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IL-33 Induces Nuocytes and Modulates Liver Injury in Viral Hepatitis

Yuejin Liang,* Zuliang Jie,* Lifei Hou,* Renan Aguilar-Valenzuela,* David Vu,* Lynn Soong,*†‡ and Jiaren Sun*†‡

Molecules containing damage-associated molecular patterns play an important role in many pathogenic processes. In this study, our aim was to investigate the role of IL-33, a damage-associated molecular pattern molecule, in adenovirus (Ad)–induced liver inflammation. Ad–infected mice exhibited a steadily increased IL-33 and its receptor IL-1R–like 1 expression in the liver during the first week of infection. Treatment of exogenous IL-33 resulted in a great decrease in the serum alanine aminotransferase levels and the number of Councilman bodies in the liver. Attenuated liver injury by IL-33 correlated with an increase in T regulatory cells but with a decrease in macrophages, dendritic cells, and NK cells in the liver. IL-33 enhanced both type 1 (IL-2 and IFN-γ) and type 2 (IL-5 and IL-13) immune responses in infected mice. However, IL-33 inhibited TNF-α expression in hepatic T cells and macrophages, and significantly reduced TNF-α levels in the liver. We found that in addition to its direct effects, IL-33 strongly induced novel nuocytes in the livers and spleens of infected mice. When cocultured with nuocytes, hepatic T cells and macrophages expressed lower levels of TNF-α. The IL-33–treated mice also demonstrated a slight delay, but no significant impairment, in eliminating an intrahepatic infection with Ad. In conclusion, this study reveals that IL-33 acts as a potent immune stimulator and a hepatoprotective cytokine in acute viral hepatitis. Its direct immunoregulatory functions and ability to induce novel nuocytes further suggest to us that it may be a potentially promising therapeutic candidate for the management of viral hepatitis. The Journal of Immunology, 2013, 190: 000–000.

Viral hepatitis is a major public health problem affecting millions of people in the world. There is no vaccine to prevent hepatitis C virus infection or to treat the disease. Recently, experimental vaccines based on adenovirus (Ad) vectors have been shown to induce sustained protective immunity against hepatitis C virus in humans (1). In addition to its potential values in vaccine development (2), Ad is also commonly used for gene delivery and cancer therapy (3, 4). Despite its utilities, Ad infection can also induce strong CD8* CTL, CD4* Th, and B lymphocyte responses (5, 6), a common characteristic among a number of hepatotropic viruses, including hepatitis A virus, hepatitis B virus, CMV, herpes simplex, and EBV. When i.v. injected, Ad preferentially targets the liver. Although most of the invading Ad is eliminated by the innate immune mechanisms, the clearance of the remnant virus is slow and variable depending on the virus-specific CTL and Th responses (5, 7, 8). Failure to constrain immune responses can turn a self-limiting infection to necroinflammatory hepatitis and/or result in treatment failure or even patient death (9, 10). Although the liver injury associated with Ad infection is known to be mediated by hepatotoxic cytokines such as TNF-α (11, 12), the key events that set off hepatic inflammation and regulate immune responses in the liver are still not totally clear.

Immune responses are often initiated by recognition of pathogen-associated molecular pattern (PAMP) molecules (13). Engagement of PAMPs in macrophages, γδ T cells, and dendritic cells (DCs) triggers their phagocytic or endocytic activities as well as cytokine and chemokine secretion. In the case of autoimmune diseases, ischemia and reperfusion injury, and chronic organ rejection events, strong immune responses are initiated and perpetuated by damage-associated molecular pattern (DAMP) molecules (14, 15). They are also known to synergize with PAMPs in infectious diseases and further enhance immune responses (16).

Among DAMPs, IL-33 is a member of the IL-1 superfamily and binds to IL-1R–like 1 (ST2), which signals the NF-κB pathway (17). IL-33 is released from necrotic cells during tissue injury and is closely correlated with serum alanine aminotransferase (ALT) and aspartate aminotransferase levels in chronic hepatitis patients (17, 18). IL-33 is a crucial amplifier of the innate immunity and drives antiviral CD8* cell responses (19, 20). However, IL-33 can also induce the expression of Th2-like cytokines (e.g., IL-5 and IL-13) as well as T regulatory (Treg) cells and prolong cardiac allograft survival (21, 22). Collectively, these reports indicate a broad function of IL-33 in infectious and noninfectious diseases, as well as its multifunctional and enigmatic mechanisms of action. IL-33 can be released from endothelial and epithelial cells (23). Lately, it has been reported that epithelial cells, hepatocytes, and hepatic stellate cells are the main sources of IL-33 in the liver (24–26), and that IL-33 protects the liver from periportal and peripheral hepatic injury caused by hepatic ischemia/reperfusion injury (27, 28).

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induction of other cell populations, such as novel nuocytes first reported in 2010 (29). Nuocytes are often referred to as innate type 2 cells, innate type 2 helper cells, or nature helper cells (29–32). These cells belong to a heterogeneous family of innate cells that do not express T or B lymphocyte markers. Nuocytes are present in human and mouse lungs, gut, and fat-associated lymphoid clusters, as well as in the liver (32–34), and they contribute to type 2 immune responses and tissue repair in asthma and parasitic diseases in an Ag-nonspecific fashion (29, 35). However, their role in viral hepatitis and hepatic inflammation is not understood.

In this study, we found a steady increase of IL-33 and ST2 expression in the liver in parallel with inflammatory cytokines during the first week of Ad infection in mice. Whereas the administration of IL-33 enhanced both type 1 and type 2 immune responses, it increased Treg cell frequencies and significantly reduced liver injury. IL-33 inhibited the TNF-α levels in the liver-derived CD4+, CD8+ T, and CD11b+ cells. More importantly, IL-33 induced a strong expansion of nuocytes in vivo, which further suppressed TNF-α expression in the hepatic lymphocytes. In conclusion, this study indicates that Damp molecule IL-33 can act as both a potent immune stimulator and a hepatoprotective cytokine in viral hepatitis. Its direct immunoregulatory functions and ability to induce novel nuocytes further suggest it as a potentially promising therapeutic candidate for the management of viral hepatitis.

Materials and Methods

Animals and treatment

Female C57BL/6 (B6) mice were purchased from The Jackson Laboratory. All mice were maintained under specific pathogen-free conditions at the University of Texas Medical Branch Animal Care Facility and used at 7–10 wk age, according to National Institutes of Health guidelines and with the approval of the Institutional Animal Care and Use Committee. Mice were i.v. injected with 3 × 10⁵ PFU Ad carrying the LacZ gene (AdLacZ) that expresses a reporter β-gal to induce hepatitis. Cytokines from BioLegend were used in our studies, unless specified otherwise. Recombinant mouse IL-33 (0.8 µg/mouse) or PBS was administered i.p. daily from 1 to 5 dpi and mice were sacrificed at the indicated time points. For the isolation of innate type 2 cells, naïve mice were i.p. injected daily with IL-33 (0.8 µg/mouse) for 5 d and euthanized at 6 dpi.

H&E and histological scores

Liver specimens were fixed in 10% buffered formalin. Paraffin-embedded sections were stained with H&E for histological evaluation by using a modified Knodell scoring system (36). Briefly, normal liver architecture without remarkable injury or cellular infiltration is scored as 0. A score of 1 represents limited infiltration of inflammatory cells in the portal triad without significant involvement in the lobular and pericentral regions. In addition to these pathological changes, a score of 2 depicts moderate involvement in the portal areas, accompanied by isolated apoptosis and necrosis in the lobular and pericentral areas. A score of 3 involves extensive lymphocyte infiltration in the portal area with widespread apoptosis and bridging necrosis throughout the liver.

Detection of AdLacZ in liver

The detection of AdLacZ was performed as previously described (37). Briefly, following fixation with 0.5% glutaraldehyde for 30 min, frozen liver sections were incubated with 0.2 mg 5-bromo-4-chloro-3-indolyl/β-D-galactopyranoside/ml at 37°C for 120 min. Infected cells expressing β-gal activity were stained blue, whereas uninfected cells were counterstained red (neutral red). Eight images of each liver section were randomly selected and captured with an Olympus BX51 microscope equipped with the Olympus DP70 video camera (Olympus Optical, Tokyo, Japan). The images were analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD). Infectivity of the hepatocytes was expressed as the average ratio of infected to total cells.

Isolation of cell populations

For intrahepatic lymphocyte (IHL) isolation, livers were perfused, digested, and purified by density gradient centrifugation with 30 and 70% Percoll (Sigma-Aldrich). For splenic lymphocyte isolation, the spleen was pressed through a 75-µm strainer. After treatment with the RBC lysing buffer (Sigma-Aldrich), mononuclear cells were collected. For lineage (Lin−) and Lin+ cell isolation, spleen cells were prepared from the IL-33-treated mice, blocked with FcR blocker (clone 2.4G2; eBioscience), and then incubated with FITC-conjugated anti-CD3, CD4, CD8, CD11b, CD11c, B220, NK1.1, Ter-119, and Gr-1 (eBioscience) for 30 min at 4°C. After washing, cell suspensions were incubated with anti-FITC microbeads, followed by a negative selection on the LD column (Miltenyi Biotec). The purities of the Lin− and Lin+ cells were 92% and 93%, respectively.

Cell culture

Lymphocytes isolated from 6-d-infected mice were seeded at 1 × 10⁶/ml in 24-well plates in complete RPMI 1640 medium at 37°C and with 5% CO₂. In some cultures, IL-2 and IL-7 (10 ng/ml) were used together with various concentrations of IL-33. After 48 h, cells were collected for FACS analysis. In some experiments, Lin− cells were seeded at 1 × 10⁶/ml in six-well plates in complete RPMI 1640 medium at 37°C and with 5% CO₂. To expand the cells, IL-2, IL-7, and IL-33 (10 ng/ml) were added respectively to the culture system. The medium was changed every 2 d, and the cytokines were supplemented. Cultured cells were analyzed for the surface markers CD43, CD44, CD45, CD69, CD25, IL-7Rα, e-Kit and MHC class II, as well as the intracellular cytokines IL-5 and IL-13, by flow cytometry to confirm the characteristics of nuocytes as reported (29).

Flow cytometry

Cells were blocked with FcγR blocker first and stained with fluorochrome-labeled Abs or biotinylated mAbs, followed by fluorochrome-conjugated streptavidin. The specific Abs and their corresponding isotype controls were purchased from BioLegend and eBioscience. The following Abs were used in combinations: FITC-anti-CD3e (145-2C11), PE-Cy7-anti-CD8a (53.6.7), PE-anti-CD127 (A7R34), FITC-anti-CD4 (GK1.5), Pacific Blue-anti-CD4 (RM-4.5), allophycocyanin-Cy7-anti-CD4 (RM-4.5), FITC-anti-CD8a (53.6.7), allophycocyanin-Cy7-anti-CD8a (53.6.7), Pacific Blue-anti-CD8 (53.6.7), FITC-anti-CD11b (M1/70), PerCP-Cy5.5-anti-CD11b (M1/70), FITC-anti-CD11c (N418), allophycocyanin-anti-CD25 (PC61.5), biotin-anti-CD43 (1B11), PE-anti-CD44 (IM7.81), PE-anti-CD45 (30-F11), FITC-anti-B220 (RA3-6B2), PE-anti-CD80 (16-10A1), biotin-anti-CD90.2 (30-H12), PE-anti-CD11c (N418), PE-Cy7-anti-CD3ε (eBMG2b), and FITC-anti-IgG2b isotype (eBMG2b).

For intracellular cytokine staining, cells were incubated for 4 h with PMA (50 ng/ml; Sigma-Aldrich), ionomycin (750 ng/ml; Sigma-Aldrich) and GolgiStop (1 µl/ml; BD Biosciences). At the end of incubation, the cells were stained with biotinylated Abs and biotinylated anti-FITC microbeads, followed by staining for intracellular cytokines by using a fix/permeabilization kit (eBioscience). Data were collected by a BD LSRFortessa (Becton Dickinson, San Jose, CA) and analyzed by using FlowJo software version 8.86 (Tree Star, Ashland, OR).

Quantitative RT-PCR

Snap-frozen liver tissues were used to extract genomic DNA and total RNA. DNA was extracted with a DNeasy blood and tissue kit (Qiagen), and total RNA was extracted with an RNeasy Mini kit (Qiagen) and digested with DNase I (Ambion). The concentrations of DNA and RNA were assessed by spectrophotometer (Eppendorf). cDNA was synthesized by using a SuperScript III first-strand synthesis system (Invitrogen). The quantitative RT-PCR (qRT-PCR) assays were performed with iQ SYBR Green Supermix and a CFX96 Touch real-time PCR detection system (Bio-Rad). 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. Melting curve analysis was also used to check the specificity of the amplification reaction. Relative quantity of mRNA expression was calculated by using the 2−ΔΔCT method. The primers are listed in Supplemental Table I.

ELISA assay

For extracting proteins from the liver, liver tissues were suspended in a RIPA lysis buffer (Cell Signaling Technology) that contains 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM NaEDTA, 1 mM EGTA, 1% Nonidet P-40, 1% sodium deoxychylate, 2.5 mM sodium pyrophosphate, 1 mM β-glycero-phosphate, 1 mM NaVO₃, and 1 µl/ml leupeptin in the presence of the
protease inhibitor mixture (Sigma-Aldrich). Protein concentrations were assayed by using a BCA kit (Pierce). The levels of IL-33 in serum and liver tissues were assayed by using an ELISA kit (eBioscience) according to the manufacturer’s instructions.

**Bio-Plex assays**

Serum IFN-γ and IL-2 were examined on a Bio-Plex platform (Bio-Rad). Briefly, colored beads coated with different Ags were mixed together with serum sample and then allowed to incubate overnight at 2–8°C. After two wash cycles, detection Ab was added and allowed to incubate for 1 h at room temperature, followed by incubation with streptavidin-PE for 30 min at room temperature. After removal of excess conjugate, 150 μl sheath fluid was added to each well. The beads were read through a bead detector based on the fluorescence of the dyes. Raw data were measured as the relative fluorescence intensity and then converted to the concentration according to the standard curve. A multiplex assay kit was purchased from Millipore.

**Adoptive cell transfer**

B6 mice were i.p. injected with IL-33 (0.8 μg/mouse) for 5 d. Lin− cells were isolated and expanded with cytokines IL-2, IL-7, and IL-33 (10 ng/ml) in vitro. Cells from 2- to 6-d culture were analyzed by flow cytometry and used for an adoptive transfer experiment as reported (29). The expanded nuocytes (2 × 10⁶ cells in 200 μl PBS) were i.v. transferred into B6 mice at 1, 3, and 5 dpi (PBS was used as a control). All of the mice were sacrificed at 6 dpi.

**Statistical analyses**

Data were shown as means ± SEM and analyzed by using the two-tailed Student t test when comparing between two groups. A p value < 0.05 was considered statistically significant. Graphs prepared from flow cytometric findings were from three independent experiments with similar results. Findings were statistically analyzed by using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA).

**FIGURE 1.** IL-33 was involved in viral hepatitis and attenuated liver damage. B6 mice were i.v. injected with AdLacZ (3 × 10⁹ PFU/mouse). (A) Mice were sacrificed at the indicated time points. IL-33 and ST2 expression was analyzed by qRT-PCR. Serum was collected for ALT detection (five to seven mice per group). (B) The liver and serum IL-33 levels in naive mice and mice at day 6 after infection were detected by an ELISA (six to seven mice per group). (C) At 24 h after infection, exogenous IL-33 (0.8 μg/mouse in PBS) was i.p. injected into the infected mice daily until sacrifice at day 6. Representative microphotographs of hepatic H&E stain (6 dpi) are shown. Scale bars, 200 μm. (D) Cumulative graphical representation of the histological scores. (E) The number of Councilman bodies was counted in the H&E slides. (F) Serum ALT was detected after IL-33 treatment (five to eight mice per group). Experiments were repeated for three times independently. Values are shown as means ± SEM. A two-tailed t test was used for statistical analysis. *p < 0.05, **p < 0.01.
Results

IL-33 attenuated T cell-mediated liver injury in viral hepatitis

To determine the expression of DAMP molecule IL-33 in viral hepatitis, we i.v. injected B6 mice with AdLacZ that expresses a reporter β-gal, as described previously (8). When AdLacZ was injected through the tail vein, the hepatotropism of the virus was evidenced by β-gal staining of the liver but not the spleen, kidneys, or lungs (data not shown). Animals were sacrificed at 0, 0.5, 1, 3, and 6 dpi, and their hepatic injury as well as IL-33 and ST2 expression levels were measured. As shown in Fig. 1A, serum ALT levels began to rise on day 3 and continued on to day 6. Hepatic IL-33 and ST2 displayed the same pattern on these time points. By 6 dpi, the levels of IL-33 in both the liver and serum of infected mice were significantly higher than those in naïve animals (Fig. 1B). These data confirm a previous report (18) showing a positive association between the IL-33 levels and liver injury in viral hepatitis.

To investigate the role of IL-33 in viral hepatitis, we treated mice with recombinant mouse IL-33 (0.8 μg/mouse, i.p.) or PBS daily from day 1 to 5 after infection. Animals were sacrificed and analyzed for liver inflammation at 6 dpi (Fig. 1C). IL-33 treatment significantly upregulated ST2 mRNA expression, although it did not change that of IL-33 (Supplemental Fig. 1A). AdLacZ injection caused prominent portal and lobular lymphocytic infiltration. Bridging necrosis accompanied by many Councilman bodies was found in all three adjacent zones (Fig. 1C, arrows). The hepatic histopathological scores of the IL-33–treated mice were comparable to those of the PBS group (Fig. 1D). Further analyses revealed a significant reduction in Councilman bodies in the IL-33–treated mice (Fig. 1E). IL-33 treatment also reduced the serum ALT levels considerably in these animals (Fig. 1F). These results suggest to us that there is a protective role of IL-33 in Ad-induced liver injury in mice.

IL-33 had differential effects on liver-infiltrated cells

To understand the mechanism of liver protection by IL-33, we analyzed the infiltration and activation of immune cells in the liver of infected mice. As shown in Fig. 2A, injection of AdLacZ caused an ~8-fold increase in IHL recruitment at 6 dpi, and one-half of these IHLs were CD8+ T cells. Consistent with the histological scores, the numbers of IHLs were not altered by IL-33 treatment. Among the infiltrating CD4+ and CD8+ T cells in the liver, the number of activated T cell subpopulations (CD44hiCD62Llo) were comparable between the IL-33 and PBS groups (Fig. 2B), demonstrating that IL-33 did not influence the migration of effector T cells to the inflamed liver.

In the liver, IL-33 treatment significantly reduced CD11b+, CD11c+, and NK cells, but it increased Treg cells (Fig. 2C). The diminished and augmented frequencies of NK and Treg cells, respectively, were also observed in the spleen (Fig. 2D). IL-33 treatment repressed the expression of MHC class II on CD11b+ and CD11c+ cells, as well as the expression of CD80 on CD11b+ CD11c+ cells (Supplemental Fig. 2), possibly indicative of subdued Ag presentation in the IL-33–treated animals (38). To gauge the effect of IL-33 treatment on AdLacZ clearance in the liver, we stained samples for β-gal activity in the liver of both groups of mice (Fig. 2E). The results from these experiments showed that both IL-33 and PBS treatments resulted in 10.6 ± 0.8 and 7.3 ± 0.7% infectivity, respectively, at 6 dpi. Both groups of mice followed similar courses of infection (0.8 ± 0.2 and 0.7 ± 0.3%, respectively, at 14 dpi), with the disappearance of most AdLacZ-infected hepatocytes by 21 dpi (<0.1% in both groups). We speculated that at 21 dpi the viral loads might draw near the threshold of the β-gal assay and thus prohibited further identification of infected cells. To provide an independent measure of viral clearance, we also determined the viral load in the liver tissue by a highly sensitive quantitative PCR. Consistent with functional loss of viral β-gal activity, there was a steady reduction of the viral genome in both
groups of mice (Fig. 2E). Taken together, these results demonstrated a slight delay but no significant impairment in the ability of the IL-33–treated mice to eliminate an intrahepatic infection with Ad.

**IL-33 induced IFN-γ but inhibited TNF-α in viral hepatitis**

To further investigate the mechanism of mitigated liver injury after IL-33 administration, we examined the cytokines secreted by inflammatory cells. Mice were i.v. injected with AdLacZ followed by IL-33 or PBS treatment by i.p. injection daily and were sacrificed at 6 dpi. As shown in Fig. 3A, the percentages of hepatic IFN-γ^+ CD4^+ and IFN-γ^+CD8^+ T cells were significantly increased in the IL-33 group compared with those of the PBS group. The serum IFN-γ and IL-2 were upregulated in IL-33 group (Fig. 3B), as well as the hepatic gene expression of IFN-γ, granzyme B, and perforin (Fig. 3C), showing that IL-33 was able to enhance the type 1 immune responses in viral hepatitis.

Next, we examined the expression of TNF-α, which is the crucial cytokine of hepatotoxicity and mediates liver damage in viral hepatitis (11). As indicated in Fig. 4A, the hepatic gene expression of TNF-α was markedly reduced in the IL-33 group. Then, we analyzed the intracellular TNF-α expression of hepatic lymphocytes from the IL-33 and PBS groups, respectively. Consistently, IL-33 treatment significantly inhibited the expression of TNF-α on hepatic CD4^+ T, CD8^+ T, and CD11b^+ cells of infected mice (Fig. 4B). To further confirm the anti-inflammatory role of IL-33 in viral hepatitis, we isolated IHLs from mice at 6 dpi and cultured them in vitro with different concentrations of IL-33 (0, 0.1, 1, and 10 ng/ml) together with IL-2 (10 ng/ml) and IL-7 (10 ng/ml) for 48 h. The results of the intracellular staining showed that the expression of TNF-α was inhibited by IL-33 in a dose-dependent manner (Fig. 4C). However, IL-33 by itself was unable to inhibit TNF-α expression in vitro (Supplemental Fig. 3), indicating that IL-33 may function differently in the distinct microenvironments of inflammation and diseases.

**IL-33 induced nuocytes in Ad-infected mice**

It is documented in autoimmune and parasitic diseases that administration of exogenous IL-33 not only promotes Th2 response but also initiates a unique innate type 2 immunity (39). It is unclear, however, whether IL-33 can promote such an innate type 2 immune response in viral hepatitis. In this study, we infected the mice with AdLacZ and i.p. injected the IL-33 or PBS daily. Animals were sacrificed at 6 dpi and liver tissues were collected for cytokine gene detection. Lymphocytes were prepared from both spleens and livers for cytokine detection. As shown in Fig. 5A, IL-33 injection induced high levels of type 2 cytokines (especially IL-5 and IL-13) as well as type 2 chemokines (CCL17 and CCL22), but not type 1 chemokines (CXCL9 and CXCL10) and anti-inflammatory cytokines IL-10 and TGF-β (Supplemental Fig. 1A). These results demonstrated that IL-33 was indeed able to initiate the type 2 immune response in viral hepatitis. Moreover, as we examined AdLacZ-infected mice, we found that the splenic Lin^− cells from these animals contained more IL-4^+, IL-5^+, IL-6^+, or IL-13^+ cells compared with those in uninfected mice, which indicated the presence of the innate type 2 cells or nuocytes (Supplemental Fig. 1B). More interestingly, exogenous IL-33 was capable of inducing an expansion of both hepatic and splenic Lin^− cells that expressed high levels of IL-5 and IL-13 (Fig. 5B, Supplemental Fig. 3B). Further analyses of surface markers on the Lin^−IL-13^+ cells showed that they expressed CD127, ICOS, c-Kit, and Sca-1, which was consistent with the surface markers of

![FIGURE 3](http://www.jimmunol.org/)
Nuocytes exhibited a potential protective role in hepatitis

To further confirm the function of the IL-33–induced nuocytes in viral hepatitis, we isolated both Lin− and Lin+ cells from the spleens at day 5 postinjection with IL-33. The purities of Lin− and Lin+ cells were up to 92 and 93%, respectively. The Lin− cells expanded robustly in the medium containing IL-2, IL-7, and IL-33 for 2–6 d (data not shown), as previously described (29). These expanded cells secreted high levels of IL-5 and IL-13 (Fig. 6A) and expressed CD43, CD44, CD45, CD69, CD25, CD127, c-Kit, and MHC class II, characteristic of nuocytes (Fig. 6B). Importantly, when the Lin− or Lin+ cells were cocultured with hepatic lymphocytes of infected mice in medium containing IL-2, IL-7, and IL-33 for 48 h, intracellular expressions of TNF-α in CD4+ T, CD8+ T, and CD11b+ cells in the Lin− group were suppressed compared with those in Lin+ group (Fig. 6C).

To explore the role of nuocytes in vivo, we adoptively transferred 2 × 10^6 of the cultured nuocytes into the AdLacZ-infected mice at 1, 3 and 5 dpi. At 6 dpi, we found increased numbers of Lin− IL-13+ or Lin+ IL-5+ cells in the livers of transferred mice (Fig. 7A). Consistent with this observation of increased nuocytes, qRT-PCR results also showed the significant upregulation of hepatic IL-13 in the transferred group (Fig. 7B). Both the serum ALT and hepatic TNF-α expression of the transferred group presented a downward trend, but with no statistical significance (Fig. 7C, 7D).

Discussion

Many hepatotropic viruses can cause liver inflammation, and viral PAMPs are an important force driving CD8+ and CD4+ T cell priming through TLRs and/or TLR-like receptors on DCs (13, 40, 41). However, it is now thought that, in addition to PAMPs, the innate immune system can also recognize DAMPs, and both PAMPs and DAMPs can serve as the on–off switch of immunity (42, 43). DAMP molecules can initiate and perpetuate inflammatory processes not only in autoimmune processes but also in infectious diseases (14, 20, 44). Among them, IL-33 was initially considered a pro-Th2 cytokine (17, 45), but is now recognized as a crucial amplifier of innate immunity as well as Th1 and Th2 immune responses (19, 21, 45). IL-33 also plays a crucial role in driving antiviral CD8+ T cell responses in lymphocytic choriomeningitis virus–infected mice (20). Moreover, IL-33 can synergize with other cytokines such as IL-12 to increase IFN-γ secretion by CD8+ T cells (46, 47). Although both the pro- and anti-inflammatory roles of IL-33 have been reported in Con A– and ischemia/reperfusion-induced liver injury, little is known about the mechanisms of IL-33 in viral hepatitis in humans or in mice (18, 27, 48).

In this study, we asked whether IL-33 is involved in Ad-induced hepatitis, and, if so, how it regulates T cell responses and inflammation in the liver. We measured cytokine expression on total T cell subpopulations, because a previous report has shown that most of the CD4+ and CD8+ T cells are virus-specific during the Ad infection (49). Our data suggest to us that IL-33 drove robust CD8+ and CD4+ T cell responses in the liver, which induced equally strong type 1 cytokines (IL-2 and IFN-γ) as well as type 2 cytokines (IL-4, IL-5, IL-6, and IL-13) in the liver and serum (Figs. 3–5). The local cytokine and chemokine microenvironment recruited large numbers of highly activated CD8+ and CD4+ T cells to the liver (Figs. 1, 2). However, the enigmatic action of the IL-33/ST2 interaction did not exacerbate liver injury, but rather it limited hepatic injury, as reflected by the greatly decreased numbers of Councilman bodies in the liver and serum ALT levels (Fig. 1). Based on our observations, we speculate that IL-33 mediates these potent hepatoprotective effects through direct and indirect mechanisms.

First, IL-33 induces strong, but divergent, immune effector functions. We have shown that IL-33 injection induced CD8+ and CD4+ T cells to secrete large amounts of IFN-γ in the liver as well as the serum; however, it greatly reduced TNF-α expression in hepatic T cells and macrophages (Figs. 3, 4). In vitro IL-33 treatment inhibited TNF-α expression in the liver-derived CD8+ and CD4+ T cells and macrophages in a dose-dependent manner.
These data are consistent with previous reports, in which TNF-α was shown to play a central role in cellular necrosis and fulminant hepatitis (11, 50, 51). Additionally, we have shown that IL-33 treatment resulted in fewer numbers of infiltrating NK cells and DCs with decreased levels of CD80 and MHC class II expression in the liver (Fig. 2, Supplemental Fig. 2). In previous reports, IL-33–conditioned DCs were found to be less potent in priming naive T cells (38). However, IL-33 has been reported to directly increase IFN-γ but decrease TNF-α in Th1 and Th2 cultures (52). Disruption of the IL-33/ST2 axis, alternatively, enhanced NK functions and exacerbated Con A–induced hepatitis (53). Finally, we have demonstrated that IL-33 could modulate immune responses by expanding CD4+Foxp3+ Treg cells in the liver and spleen (Fig. 2). Disruption of the IL-33/ST2 signaling pathway prevented the generation of CD4+Foxp3+ Treg cells and exacerbated Con A–induced hepatitis (27). Additionally, according to a previous report, the viral clearance of Ad was Fas- and TNFR1-dependent (54). Our result showed that although the viral clearance was delayed owing to IL-33 treatment in the early stages, it was not compromised in the intermediate and late stages of Ad infection (Fig. 2E). Collectively, these results suggested to us that IL-33 was able to directly engage multiple arms of immune mechanisms and limit liver injury in T cell–mediated hepatitis.

IL-33 is a potent inducer of a new population of innate type 2 leukocytes, namely nuocytes (29–32). These cells belong to a heterogeneous family of innate cells that do not express T or B lymphocyte markers (32–34). They are critically involved in intestinal parasite expulsion, influenza infection, and related airway hypersensitivity (29, 34, 35, 55, 56). In this study, we found that IL-33 strongly induced nuocytes in the liver and spleen of infected mice. In vitro, nuocytes expressed high levels of IL-5 and IL-13 and were capable of inhibiting TNF-α expression in liver-derived lymphocytes in infected mice (Figs. 5, 6). This result is consistent with previous reports that IL-13 could inhibit TNF-α in mice (57) and reduced liver injury induced by ischemia/reperfusion (58). Furthermore, we found that IL-33 treatment–mediated ST2 up-expression

**FIGURE 5.** Nuocytes were induced by IL-33 in the liver of viral hepatitis. B6 mice were i.v. injected with AdLacZ (3 × 10^9 PFU/mouse) and at 24 h after infection; exogenous IL-33 (0.8 μg/mouse in PBS) was i.p. injected into the infected mice daily until sacrifice at day 6 (three to four mice per group). (A) Liver tissues were collected, and hepatic gene expression of type 2 cytokines (IL-4, IL-5, IL-6, IL-13) and chemokines (CXCL9, CXCL10, CCL17, and CCL22) were detected by qRT-PCR. (B) IHLs were isolated and stimulated in the complete RPMI 1640 medium with PMA/ionomycin in the presence of GolgiStop for 4 h. Cells were collected, and lineage markers (CD3, CD4, CD8, CD11b, CD11c, NK1.1, B220, Gr-1, and Ter-119) as well as intracellular IL-5 and IL-13 were analyzed by flow cytometry. The numbers represent, respectively, the percentages and the absolute numbers of detected cells in the boxes. (C) The Lin^− IL-13^+ cells in the IL-33 group were gated for the further detection of surface markers (CD127, ICOS, c-Kit, and Sca-1). Solid lines represent the isotype control, and gray lines represent target Ab staining.
Supplemental Fig. 1) and the IL-33/ST2 pathway activation led to an enhanced expression of type 2 chemokines CCL17 and CCL22 in the liver (Fig. 5). Despite its ability to induce a broad array of T cell effector molecules, cytokines, and chemokines, IL-33 did not induce IL-10 and TGF-β or CXCR3 ligands (CXCL9 and CXCL10) in the liver (Supplemental Figs. 1, 5). As previously reported (29), we used i.v. delivered Lin<sup>+</sup> cells or PBS in the adoptive transfer experiments, which may raise some concerns for an ideal control for nuocytes in such a study. Nevertheless, our adoptive transfer experiments clearly indicated that Lin<sup>−</sup> nuocytes persisted in the liver and secreted IL-5 and IL-13 in the liver of Ad-infected mice at 6 dpi (Fig. 7). Although the mice receiving nuocytes had lower serum ALT compared with the levels in the mock-transferred group, the difference between the two groups was not statistically significant (Fig. 7). These results suggested to us that nuocyte induction was merely one facet of the complex mechanisms of IL-33 action. More studies are needed to fully appreciate the direct and indirect actions of IL-33 in the pathogenesis of viral hepatitis.

In summary, we revealed that DAMP molecule IL-33 is a potent hepatoprotective cytokine in Ad-induced hepatitis. Exogenous IL-33-induced strong type 1, type 2, and Treg cell responses. However, it significantly inhibited TNF-α expression in T cells and macrophages and limited liver injury. In addition to its direct effect, IL-33 could also induce a strong expansion of IL-5/IL-13–expressing Lin<sup>−</sup> nuocytes, further downmodulating TNF-α. Its direct immunoregulatory functions and ability to induce novel nuocytes also may indicate that IL-33 is a potential therapeutic candidate for the management of liver injury and viral hepatitis.
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Disclosures

The authors have no financial conflicts of interest.

References


Figure S1. Nuocytes were induced in the spleen of virus-infected and IL-33 injected mice. After 24 h post-infection, IL-33 (0.8 μg/mouse) or PBS was i.p. injected into the infected mice daily until sacrifice at day 6 (3-5 mice per group). A) Hepatic gene expressions of IL-10 and TGF-β, as well as IL-33 and ST2, were detected by qRT-PCR. B) Splenic lymphocytes were isolated and stimulated with PMA and Ionomycin in the presence of GolgiStop for 4 h. Lineage markers (CD3, CD4, CD8, CD11b, CD11c, NK1.1, B220, Gr-1 and Ter-119) and intracellular IL-4, 5, 6 and 13 were analyzed by flow cytometry. Both the percentages and the absolute numbers of cells are shown on the flow cytometry figures. Graphs of flow cytometry were from three independent experiments with similar results. A Two-tailed T-test was used for statistical analysis. **, P<0.01;
Figure S2. IL-33 reduced the numbers of antigen-presenting cells and altered the expression of co-stimulatory molecules in the livers of infected mice. After 24 h post-infection, IL-33 (0.8 μg/mouse) or PBS was i.p. injected into the infected mice daily until sacrifice at day 6 (3-5 mice per group). A) In mice with viral hepatitis, the number of CD11b^+MHCII^+ or CD11c^+MHCII^+ cells in the liver dropped by approximately one-half after IL-33 injection, compared with that in PBS controls. B) The number of CD11b^+CD11c^+ cells markedly decreased in the IL-33 group compared with that in the PBS group. The CD11b^-CD11c^- cells were gated for analysis of CD80 expression. In the IL-33 group (gray), CD11b^-CD11c^- cells expressed lower levels of CD80 compared with those in the PBS group (solid lines). Graphs of flow cytometry were from three independent experiments with similar results.
Figure S3. IL-33 was not able to inhibit TNF-α of hepatic lymphocytes in vitro without stimulation by IL-2 and IL-7. Hepatic lymphocytes were isolated from the mice at 6 dpi and cultured with increased concentrations of IL-33 (0, 0.1, 1, 10 ng/ml) in complete RPMI medium for 48 h. PMA and ionomycin were used to stimulate the cells in the presence of GolgiStop at the last 4 h of the culture. Cells were collected and expression of TNF-α on CD4⁺ T, CD8⁺ T and CD11b⁺ cells was detected by flow cytometry. This experiment was repeated three times and the representative graphs are shown.
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