and contained CD4γδ, CD8γδ, and NKT cells. Compared with the high percentage of IL-17+ γδ T cells (42%, Fig. 4B), the IL-17+ γδ T cell subsets were all below 0.9% within their respective populations (Supplemental Fig. 1B). Unlike the conventional Th17 effectors (13), the IL-17–producing T cells in the liver do not require a lengthy Ag presentation process, but rather behaved more as the sentinels of the immune system (32). On the basis of these data, we conclude that early IL-17 production from γδ T cells is critical for the development of virus-specific adaptive immune responses in the liver.
As with CD4+ helper T cells, subsets of γδ T cells could also be defined based on distinct cytokine profiles, including IFN-γ-producing and IL-17–producing γδ T cells. Recent reports showed that IL-17–producing γδ T cells express CCR6, IL-23R, and CD25, whereas IFN-γ-producing γδ T cells preferentially express CD27 and NKI.1 (reviewed in Ref. 51). Given that the ligand of CD27 is CD70, which is mostly expressed on the peripheral APCs, it is not surprising that there is an expansion of IFN-γ-producing γδ T cells at 6 d after Ad infection (Fig. 4). Furthermore, in this study, we found that IL-17–producing γδ T cells expressed high levels of IL-7Rα, meaning possibly that IL-7 plays a critical role in the activation of IL-17–producing γδ T cells, as it is known to do in the expansion of IL-17+ γδ T cells in autoimmune diseases and bacterial sepsis in animals (40, 41, 52).

The liver is a metabolically active, but immunologically quiescent, organ. Previously, we and others have shown that hepatocytes are capable of activating and altering homeostasis and effector function of Th cells and CTLs in the liver (4, 39, 53). Moreover, there is recent evidence that liver tissues can produce large amounts of IL-7 in response to TLR4 activation (40). Also, in mice infected with lymphocytic choriomeningitis virus, IL-7 injections controlled viral infection by augmenting T cell responses, leading to an increase in serum IL-7 levels (54). However, it is unclear in that infection model whether hepatocytes are capable of producing IL-7 directly. In this study, by using Ad-induced hepatitis and primary hepatocyte cultures (Figs. 5, 7), we provide the first evidence, to our knowledge, that hepatocytes are capable of producing IL-7 during viral hepatitis infection. Thus, hepatocytes can regulate neighboring cells through cytokine production and promote Ag presentation and T cell activation. We propose that the liver parenchymal cells and intrahepatic lymphocytes mounted a highly coordinated immune response soon after viral infection. As hepatocytes produced IL-7, a group of γδ T cells began to upregulate their surface IL-7Rα and secreted IL-17 in situ.

Ad infection has been reported to induce quick and successive IFN-β and IFN-α responses in the liver (55). The binding of IFN-β and IFN-α to a shared IFNAR can induce IFN-stimulated genes and chemokine/cytokine production, resulting in the establishment of an anti-viral state in the liver. IFN-α could also induce the development of anti-viral CTL responses through the activation of APCs (Supplemental Fig. 3A). In this study, we found that Ad infection resulted in a sharp IL-7 response only in the livers of wild-type mice, but not in those of the IFNAR−/− animals (Fig. 7B). Using in vitro primary hepatocyte cultures, we have, for the first time, to our knowledge, demonstrated that hepatocytes are capable of producing IL-7 in response to IFN-β stimulation. Because IFN-β itself did not directly change γδ T cell functions, as judged by the levels of IL-17 production or cell surface expression of IL-7Rα (Supplemental Fig. 3B, 3C), we believe that hepatic IL-7 serves as a crucial player in orchestrating cross talk between type I IFN signaling and IL-17+ γδ T cells, albeit type I IFN signaling was reported to suppress Th17 development in some autoimmune diseases (56).

Ad is a prototypical DNA virus and an important pathogen. It is also one of the preferred vectors for gene therapy, cancer therapy, and experimental vaccines (1). When i.v. injected in mice, a majority of the viruses was eliminated quickly by the innate immune mechanisms within 24 h (2). However, subsequently, they were slowly cleared by the virus-specific CTL and Th responses (3, 4, 6, 7). Surprisingly, γδ T cell–deficient mice developed significantly less severe liver inflammation but showed no significant difference in viral clearance (11). In contrast, overzealous T cell responses may result in increased necroinflammatory hepatitis without accelerating viral elimination in vivo (4, 39). Thus, further investigations using chronic infection models are needed to define the role of IL-17 in virus clearance.

In summary, our in vivo and in vitro studies indicated that shortly after adenoviral infection, hepatocytes secreted IL-7, and that IFN-I could promote hepatocyte activation and IL-7 production. Hepatocyte-derived IL-7 selectively regulated the expansion of IL-17–producing T cells in the liver, especially those IL-7Rα–expressing T cells. Moreover, at the early stages of infection in the liver, the majority of IL-17+ cells are γδ T lymphocytes, and their IL-7 secretion is critical for the subsequent anti-viral Th and CTL responses. Thus, the highly coordinated events taking place among hepatocytes, as well as innate and adaptive immune cells, eventually lead to viral clearance and disease resolution in the liver. While this study clearly revealed an essential role for the IFN-β/IL-7/IL-17 cascade in regulating innate and Ad-specific immune responses in the liver, it will be important to investigate the potential cross talk of these cytokines in other types of viral hepatitis.

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Disclosures

The authors have no financial conflicts of interest.

References

### Supplementary Materials

**Supp. Table 1. Primer pairs for qRT-PCR assays**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>IL-17</td>
<td>Forward 5’-TTTAACTCCCTTTGGGCGCAAAA-3’&lt;br&gt;Reverse 5’-CTTTCCCTCCGGCATTGACAC-3’</td>
</tr>
<tr>
<td>IL-23</td>
<td>Forward 5’-AGCAACTTCACACCTCCCTAC-3’&lt;br&gt;Reverse 5’-ACTGCTGACTAGAAGTCAGGC-3’</td>
</tr>
<tr>
<td>IL-12</td>
<td>Forward 5’-AGACATCACACGGGACCAAAC-3’&lt;br&gt;Reverse 5’-GGAAGCTCTCTGTTTTTGTAGTA-3’</td>
</tr>
<tr>
<td>IL-7</td>
<td>Forward 5’-TTCCTCCACTGATCCTTGCTTTC-3’&lt;br&gt;Reverse 5’-AGCAGCTCTCTTTGTGGTCAC-3’</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward 5’-GCAACTGTTCCTGAATCTCAACT-3’&lt;br&gt;Reverse 5’-ATCTTTTGAGGTCGTTAC-3’</td>
</tr>
<tr>
<td>IL-10</td>
<td>Forward 5’-GCTCTTACCTGACTGGCATG-3’&lt;br&gt;Reverse 5’-CGCAGCTGAGAAGCAGTG-3’</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Forward 5’-ATGAACGCTACACTCGATC-3’&lt;br&gt;Reverse 5’-CCATCCTCTGTGCCGTTCCT-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward 5’-CCCTCAACTTCAGATCATCTTC-3’&lt;br&gt;Reverse 5’-CTTTGACGATCCCGGTTG-3’</td>
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Supp. Figure 1

Supp. Fig. 1. IL-17- and IFN-γ-producing cells following AdlacZ infection. Wild-type C57BL/6 mice were injected i.v. with $2 \times 10^9$ pfu of AdLacZ and euthanized at 0, 1 and 6 days post-infection. A) Splenocytes were prepared and stimulated with PMA and ionomycin for 4 h. Shown are representative flow cytometric analysis of IL-17- and IFN-γ-producing splenocytes. Results showed that there were no significant expansions of IL-17- or IFN-γ-producing cells in splenocytes following infection. B) To further dissect the IL-17-producing γδ T cells in the liver at 24 h post AdLacZ infection, we gated on different cell subsets, including CD4+ T, CD8+ T, NK (CD3− NK1.1+), and NKT (CD3+ NK1.1+) cells to examine their IFN-γ and IL-17 production through intracellular staining. Intrahepatic lymphocytes were isolated and stimulated with PMA and ionomycin for 4 h. Shown are representative flow cytometric analysis of IL-17- and IFN-γ-producing dot plots with different cell subset gates. Data shown that only minor fractions (<0.9%) of them secreted IL-17. One exception is intrahepatic NK cells that only secreted IFN-γ but not IL-17. For A and B, n = 3 at each time point, and experiments were repeated at least 3 times with similar patterns.
Supp. Figure 2

**Supp. Fig. 2. γδ T subsets and IL-17-producing cells in mice.** A) C57BL/6 mice were injected i.v. with $2 \times 10^9$ pfu of AdLacZ and euthanized at 0 and 1 day post-infection (3 mice per group). IHLs were prepared and stimulated with PMA and ionomycin for 4 h. Then, stimulated cells were collected and stained with surface CD3, TCRγδ, Vγ4, and intracellular IL-17. Shown were representative flow cytometric dot plots. Experiments were repeated at least 3 times with the same pattern. Results showed that the Vγ4 subset was the major IL-17-producing γδ T cells in the liver following Ad infection. B) Flow cytometric examination of subsets of intrahepatic and splenic γδ T cells in naïve B6 mice (n = 3). Shown were representative results. The liver and spleen had similar proportions of Vγ1, Vγ4 and Vγ5 subsets. C) Splenic γδ T cells were isolated from naïve B6 mice through magnetic purification and then were cultured with or without IL-7 for 24 h. At the end of culture, cells were collected and stained with PE-Annexin V (BD Bioscience). Experiments were repeated twice with similar patterns; shown were representative flow cytometry histograms. Data showed that most of the γδ T cells were Annexin V+ (70%) without IL-7. However, the presence of IL-7 reduced apoptotic cells (47% Annexin V+) and maintained the survival of γδ T cell in vitro.
**Supp. Figure 3**

**Supp. Fig. 3. IFN-β exerted no direct influence on IL-17 production or IL-7R expression on γδ T cells but was critical for adaptive anti-virus immune response.**

A) Both wild-type B6 mice and IFNαR-/- mice were injected i.v. with 2 × 10⁹ pfu of AdLacZ and euthanized at day 6 post-infection (n = 5 in both groups). IFNαR-/- mice developed less severe hepatitis when compared to wild-type mice. Consistent with the lower serum ALT levels, CD8⁺ T cell effector functions were significantly impaired in IFNαR-/- mice. Left, Serum ALT level; Right-up, IHLs were isolated and stimulated with anti-CD3 for 4 h in the presence of GolgiStop. The cells were then stained for surface markers and intracellular cytokines and examined by flow cytometry. Shown were representative flow cytometric dot plots; right-bottom, statistical results of 5 mice in both groups. **, p < 0.01.

B and C, Splenic γδ T cells were purified from naïve mice by using magnetic positive selection. B) Splenic γδ T cells were cultured with IL-1, IL-23, or IFN-β either individually or in combinations for 48 h. Supernatants were analyzed for IL-17 by ELISA. Our results showed that IFN-β did not influence the ability of IL-23 to induce IL-17 in γδ T cells. C) Splenic γδ T cells were cultured with IFN-β, IL-1β, IL-7 and IL-23 respectively, for 24 h, and subsequently examined for surface IL-7R expression. Shown were representative data from 3 independent experiments. Data showed that IFN-β had no direct influence on IL-7R expression on γδ T cells *in vitro.*