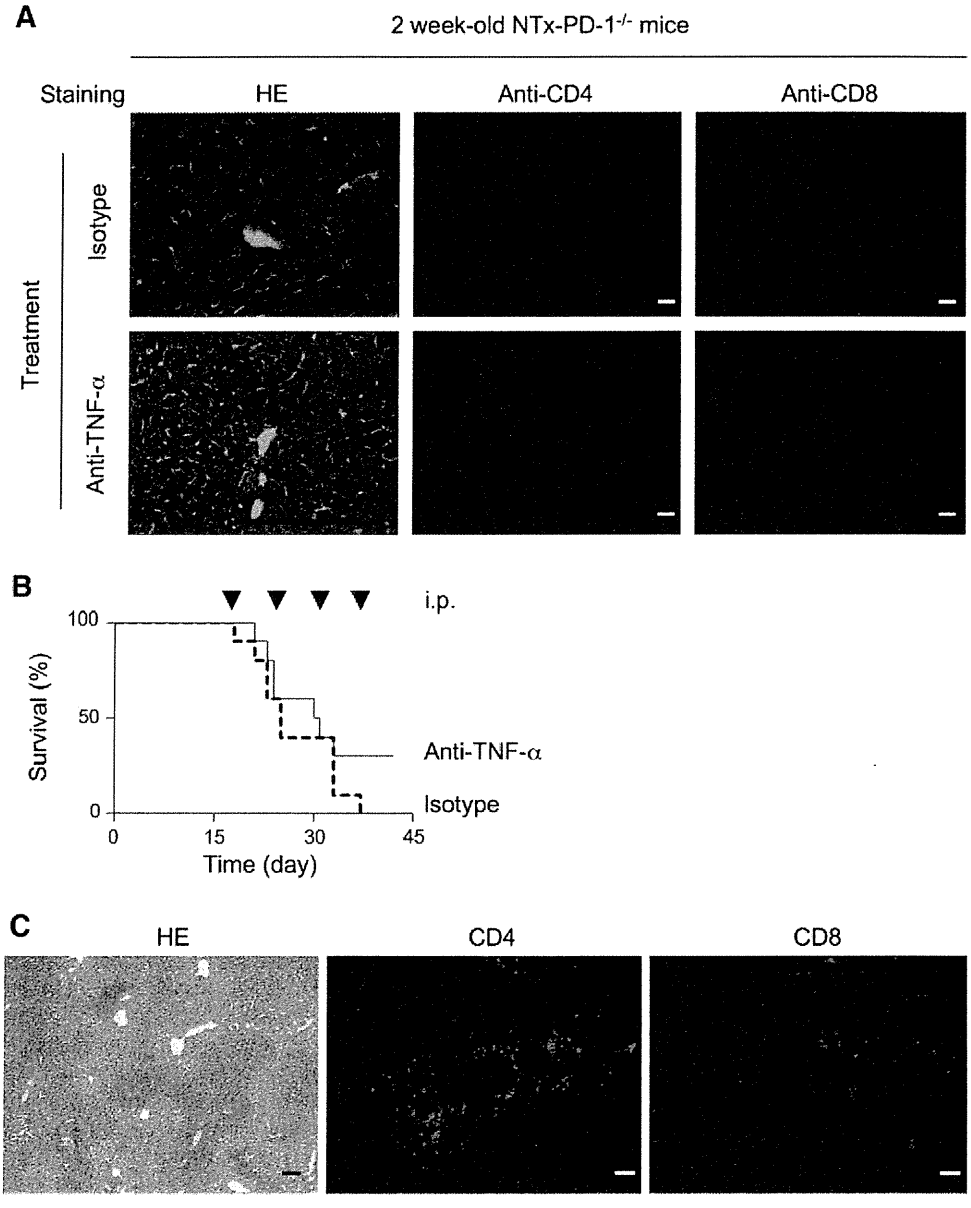
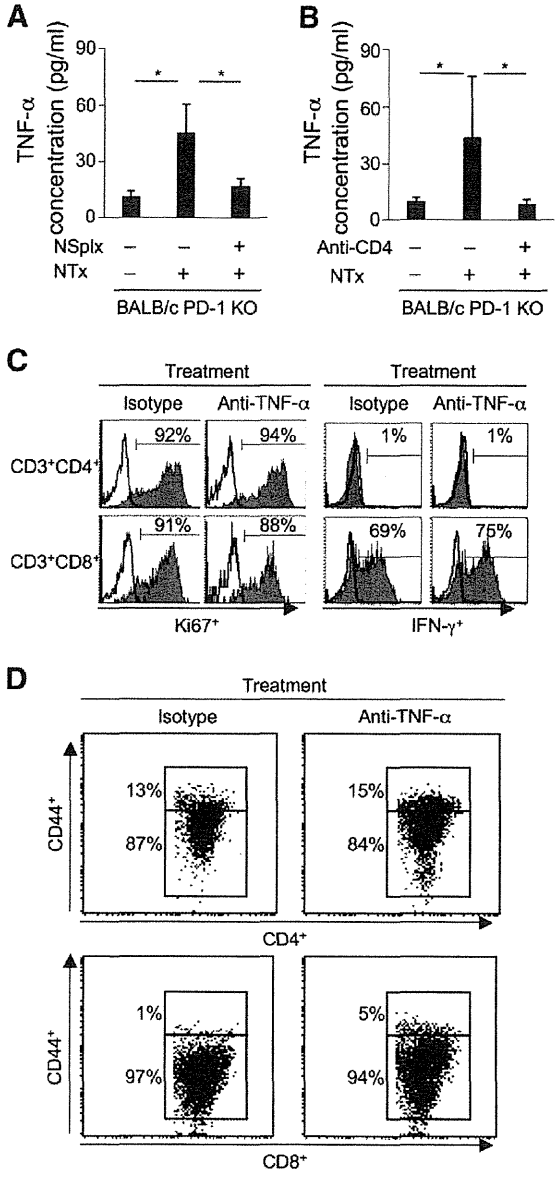


Figure 2 Neutralization of TNF-α inhibits the infiltration of T cells into the liver in the induction phase of AIH, whereas it does not then suppress the progression to fatal AIH. (A) NTx-PD-1$^{-/-}$ mice at one day after thymectomy were injected intraperitoneally every week with 100 μg of neutralizing anti-mouse TNF-α or the isotype control mAb. After two injections, mice at two weeks of age were sacrificed, and their livers were harvested. Staining of the liver for hematoxylin and eosin (HE), CD4 and CD8 in 2-week-old NTx-PD-1$^{-/-}$ mice with neutralizing anti-TNF-α or the isotype control. Scale bars, 100 μm. (B and C) NTx-PD-1$^{-/-}$ mice at 14 days after thymectomy were injected intraperitoneally every week with 100 μg of neutralizing anti-mouse TNF-α or the isotype control mAb. Survival of NTx-PD-1$^{-/-}$ mice treated with anti-TNF-α (solid line, n=10), or the isotype controls (dotted lines, n=10) at four injections (B). After two injections, mice at 4 weeks of age were sacrificed, and their livers were harvested. Histological and immunohistological analyses of the liver in NTx-PD-1$^{-/-}$ mice treated with anti-TNF-α. The sections of tissues were stained with hematoxylin and eosin (HE), CD4 and CD8. All *scale bars*, 100 μm (C).

NTx-PD-1$^{-/-}$ mice (Figs. 4D and E). However, the treatment with anti-CCL20 did not significantly suppress elevated serum levels of TNF-α at two or four weeks of age (Fig. 4F). These data indicate that administering anti-CCL20 suppressed the development of fatal AIH but did not alter elevated serum levels of TNF-α.

3.7. TNF-α stimulation induces enhanced CCL20 expression in hepatocytes *in vivo* and *ex vivo*

Finally, we examined whether TNF-α can induce upregulated expression of CCL20 in hepatocytes. Four-week-old PD-1$^{-/-}$ mice were injected intraperitoneally with 10 μg/kg of rTNF-α.



One hour after injection, mice showed a significantly elevated serum level of TNF-α, reaching the maximal level (Fig. 5A upper panel). In addition, 2 h after injection of rTNF-α, we found upregulated mRNA expression of CCL20 in the liver (Fig. 5A lower panel). Previously we had shown that $CD4^+$ T cells in the spleen and liver expressed chemokine receptor $CCR6^+$ and, to a lesser extent, $CCR9^+$ cells in NTx-PD-$1^{-/-}$ mice [19]. However, 2 h after injection of rTNF-α, we found upregulated mRNA expression of CCR6 ligand, CCL20 but not CCR9 ligand, CCL25 in the liver (Fig. 5B). Upregulated mRNA expression of CCL20 in the liver was confirmed by immunohistology. Hepatocytes from four-week-old PD-$1^{-/-}$ mice revealed CCL20 staining. Importantly, after intraperitoneal injection of 10 μg/kg of rTNF-α four times every 3 h into four-week-old PD-$1^{-/-}$ mice, hepatocytes from these mice at 3 h after the last injection revealed enhanced CCL20 staining (Fig. 5C). Taken together, these data suggest that TNF-α upregulates CCL20 expression in hepatocytes *in vivo*.

Furthermore, to examine whether TNF-α directly induces upregulated secretion of CCL20 from hepatocytes, we isolated hepatocytes from PD-$1^{-/-}$ mice and cultured in media with or without rTNF-α. Protein ELISA for CCL20 showed that rTNF-α stimulation directly induced enhanced CCL20 secretion from hepatocytes in culture (Fig 5D). These data indicate that TNF-α directly induces upregulated secretion of CCL20 from hepatocytes *ex vivo*.

4. Discussion

In the present study, we examined the roles of TNF-α in the development of AIH in mice. We found that not only mice with severe AIH, but also NTx-PD-$1^{-/-}$ mice in the induction phase, showed elevated serum levels of TNF-α. *In vivo* injection of rTNF-α induced upregulated expression of CCL20 in hepatocytes. Administration of anti-TNF-α suppressed CCL20 expression in the hepatocytes and the infiltration of splenic $CD4^+$ and $CD8^+$ T cells into the liver in the induction phase, preventing the development of fatal AIH. In contrast, administration of anti-CCL20 suppressed fatal AIH but not elevated serum levels of TNF-α, and anti-TNF-α treatment did not significantly suppress the progression of fatal inflammation after the induction of AIH. From these findings, we concluded that TNF-α is essential for the development of AIH in mice and is critically involved in the induction of AIH through the upregulation of hepatic CCL20 expression. Although some roles of cytokines had been demonstrated in several mouse models of AIH [9–14], in these models, the function of TNF-α in the development of hepatitis was not clear. Even in Con A-induced acute hepatic injury, it is still controversial whether TNF-α has an essential role in inducing hepatic injury [15–17]. Therefore, our results offer insights into the potentially important roles of TNF-α in the pathogenesis of human AIH.

Figure 3 Increased TNF-α production depends on activation of $CD4^+$ T cells in the spleen. Activation of T cells in the spleen is not suppressed by neutralization of TNF-α in the induction phase of fatal AIH. (A) Serum TNF-α levels of 2-week-old NTx-PD-$1^{-/-}$ mice with or without neonatal splenectomy (NSplx) and control PD-$1^{-/-}$ mice were measured by ELISA. Data are shown as the mean of four mice. *Error bars* represent SD. *; $P<0.05$. (B) PD-$1^{-/-}$ mice at one day after thymectomy were injected intraperitoneally every week with or without anti-CD4. After two injections, mice at two weeks of age were sacrificed, and their sera were harvested. Serum TNF-α levels of 2-week-old indicated mice were measured by ELISA. Data are shown as the mean of five mice. *Error bars* represent SD. *; $P<0.05$. (C–D) Flow cytometric analysis of cells in the spleen of indicated 2-week-old mice. Phenotypes of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were shown. Data shown represent one of three separate experiments. Filled histograms represent staining of anti-Ki-67 and intracellular staining of IFN-γ. Open histograms represent the isotype controls. Numbers indicate percent of positive cells in indicated markers (C). Dot plots represent CD44 staining. Numbers indicate the percentage of $CD44^{int+}$ and $CD44^{high+}$ (D).

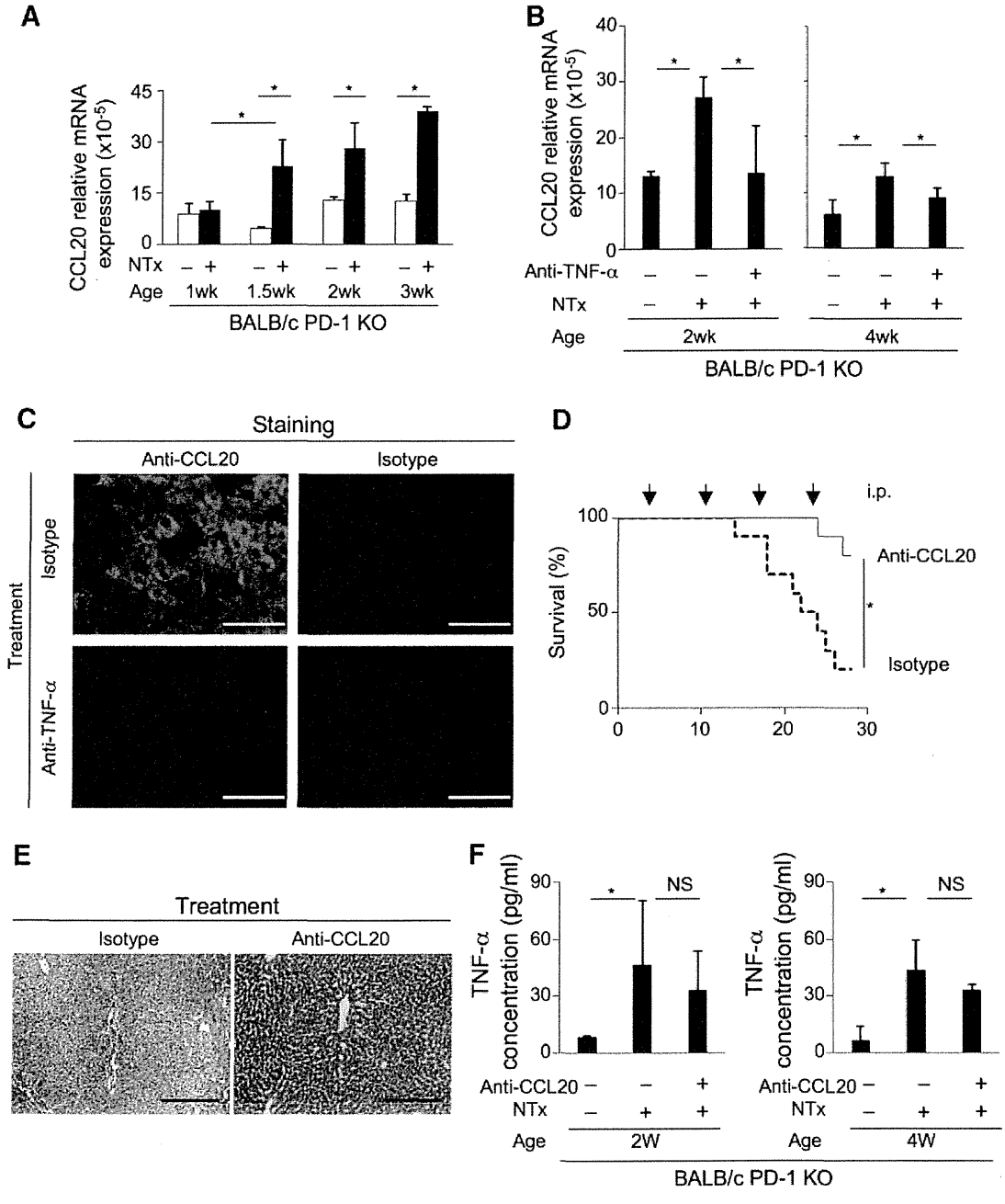


Figure 4 Neutralization of TNF-α suppresses upregulated CCL20 expression in the liver, whereas administration of anti-CCL20 does not alter elevated serum levels of TNF-α. (A) Relative mRNA CCL20 expressions in the liver of indicated mice. Data are shown as the mean of at least five mice. (B–F) NTx-PD-1$^{-/-}$ mice at one day after thymectomy were injected intraperitoneally every week with 100 μg of neutralizing anti-mouse TNF-α, anti-mouse CCL20 or the isotype control mAbs. After two or four injections, five of each group mice at two or four weeks of age were sacrificed, respectively, and their livers were harvested. Relative mRNA CCL20 expressions in the liver of indicated mice. Values are expressed as arbitrary units relative to GAPDH. Bars indicate the mean of each group. Horizontal short bars indicate the SD. *; $P<0.05$ (B). Immunostaining with anti-CCL20 or the isotype control. The livers from four-week-old NTx-PD-1$^{-/-}$ mice treated with anti-TNF-α or the isotype control were used. Scale bars, 50 μm (C). Survival of NTx-PD-1$^{-/-}$ mice treated with anti-CCL20 (solid line, n=10), or the isotype controls (dotted lines, n=10) at four weeks of age. *; $P<0.05$ (D). Staining of the liver from four-week-old NTx-PD-1$^{-/-}$ mice for hematoxylin and eosin. *Scale bars*, 100 μm (E). After two or four injections, five of each group mice at two or four weeks of age were sacrificed. Serum TNF-α levels of indicated mice were measured by ELISA. Data are shown as the mean of at least four mice. Horizontal short bars indicate the SD. *; $P<0.05$ (F).

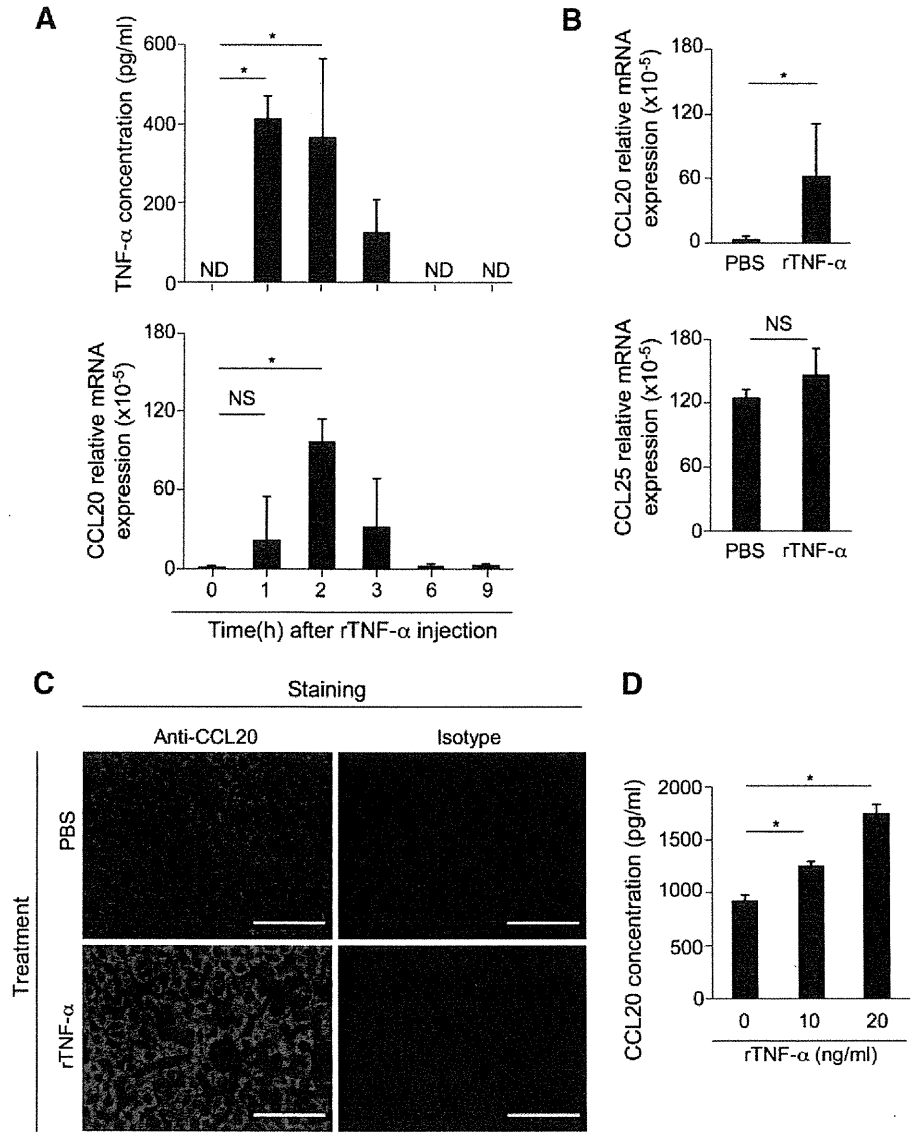


Figure 5 TNF-α induces upregulated CCL20 production of hepatocytes *in vivo* and *ex vivo*. (A–C) Four-week-old PD-1$^{-/-}$ mice were injected intraperitoneally with 10 μg/kg of mouse rTNF-α or PBS. Serum TNF-α levels and CCL20 mRNA expressions in the liver of four-week-old PD-1$^{-/-}$ mice at the indicated time after injection of rTNF-α. Data are shown as the mean of at least three mice. Horizontal short bars indicate the SD. *; $P<0.05$ (A). Relative mRNA expressions of CCL20 or CCL25 in the liver of PD-1$^{-/-}$ mice 2 h after injection of rTNF-α or PBS. Values are expressed as arbitrary units relative to GAPDH. Data are shown as mean of at least three mice. Horizontal short bars indicate the SD. *; $P<0.05$ (B). Immunostaining with anti-CCL20 or the isotype control. The livers used were from four-week-old PD-1$^{-/-}$ mice injected with rTNF-α or PBS four times every three hours and sacrificed 3 h after the last injection. All scale bars, 50 μm (C). Hepatocytes were isolated from PD-1$^{-/-}$ mice and cultured for 72 h with or without 10 or 20 ng/ml of rTNF-α. Concentrations of CCL20 in culture supernatants were measured by ELISA. Bars indicate the mean of triplicate wells of each group. Horizontal short bars indicate the SD. *; $P<0.05$ (D).

We still lack a detailed understanding of the complex roles of TNF-α in the pathophysiology of liver diseases. Although in human, TNF-α is involved in the pathophysiology of various conditions such as viral hepatitis, alcoholic liver disease, nonalcoholic fatty liver disease, and ischemia-reperfusion injury, TNF-α exerts pleiotropic effects on the liver [5]. TNF-α directly and indirectly induces cell death of hepatocytes, whereas it can mediate production of inflammatory mediators, hepatocyte proliferation, and liver regeneration [5]. Although it is unclear whether TNF-α is critical in AIH development, the serum TNF-α level was elevated in untreated children with type 1 AIH and the polymorphism in a promoter region of TNF-α is associated with the severity of type 1 AIH [6]. In this study using a mouse model of AIH, we showed that TNF-α is essential for its development and is critically involved in its induction through upregulation of hepatic CCL20 expression. Therefore, TNF-α may be critically involved in the induction of human AIH, especially type 1.

In a previous study, we had shown that in NTx-PD-1$^{-/-}$ mice, induction of AIH triggered CCR6, expressing splenic CD4$^+$ T cells that migrated into the CCL20-expressing liver [19]. However, it had been unclear how upregulated CCL20 expression was triggered in the liver in the induction phase. In this study, we demonstrated that anti-TNF-α suppressed CCL20 expression in hepatocytes in the induction phase of AIH and that rTNF-α upregulated hepatic CCL20 expression. CCL20 expression is found in the normal liver, Peyer's patches [23], and epithelial cells of the chroid plexus in the brain [24]. In addition, CCL20 expression is highly induced in the inflamed liver after *Propionibacterium acnes* priming plus LPS challenge in a TNF-α-dependent manner. NF-κB activation was crucial for induction of CCL20 expression by TNF-α [25]. Thus, it is likely that increased TNF-α production may directly trigger NF-κB activation to upregulate CCL20 expression in the hepatocytes in the induction phase of AIH in NTx-PD-1$^{-/-}$ mice.

We showed the essential roles of upregulated expression of hepatic CCL20 by TNF-α in the development of AIH. In previous studies, CCL20 expression is highly induced in the inflamed liver after *P. acnes* priming plus LPS challenge [25]. In addition, we showed that rTNF-α injection induced CCL20 expression in the live of PD-1$^{-/-}$ mice without liver inflammation. Importantly, the TNF-α-dependent CCL20 upregulation occurs not only in PD-1$^{-/-}$ mice but also in normal mice (data not shown). Furthermore, CCL20 expression was detected in patients with not only AIH but also other types of chronic viral hepatitis [26]. Upregulated expression of CCL20 in the liver triggered by TNF-α may be involved in the induction of other inflammatory liver diseases.

In this mouse model of AIH, neutralizing TNF-α was effective only as a preventive strategy for the induction phase of AIH, not during progression. Although increased serum levels of TNF-α was observed in both phases, roles of TNF-α may be limited in the progression phase of AIH. In fatal AIH, TNF-α independent CCL20 upregulation by other cytokines might be predominant, or other chemokine–chemokine receptor systems might be involved in the progression. Importantly, cytotoxic CD8$^+$ T cells can induce Fas ligand- and perforin-mediated destruction of hepatocytes [27]; in this mouse model, CD8$^+$ T cells are crucially involved in the progression to fatal hepatic damage. Thus, although TNF-α directly and indirectly induces cell death of hepatocytes [5], in the progression phase of fatal AIH, TNF-α independent CD8$^+$ T-cell mediated destruction of hepatocytes may have major roles in the fatal hepatic damage.

In a clinical scenario in human AIH, our data imply that anti-TNF-α may be a new therapeutic option to prevent recurrence for patients undergoing liver transplantation for AIH, who have a high potential of its recurrence [28,29]. However, the use of anti-TNF-α for patients with initial presentation of AIH may be limited to cases of mild to moderate severity or AIH before progression to fulminant hepatic failure.

In humans, a case report showed that a refractory AIH patient was successfully treated by therapy combining prednisolone, azathioprine, and infliximab, even though accompanied by recurrent infection probably related to the addition of infliximab [30]. In this study, we demonstrated that although neutralizing TNF-α suppressed the induction of AIH, the neutralization altered neither differentiation of T_{FH} cells nor activation of CD8$^+$ T cells at the induction site of AIH. The combination of prednisolone and azathioprine may suppress T-cell activation at the AIH induction site, increasing the efficacy of infliximab to suppress the upregulation of CCL20 production in the liver. In this regard, it may be noted that in addition to the potentially increased infection and hepatotoxic side effects of anti-TNF agents, it is unknown at present whether anti-TNF-α monotherapy has significant therapeutic efficacy for human AIH. Clearly, anti-TNF-α monotherapy for refractory AIH should be handled with care in practice.

In addition, although anti-TNF-α suppressed the induction of AIH in mice, several case reports showed that AIH-like hepatitis was induced by the administration of TNF antagonists [31]. It is unknown at present why TNF antagonists can induce human AIH-like hepatitis. Notably, the induction of AIH-like hepatitis was predominantly observed in the use of infliximab [31]. Because infliximab is called a chimeric monoclonal antibody, a combination of mouse and human antibody [32] and another chimeric monoclonal antibody, rituximab was reported to induce hepatitis [33], chimeric monoclonal antibodies might have a potential to induce immune-mediated liver injury.

In conclusion, we have demonstrated that in AIH in NTx-PD-1$^{-/-}$ mice, TNF-α is critically involved in the induction of fatal AIH through the upregulation of hepatic CCL20 expression. Although AIH in NTx-PD-1$^{-/-}$ mice shares some key features with human AIH, it is not an exact copy; nevertheless, the data in this study imply that TNF-α antagonists might offer a new therapeutic approach to human AIH. It may turn out that the use of TNF-α antagonists is limited to the induction phase of AIH, before fatal progression is underway.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2012.10.008.

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IFN-γ is reciprocally involved in the concurrent development of organ-specific autoimmunity in the liver and stomach

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Abstract

Interferon (IFN)-γ acts as a critical proinflammatory mediator in autoimmune processes, whereas it exerts regulatory functions to limit tissue damage associated with inflammation. However, a detailed understanding of the complex roles of IFN-γ in the development of organ-specific autoimmunity is still lacking. Recently, we found that programmed cell death 1-deficient mice thymectomized 3 days after birth (NTx–PD-1$^{-/-}$ mice) concurrently developed autoimmune hepatitis (AIH) and autoimmune gastritis (AIG). In this study, we investigated the roles of IFN-γ in the development of AIH and AIG in this mouse model. In NTx–PD-1$^{-/-}$ mice, serum levels of IFN-γ were markedly elevated. Neutralization of IFN-γ prevented the development of AIG. However, the same treatment exacerbated hepatic T-cell infiltration in AIH. Because of the loss of anti-proliferative effects by IFN-γ, neutralization of IFN-γ increased T-cell proliferation in the spleen and liver, resulting in exacerbated T-cell infiltration in the liver. On the other hand, in the development of AIG, CD4$^+$ T-cell migration into the gastric mucosa is essential for induction. CCL20 expression was up-regulated in the gastric mucosa, and anti-CCL20 suppressed CD4$^+$ T-cell infiltration into the gastric mucosa. Importantly, anti-IFN-γ suppressed CCL20 expression and infiltration of CD4$^+$ T cells in the gastric mucosa, whereas *in vivo* injection of recombinant IFN-γ up-regulated CCL20 expression in the stomach, suggesting that IFN-γ is critically involved in CD4$^+$ T-cell accumulation in AIG by up-regulating local CCL20 expression. In conclusion, IFN-γ is involved differently in the development of AIH and of AIG. IFN-γ negatively regulates T-cell proliferation in fatal AIH, whereas it initiates development of AIG. These findings imply that increased production of IFN-γ induced by an organ-specific autoimmunity may trigger the concurrent development of another organ-specific autoimmune disease.

Keywords: *IFN-γ, proinflammatory mediator, autoimmune gastritis, CCL20, regulatory function, autoimmune hepatitis*

Abbreviations: *AIG, autoimmune gastritis; AIH, autoimmune hepatitis; ANA, anti-nuclear antibody; IFN, interferon;* H. pylori, Helicobacter pylori*; NTx, neonatal thymectomy; NTx mice, mice thymectomized 3 days after birth; PD-1, programmed cell death 1; Th1, T helper 1; Treg, CD4$^+$CD25$^+$ regulatory T*

Introduction

Interferon (IFN)-γ exerts pleiotropic effects on the immune system [1–3]. IFN-γ acts as a critical proinflammatory mediator in immunity and inflammation. IFN-γ induces macrophage activation and T helper 1 (Th1) cell response and is critically involved in inflammation as well as in host defense against intracellular pathogens, tumor surveillance, and Th1-dominated autoimmune diseases [1–3]. In contrast, IFN-γ exerts regulatory functions to limit tissue damage associated with inflammation [1–3].

In addition, IFN-γ is essential to regulate the optimal population expansion of activated CD4$^+$ T cells and to maintain CD4$^+$ T-cell homeostasis during

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immune responses. IFN-γ induces apoptosis and/or suppression of proliferation in activated $CD4^+$ T cells [4–7]. Moreover, it has been reported to be critically required for the conversion of $CD4^+CD25^-$ T cells to regulatory T cells during experimental autoimmune encephalomyelitis [8]. Therefore, IFN-γ can either augment or suppress autoimmunity and associated tissue damage. However, we still lack a detailed understanding of the complex roles of IFN-γ in individual, organ-specific autoimmune diseases.

In the areas of gastroenterology and hepatology, autoimmune gastritis (AIG) and autoimmune hepatitis (AIH) are typical organ-specific autoimmune diseases. The histological findings of AIG are characterized by a chronic mononuclear cell infiltration affecting only or predominantly the corpus mucosa and causing loss of parietal and chief cells from the gastric gland [9]. Its serologic hallmark is the production of antibody against H^+K^+-ATPase in the parietal cells of the stomach [10,11].

Mouse models of AIG share many pathological and clinical features with human AIG and help clarify the mechanisms involved in its development [12–14]. Mouse models of AIG are characterized by a marked infiltration of $CD4^+$ T cells, which produce large amounts of IFN-γ. In mice, depleted $CD4^+$ but not $CD8^+$ T cells or administrated blocking Abs to IFN-γ severely impair the development of AIG [15,16]. However, the precise roles of IFN-γ in the development of $CD4^+$ T cell-dependent AIG are still unclear.

On the other hand, human AIH appears to be a T-cell-mediated autoimmune disease and is characterized by a mononuclear cell infiltration invading the parenchyma with the production of a variety of characteristics circulating autoantibodies [17,18]. Recently, we developed the first mouse model of spontaneous fatal AIH [19]. As adults, both programmed cell death 1-deficient mice ($PD\text{-}1^{-/-}$ mice) and BALB/c mice thymectomized 3 days after birth (NTx mice), a process severely reducing the number of naturally arising $Foxp3^+$ regulatory T cells (Tregs) in periphery, developed AIG but not AIH.

However, $PD\text{-}1^{-/-}$ BALB/c mice with neonatal thymectomy (NTx–$PD\text{-}1^{-/-}$ mice) developed AIG with fatal AIH characterized by $CD4^+$ and $CD8^+$ T-cell infiltration, with massive lobular necrosis in the liver and autoantibody production against nuclear antigens [19]. In NTx–$PD\text{-}1^{-/-}$ mice, both $CD4^+$ and $CD8^+$ T cells are indispensable for the development of fatal AIH. However, the infiltration of $CD8^+$ T cells in the liver is regulated by $CD4^+$ T cells, and $CD8^+$ T cells are mainly involved in progression to fatal hepatic damage [19,20]. In contrast, $CD4^+$ T cells are responsible for induction of fatal AIH, and initial activation of $CD4^+$ T cells occurs in the spleen [20]. In addition, AIG- and AIH-bearing NTx–$PD\text{-}1^{-/-}$ mice at 3 weeks old showed markedly increased levels of IFN-γ in the serum [19]. However, it is not clear whether, in this mouse model, IFN-γ is essential in the development of AIG and/or fatal AIH.

In this study, we examined the roles of IFN-γ in the development of a mouse model of spontaneous AIG and AIH. We found that in NTx–$PD\text{-}1^{-/-}$ mice, serum levels of IFN-γ were markedly elevated. However, neutralization of IFN-γ prevented the development of AIG, whereas it intensified hepatic T-cell infiltration in AIH. Because of the loss of anti-proliferative effects by IFN-γ, neutralization of IFN-γ increased T-cell proliferation in the spleen and liver, resulting in exacerbated T-cell infiltration in the liver. On the other hand, anti-IFN-γ suppressed up-regulated CCL20 expression in the gastric mucosa and infiltration of $CD4^+$ T cells in the stomach, resulting in the impairment of the development of AIG. Taken together, our results showed that although AIG and AIH were simultaneously and sequentially progressed in NTx–$PD\text{-}1^{-/-}$ mice, IFN-γ is involved differently in the development of AIG and AIH.

Materials and methods

Mice

BALB/c mice were purchased from Japan SLC (Shizuoka, Japan), and PD-1-deficient mice on a BALB/c background were generated as described previously [21]. These mice were bred and housed under specific pathogen-free conditions. Thymectomy of the mice 3 days after birth was performed as described previously [22]. All mouse protocols were approved by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

Real-time quantitative reverse transcription polymerase chain reaction

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed as described previously [23]. Liver and gastric tissues were frozen in RNA later (Qiagen, Hilden, Germany). RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Single-stranded complementary DNA was synthesized with SuperScript™II RT (Invitrogen, Carlsbad, CA, USA). Real-time quantitative RT-PCR was performed using SYBR Green-Master (Roche Applied Science, Basel, Switzerland). Real-time quantitative reactions were performed with LightCycler 480 Instrument (Roche Applied Science) according to the manufacturer's instructions. Values are expressed as arbitrary units relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following primers were used: *GAPDH*: 5′-CAACTTTGTCAAGCTCATTTCC-3′ and 5′-GGTCCAGGGTTTCTTACTCC-3′; *Foxp3*: 5′-TCAGGAGCC-

CACCAGTACA-3′ and 5′-TCTGAAGGCAGAGTCAGGAGA-3′; *IL-10*: 5′-TTTGAATTCCCTGGGTGAGAA-3′ and 5′-GGAGAAATCGATGACAGCGC-3′; *T-bet*: 5′-TCAACCAGCACCAGACAGAG-3′ and 5′-AAACATCCTGTAATGGCTTGTG-3′; *IFN-γ*: 5′-GGATGCATTCATGAGTATTGC-3′ and 5′-CCTTTTCCGCTTCCTGAGG-3′; *GATA-3*: 5′-TTATCAAGCCCAAGCGAAG-3′ and 5′-TGGTGGTGGTCTGACAGTTC-3′; *IL-4*: 5′-TCATCGGCATTTTGAACGAG-3′ and 5′-CGTTTGGCACATCCATCTCC-3′; *IL-13*: 5′-TGGGTGACTGCAGTCCTGGCT-3′ and 5′-GTTGCTTTGTGTAGCTGAGCA-3′; *ROR-γt*: 5′-CCGCTGAGAGGGCTTCAC-3′ and 5′-TGCAGGAGTAGGCCACATTACA-3′; *IL-17A*: 5′-CTCCAGAAGGCCCTCAGACTAC-3′ and 5′-AGCTTTCCCTCCGCATTGACACAG-3′; *IL-22*: 5′-AGAAGGCTGAAGGAGACAGT-3′ and 5′-GACATAAACAGCAGGTCCAGTT-3′; *Bcl-6*: 5′-CTGCAGATGGAGCATGTTGT-3′ and 5′-GCCATTTCTGCTTCACTGG-3′; *IL-21*: 5′-GACATTCATCATCGACCTCGT-3′ and 5′-TCACAGGAAGGGCATTTAGC-3′; and *CCL20*: 5′-ATGGCCTGCGGTGGCAAGCGTCTG-3′ and 5′-TAGGCTGAGGAGGTTCACAGCCCT-3′.

Enzyme-linked immunosorbent assay

Serum cytokine concentrations were measured with a mouse IFN-γ enzyme-linked immunosorbent assay (ELISA) set (eBioscience, San Diego, CA, USA) according to the manufacturer's protocols.

In vivo neutralization and injection of cytokines

NTx–PD-1$^{-/-}$ mice at 1 day after thymectomy were intraperitoneally injected every week with 100 μg of monoclonal antibodies (mAbs). Anti-CD4 and anti-CD8a for depletion of CD4^{+} T cells and CD8^{+} T cells, respectively, and neutralizing Abs to mouse IFN-γ were from eBioscience. Neutralizing Abs to mouse CCL20 were from R&D Systems (Minneapolis, MN, USA). All isotypes were from eBioscience or R&D Systems. After injections, mice at day 21 or 28 after birth were sacrificed, and the liver, stomach, spleen and serum were harvested. PD-1$^{-/-}$ mice at 4 weeks of age were injected intraperitoneally with 10 μg/kg of recombinant mouse IFN-γ (eBioscience). Before and after 2, 4, 6, or 8 h post-injection, mice were sacrificed, and the liver and stomach were harvested.

Histologic and immunohistologic analysis

Organs were fixed in neutral buffered formalin and embedded in paraffin wax. Sections were stained with hematoxylin and eosin (HE) for histopathology. Fluorescence immunohistology was performed on frozen sections as described previously [19], using FITC-conjugated anti-CD4, anti-CD8, or anti-CD11c (eBioscience), The degree of gastritis was determined according to the semiquantitative scoring system as described previously [24].

Chronic inflammation, characterized by the infiltration of mononuclear cells, was graded from 0 to 3, where 0 = no increase in the number of inflammatory cells, 1 = slight infiltration of the lamina propria by lymphocytes and plasma cells, 2 = moderately dense infiltration of the lamina propria by lymphocytes and plasma cells, and 3 = very dense lymphoplasma cell infiltration in the lamina propria. Atrophic changes were graded from 0 to 3 according to the loss of specialized cells, chief and parietal cells (0 = no loss, 1 = mild loss, 2 = moderate loss, and 3 = severe loss of specialized cells). Hyperplastic changes were graded from 0 to 3 according to hypertrophy of total gland cells (0 = no hypertrophy, 1 = mild hypertrophy, 2 = moderate hypertrophy, and 3 = severe hypertrophy of total gland cells).

Flow cytometry analysis

Single-cell suspensions from livers and spleens were prepared as described previously [25,26]. Cells were stained with PE-conjugated anti-CD3e (eBioscience) and either APC-conjugated anti-CD8a (eBioscience) or APC-Cy7-conjugated anti-CD4 (BD Biosciences, San Jose, CA, USA). Stained cells were fixed and permeabilized using Foxp3 staining buffer (eBioscience), and stained with FITC-conjugated anti-Ki-67 antigen. For 7-AAD staining, cells were stained with FITC-conjugated anti-CD3e (eBioscience) and 7-AAD (BD Biosciences) and either PE-Texas Red-conjugated anti-CD4 (Abcam, Cambridge, UK) or PE-Texas Red-conjugated anti-CD8 (Abcam). Stained cells were analyzed with FACSCanto^{TM} II (BD Biosciences). Data were analyzed using CellQuest Pro^{TM} (BD Biosciences).

Statistical analysis

The data are presented as the mean values ± standard deviations. Statistical analysis was performed by the Student's *t*-test for pairwise comparisons and analysis of variance with the Tukey–Kramer test for multiple comparisons. *P*-values below 0.05 were considered significant.

Results

Serum levels of IFN-γ are elevated in NTx–PD-1$^{-/-}$ mice

Previously we showed that, in NTx–PD-1$^{-/-}$ mice, hepatitis and gastritis were simultaneously initiated and then progressed rapidly [19]. Four days after thymectomy, 1-week-old NTx–PD-1$^{-/-}$ mice had

mononuclear cell infiltration neither of the stomach nor the liver. In contrast, 2-week-old NTx–PD-1$^{-/-}$ mice showed moderate mononuclear cell infiltrations, predominantly in the portal area of the liver and in the lamina propria of the gastric gland. These mononuclear cell infiltrations rapidly progressed and were followed by massive destruction of the parenchyma of the liver and parietal cells in the gastric gland in 3-week-old NTx–PD-1$^{-/-}$ mice [19].

In this study, first, to evaluate the roles of IFN-γ in the development of AIH and AIG, we examined serum levels of IFN-γ at 1–3 weeks of age. Previously we showed that severe AIG- and AIH-bearing NTx–PD-1$^{-/-}$ mice at 3 weeks old showed increased levels of IFN-γ in the serum [19]. In this study, we found that the serum levels of IFN-γ were also elevated from 1 to 2 weeks of age before the development of autoimmunity and that the elevated serum level of IFN-γ gradually decreased during the progression of autoimmunity (Figure 1). These data suggest that in NTx–PD-1$^{-/-}$ mice, increased production of IFN-γ may be involved in the development of AIG and/or AIH.

Neutralization of IFN-γ prevents the development of AIG but exacerbates inflammation of the liver in AIH

Next, we evaluated whether IFN-γ is essential in the development of AIG and AIH. NTx–PD-1$^{-/-}$ mice were injected intraperitoneally with 100 μg of neutralizing mAb to mouse IFN-γ or the isotype control at 1 day after thymectomy and then once a week. After four injections, mice at 4 weeks of age were sacrificed, and the stomach and liver were harvested (Figure 2A). Although 4-week-old NTx–PD-1$^{-/-}$ mice injected with the isotype control developed severe AIG with a mononuclear cell infiltration and a loss of parietal and chief cells in the gastric mucosa (Figure 2B, left panel), four injections of anti-IFN-γ suppressed both events (Figure 2B, right panel).

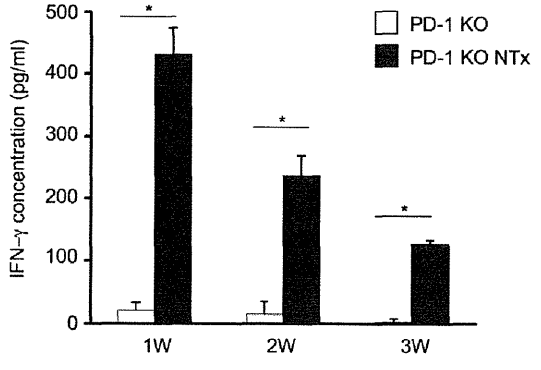

Figure 1. NTx–PD-1$^{-/-}$ mice showed increased serum levels of IFN-γ. Serum IFN-γ levels of PD-1$^{-/-}$ mice with (*closed bars*) or without (*open bars*) NTx at 1 to 3 weeks of age were measured by ELISA. Data are shown as the mean of at least three mice. *Error bars* represent SD. *$P < 0.05$.

These findings were further confirmed by a gastritis scoring system that evaluates (1) chronic inflammation, characterized by the infiltration of mononuclear cells; (2) atrophic changes based on the loss of parietal and chief cells; and (3) hyperplastic changes of foveolar mucus neck cells (Figure 2C). These data indicated that IFN-γ plays a critical role in the development of AIG in NTx–PD-1$^{-/-}$ mice.

In contrast to AIG, neutralization of IFN-γ did not suppress, but rather exacerbated inflammation of the liver in AIH. Four-week-old NTx–PD-1$^{-/-}$ mice with injection of the isotype control developed severe mononuclear cell infiltration in the liver and a massive degeneration of hepatocytes (Figure 2D, left panel), whereas injection of anti-IFN-γ showed more severe mononuclear cell infiltration in the liver with a massive degeneration of hepatocytes (Figure 2D, right panel). In addition, injection of anti-IFN-γ revealed significantly increased serum concentrations of AST at 4 weeks of age (Figure 2E).

Because we found the most elevated serum level of IFN-γ at 1 week of age (Figure 1), we could not exclude the possibility that anti-IFN-γ might not neutralize very early production of IFN-γ just after the NTx, resulting in the inconsistent effects of anti-IFN-γ in the development of AIG and AIH. We again performed neutralizing experiments, adding an intraperitoneal injection of 100 μg of anti-IFN-γ or the isotype control at 1 day before thymectomy. These mice were further injected 1 day after thymectomy and then once a week with anti-IFN-γ. After five injections, mice at 4 weeks of age were sacrificed and the stomach and liver were harvested (Figure 3A). Five injections of anti-IFN-γ also suppressed the mononuclear cell infiltration and loss of parietal and chief cells in the gastric mucosa (Figure 3B). These findings were further confirmed by a gastritis scoring system (Figure 3C).

In contrast, five injections of anti-IFN-γ again induced more severe mononuclear cell infiltration in the liver with higher concentrations of AST, ALT, and total bilirubin in the serum of the mice at 4 weeks of age (Figure 3D and E). These data suggest that neutralization of IFN-γ suppresses the development of AIG but worsens inflammation of the liver in AIH, implying that IFN-γ is essential in the development of AIG, whereas it reciprocally acts as negative regulator for the development of AIH in NTx–PD-1$^{-/-}$ mice.

Neutralization of IFN-γ exacerbates T-cell infiltration in the liver

In the previous study, we showed that in the liver of AIH-developed NTx–PD-1$^{-/-}$ mice, infiltrating cells are mainly CD3$^+$ T cells [19]. Infiltrating CD4$^+$ T cells are predominantly localized in the portal area, whereas mainly increased CD8$^+$ T cells are widely diffused in the parenchyma of the

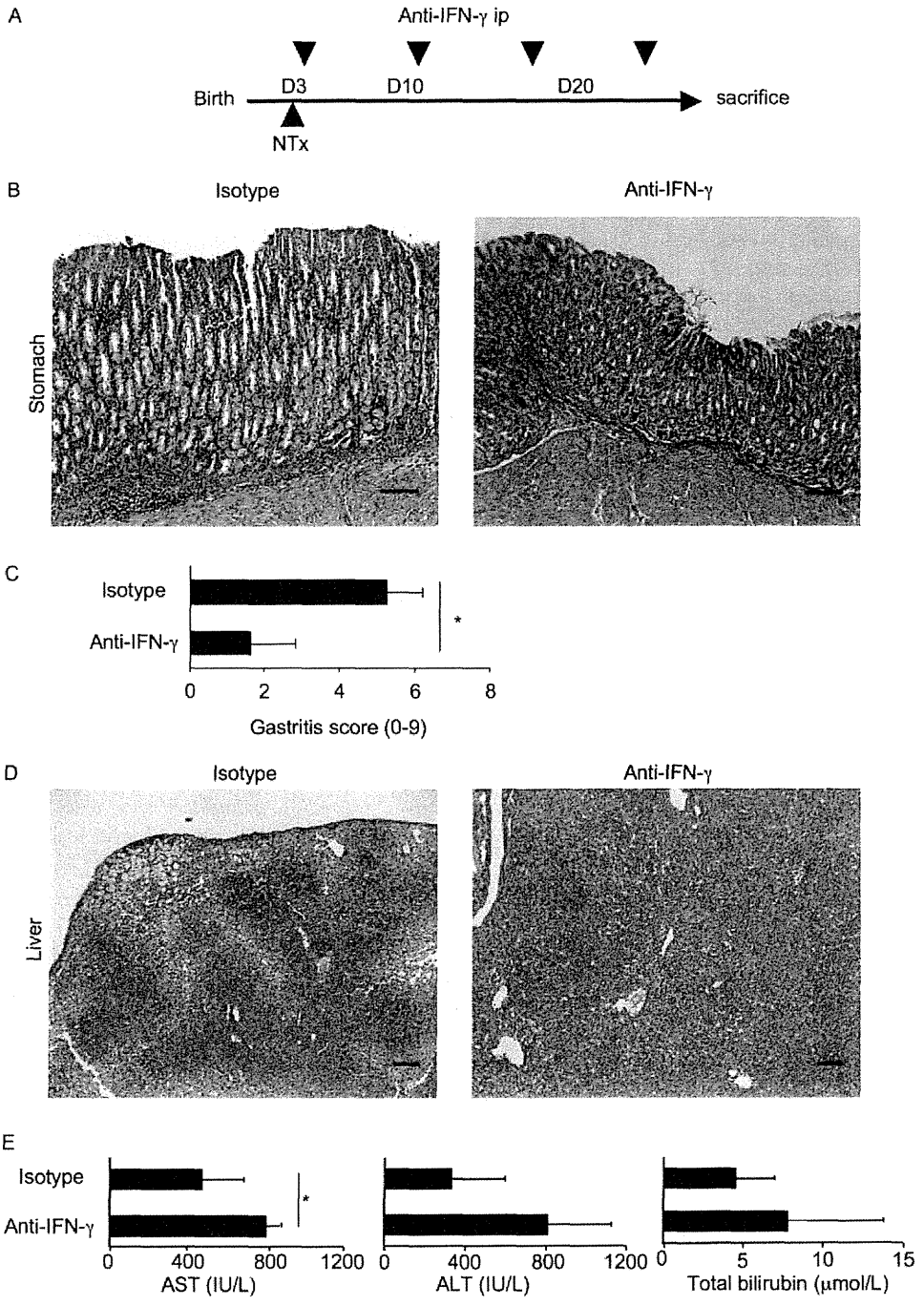

Figure 2. Neutralization of IFN-γ suppressed the development of AIG but exacerbated inflammation of the liver in AIH. (A) Protocol of *in vivo* neutralization of IFN-γ. NTx–PD-1$^{-/-}$ mice at 1 day after thymectomy were injected intraperitoneally (ip) every week with 100 μg of neutralizing anti-mouse IFN-γ ($n = 5$) or the isotype control mAb ($n = 10$). After four injections, mice at 4 weeks of age were sacrificed, and the stomach, liver, and serum were harvested. (B) Histological analysis of the stomach. The sections of tissues were fixed in formalin and stained with HE. (C) Gastritis score of NTx–PD-1$^{-/-}$ mice at 4 weeks of age treated with anti-IFN-γ or the isotype control. (D) Histological analysis of the liver. The sections of tissues were fixed in formalin and stained with HE. (E) Serum levels of the liver transaminase, AST and ALT, and total bilirubin. Sera from NTx–PD-1$^{-/-}$ mice at 4 weeks of age treated with anti-IFN-γ or the isotype control were measured. Data are shown as the mean of at least three mice. Error bars represent SD. *$P < 0.05$. All *scale bars*, 100 μm.

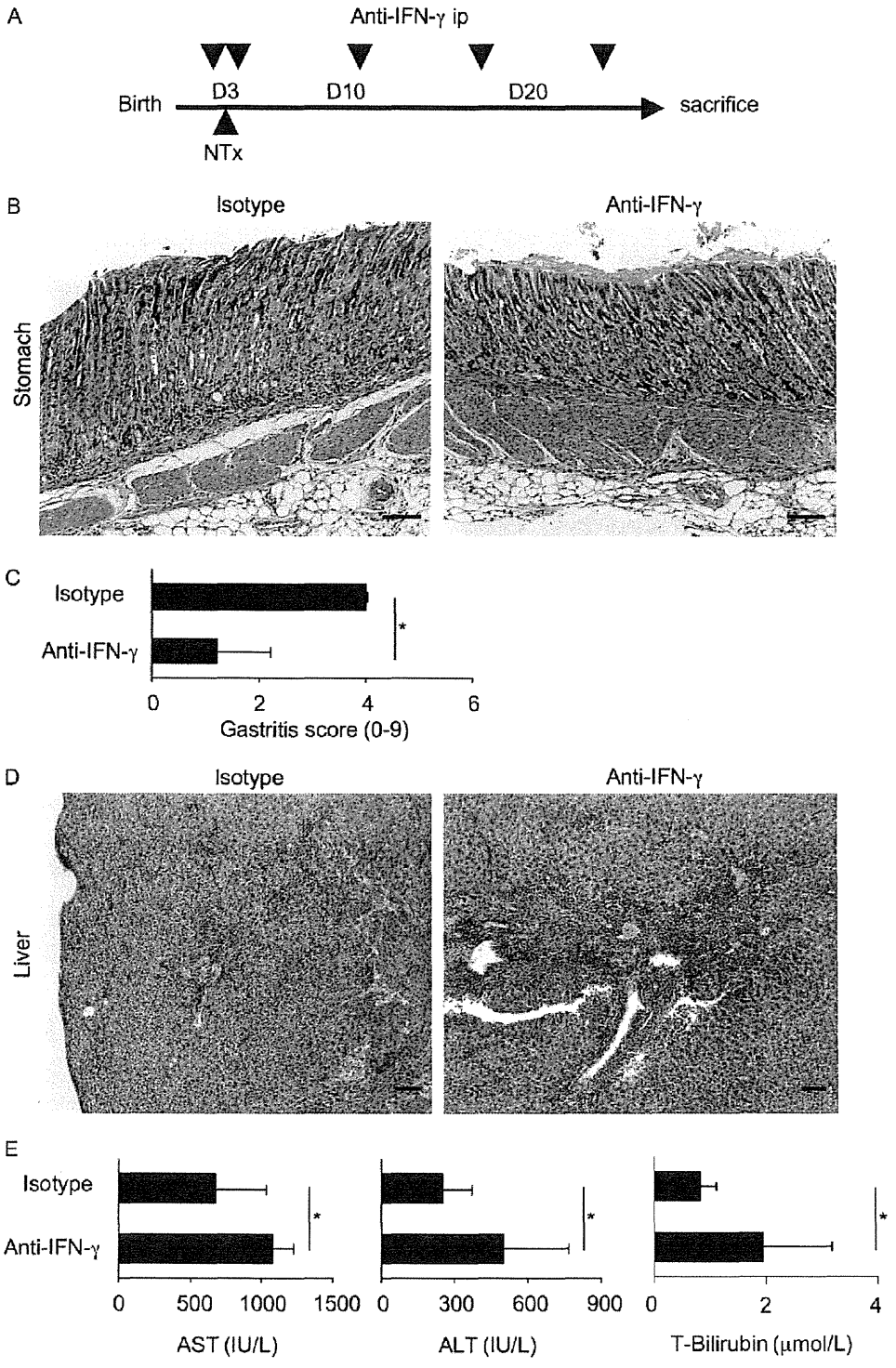

Figure 3. Neutralization of IFN-γ from 1 day before thymectomy suppressed the development of AIG but exacerbated inflammation of the liver in AIH. (A) Protocol of *in vivo* neutralization of IFN-γ. NTx–PD-1$^{-/-}$ mice were injected intraperitoneally (ip) 1 day before thymectomy and every week from 1 day after thymectomy with 100 μg of neutralizing anti-mouse IFN-γ ($n = 5$) or the isotype control mAb ($n = 10$). After five injections, mice at 4 weeks of age were sacrificed, and the liver, stomach, and serum were harvested. (B–E) Data shown as in Figure 2. Histological analysis of the stomach (B); Gastritis score (C); Histological analysis of the liver (D); Serum levels of the liver transaminase, AST and ALT, and total bilirubin (E). Data are shown as mean of at least three mice. Error bars represent SD. $^*P < 0.05$. All *scale bars*, 100 μm.

liver [19]. In this study, NTx–PD-1$^{-/-}$ mice were intraperitoneally injected, neutralizing anti-IFN-γ as described in Figure 2. Infiltrating cells in the liver were examined by immunohistology. We found that infiltrating CD4^{+} and CD8^{+} T cells were more widely and massively diffused in the parenchyma under neutralization of IFN-γ in NTx–PD-1$^{-/-}$ mice (Figure 4A).

Neutralization of IFN-γ does not induce aberrant differentiation into other T-cell subsets

IFN-γ exerts many effects on T-cell differentiation in the immune responses *in vivo* [1–3]. To investigate whether neutralization of IFN-γ induces aberrant differentiation of expanded T cells in the liver, we performed a global quantitative mRNA screening of

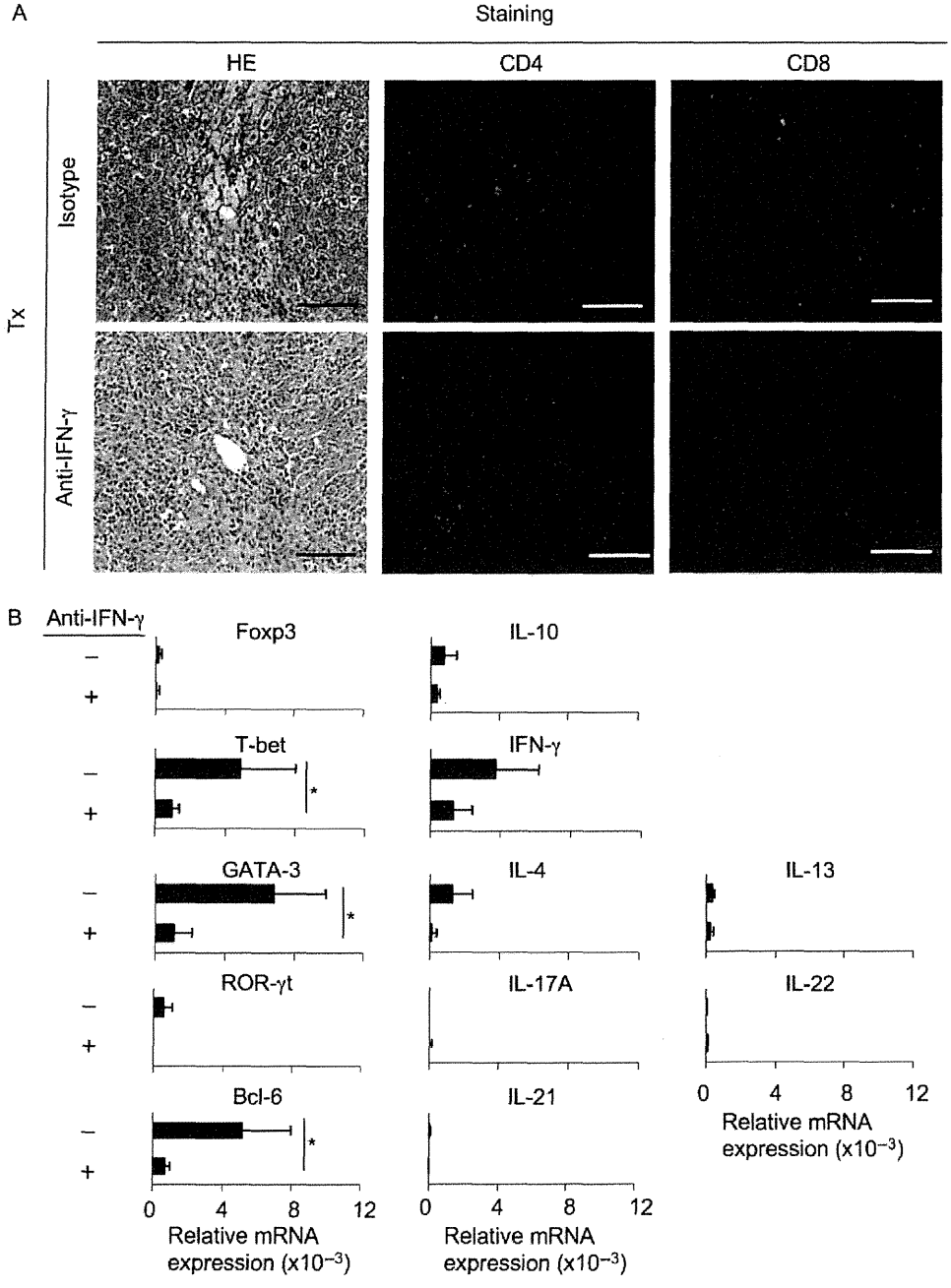

Figure 4. Neutralization of IFN-γ exacerbated T-cell infiltration in the liver, but did not induce aberrant differentiation into other T-cell subsets. NTx–PD-1$^{-/-}$ mice were injected with or without anti-IFN-γ as described in Figure 2. After four injections, mice at 4 weeks of age were sacrificed, and the livers were harvested. (A) Staining of the livers for HE, CD4, and CD8. Scale bars, 100 μm. (B) Real-time quantitative RT-PCR analyses for mRNA expressions of the indicated molecules in the liver. Data are shown as the mean of at least three mice. *Error bars* represent SD. $^{*}P < 0.05$.

master regulators for T-cell subsets and the related cytokines—Foxp3, T-bet, GATA-3, RORγt, Bcl-6, IFN-γ, IL-4, IL-10, IL-13, IL-17A, IL-21, and IL-22 in the hepatic tissues of NTx–PD-1$^{-/-}$ mice at 4 weeks of age.

Previously we showed that in hepatic CD4$^+$ T cells in the progression phase of AIH at 3 weeks, T-bet and Bcl-6 expressions were up-regulated [20]. In this study, we found neutralization of IFN-γ suppressed expression of T-bet, Bcl-6, and GATA-3 in the inflamed liver tissues (Figure 4B). However, we could not detect increased expression of any other molecules in the inflamed liver tissues under the neutralization of IFN-γ (Figure 4B). These data suggest that neutralization of IFN-γ did not induce any aberrant differentiation of T cells in the inflamed liver.

Neutralization of IFN-γ exacerbates T-cell proliferation, increasing the number of T cells in the spleen and liver

During an immune response, IFN-γ optimizes the population expansion of activated CD4$^+$ T cells and maintains CD4$^+$ T-cell homeostasis [1–7]. IFN-γ suppresses proliferation of activated CD4$^+$ T cells, whereas IFN-γ induces apoptosis of activated CD4$^+$ T cells [4–7]. Previously, we showed that CD4$^+$ and CD8$^+$ T cells in the spleen and liver of 3-week-old NTx–PD-1$^{-/-}$ mice showed a CD44^{high}CD62L$^-$ Ki-67^{high} effector memory T-cell phenotype with highly proliferating potential [19].

In this study, we examined by flow cytometry whether neutralization of IFN-γ enhanced proliferation and/or reduced apoptosis of activated T cells in the liver and/or spleen of 3-week-old NTx–PD-1$^{-/-}$ mice. We found that neutralization of IFN-γ increased the cell numbers of CD4$^+$ T cells in the liver (Figure 5A). Although neutralization increased the number of Ki-67$^+$ proliferating cells in CD4$^+$ T cells of the liver, it did not reduce but rather increased the numbers of 7-ADD$^+$ apoptotic cells in CD4$^+$ T cells of the liver (Figure 5A). These data are further confirmed and more significant in CD4$^+$ and CD8$^+$ T cells in the spleen (Figure 5B). These data suggest that in the progression of the diseases, neutralization of IFN-γ enhanced proliferation of T cells rather than reduction of apoptotic T cells, resulting in the effector T-cell expansion.

Inflamed gastric mucosa in AIG contains CD4$^+$ T cells, and depletion of CD4$^+$ T cells suppresses AIG

Next, we examined why the neutralization of IFN-γ did not allow expanded effector T cells to infiltrate into the gastric mucosa. To investigate the involvement of the CD4$^+$ and/or CD8$^+$ T cells in the development of AIG, NTx–PD-1$^{-/-}$ mice were injected intraperitoneally at 1 day after NTx and

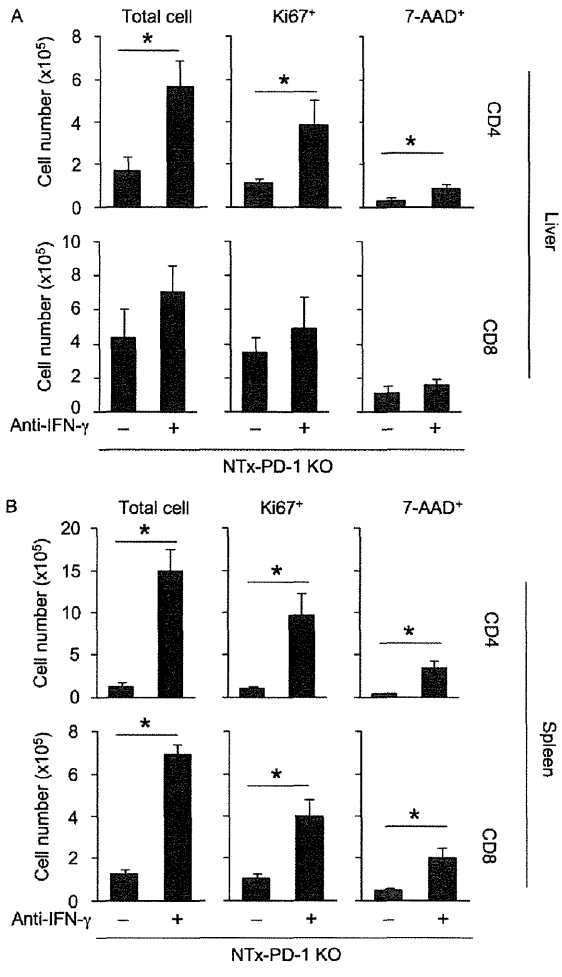

Figure 5. Neutralization of IFN-γ exacerbated T-cell proliferation, resulting in the increased number of T cells in the spleen and liver. NTx–PD-1$^{-/-}$ mice were injected with or without anti-IFN-γ as described in Figure 2. After three injections, mice at 3 weeks of age were sacrificed, and the cells were isolated from the liver (A) and the spleen (B) and stained with Abs against CD3, CD4, CD8, and Ki-67 or 7-ADD. Percentages of indicated cells were determined by flow cytometry. *Numbers* of total cells, Ki-67$^+$ cells, or 7-ADD$^+$ cells in CD4$^+$ or CD8$^+$ T cells were calculated by (percentage of the cells in viable cells) × (number of viable cells). Data are shown as the mean of at least three mice. *Error bars* represent SD. *$P < 0.05$.

then once a week with either depletion antibodies to CD4 or CD8. After four injections of anti-CD4 or anti-CD8, the number of CD4$^+$ or CD8$^+$ T cells in the periphery was greatly reduced, respectively (data not shown). Previously, we had shown that either depletion of CD4$^+$ T cells or CD8$^+$ T cells suppressed fatal AIH [20]. In this study, we found that depletion of CD4$^+$ T cells inhibited the development of AIG, whereas depletion of CD8$^+$ T cells did not suppress AIG development (Figure 6A and B). These data suggest that CD4$^+$ T cells but not CD8$^+$ T cells are indispensable for the development of AIG.

Because the gastric mucosa originally does not have a lymphoid apparatus, CD4$^+$ T cells may start to

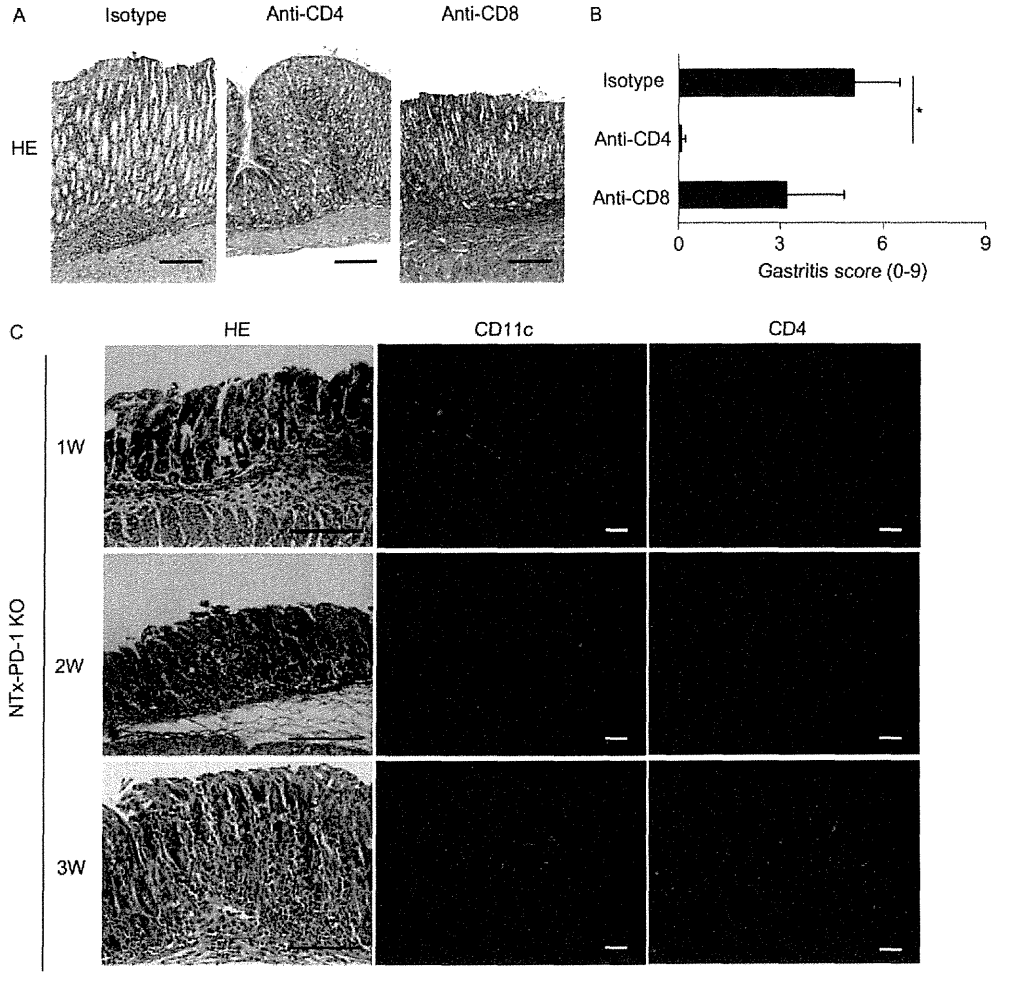

Figure 6. Infiltrating $CD4^+$ T cells in the gastric mucosa are essential for the induction of AIG. (A and B) NTx–PD-$1^{-/-}$ mice at 1 day after thymectomy were injected intraperitoneally every week with 100 μg of depletion Abs for $CD4^+$ T cells ($n = 5$), $CD8^+$ T cells ($n = 5$), or the isotype control ($n = 5$). After four injections, mice at 4 weeks of age were sacrificed, and the stomachs were harvested. Histological analysis of the stomach: The sections of tissues were fixed in formalin and stained with HE. All *scale bars*, 100 μm (A). Gastritis score. Data are shown as the mean and error bars represent SD. $*P < 0.05$ (B). (C) Staining of the stomach for HE, CD11c, and CD4 in NTx–PD-$1^{-/-}$ mice at indicated age in weeks. *Scale bars*, 50 μm (left panels) and 100 μm (middle and right panels).

infiltrate into the gastric mucosa after the induction of AIG. Next, we monitored immunohistological findings of the stomach from 1 to 3 weeks (Figure 6C). Four days after thymectomy, 1-week-old NTx–PD-$1^{-/-}$ mice did not have any inflammation in the immature gastric gland. Although $CD4^+$ T cells did not infiltrate into the gastric mucosa, $CD11c^+$ cells existed in the lamina propria of the gastric gland (Figure 6C, upper panels), where 2-week-old NTx–PD-$1^{-/-}$ mice showed moderate mononuclear cell infiltrations. These infiltrations were associated with the increased number of $CD11c^+$ cells and $CD4^+$ T cells within the gastric mucosa (Figure 6C, middle panels).

Three-week-old NTx–PD-$1^{-/-}$ mice revealed that infiltrations of $CD11c^+$ cells and $CD4^+$ T cells progressed in the gastric mucosa (Figure 6C, lower panels). These data suggest that in NTx–PD-$1^{-/-}$ mice, infiltrating $CD4^+$ T cells in the gastric mucosa is essential for the induction of AIG.

Inflamed gastric mucosa up-regulates gene expression of CCL20, and anti-IFN-γ treatment suppresses CCL20 expression in the stomach

Previous studies demonstrated that *Helicobacter pylori* (*H. pylori*) infection in the stomach induces up-regulation of CCL20 gene expression in gastric epithelial cells in humans and mice [23,27]. Because local production of chemokine CCL20 is critical for the migration of $CCR6^+$ immune cells in the inflamed lesions [20,28–30], we hypothesized that IFN-γ might trigger the up-regulation of CCL20 gene expression in the stomach. To test this hypothesis,

NTx–PD-1$^{-/-}$ mice were intraperitoneally injected with neutralizing anti-IFN-γ as described in Figure 2. In comparison with normal gastric mucosa in 3-week-old PD-1$^{-/-}$ mice, inflamed gastric mucosa in same-aged NTx–PD-1$^{-/-}$ mice showed up-regulation of CCL20 gene expression (Figure 7A). In addition, neutralization of IFN-γ suppressed up-regulation of CCL20 gene expression in the stomach

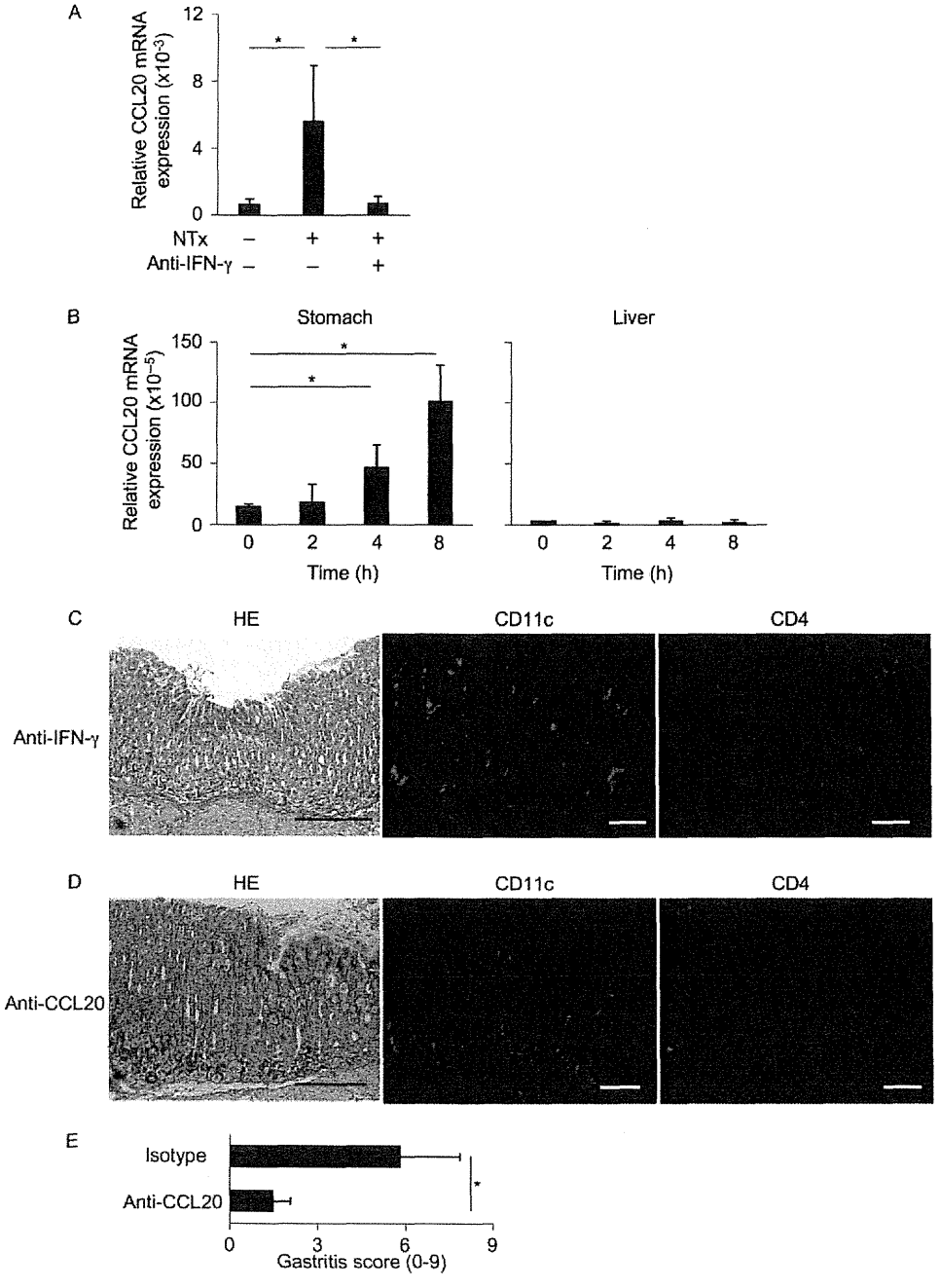

Figure 7. IFN-γ-dependent induction of CCL20 expression in the gastric mucosa is critical for the development of AIG. (A) NTx–PD-1$^{-/-}$ mice were injected with or without anti-IFN-γ as described in Figure 2. After three injections, mice at 3 weeks of age were sacrificed, and the stomachs were harvested. Real-time quantitative RT-PCR analyses for mRNA expressions of CCL20 in the stomachs: Data are shown as the mean of three mice. (B) Four-week-old PD-1$^{-/-}$ mice were injected intraperitoneally with 10 μg/kg of recombinant mouse IFN-γ. At the indicated time after injection, mice were sacrificed. CCL20 mRNA expressions in the stomach and liver were shown. Data are shown as the mean of three mice. (C and D) NTx–PD-1$^{-/-}$ mice were injected with anti-IFN-γ ($n = 5$, C) or anti-CCL20 ($n = 5$, D) as described in Figure 2. After four injections, mice at 4 weeks of age were sacrificed, and the stomachs were harvested. Staining of the stomachs for HE, CD11c, and CD4. *Scale bars*, 100 μm. (E) Gastritis score in NTx–PD-1$^{-/-}$ mice at 4 weeks of age injected with anti-CCL20 ($n = 5$) or isotype control ($n = 10$). Data are shown as the mean and *error bars* represent SD. $*P < 0.05$.

(Figure 7A), suggesting that in the development of AIG, IFN-γ may be involved in up-regulating CCL20 gene expression in the stomach.

In vivo administration of recombinant IFN-γ induces CCL-20 expression in the stomach

Next, to examine whether IFN-γ can induce up-regulated expression of CCL20 in the stomach *in vivo*, 4-week-old PD-1$^{-/-}$ mice were injected intraperitoneally with 10 μg/kg of recombinant mouse IFN-γ. Four hours later, we found up-regulated mRNA expression of CCL20 in the stomach but not in liver (Figure 7B). These data confirmed that IFN-γ can induce up-regulated expression of CCL20 in the stomach *in vivo*.

Neutralization of either IFN-γ or CCL-20 suppresses the infiltration of CD4$^+$ cells into the gastric mucosa and the development of AIG

Finally, we confirmed that neutralization of either IFN-γ or CCL-20 suppresses the infiltration of CD4$^+$ cells into the gastric mucosa by immunohistology. NTx–PD-1$^{-/-}$ mice were intraperitoneally injected with neutralizing anti-IFN-γ or anti-CCL20, as described in Figure 2. After four injections, mice at 4 weeks of age showed that neutralization of both IFN-γ and CCL-20 suppressed the infiltration of CD4$^+$ cells but not CD11c$^+$ cells into the gastric mucosa (Figure 7C–E). Our results, taken together, indicate that increased production of IFN-γ induces up-regulation of CCL20 expression in the stomach to trigger the infiltration of CD4$^+$ T-cell in the gastric mucosa, implying that IFN-γ is indispensable in the development of AIG.

Discussion

In the present study, we examined the roles of IFN-γ in the development of spontaneous AIG and AIH in a mouse model. We found that neutralization of IFN-γ prevented the development of AIG, for which CD4$^+$ T-cell migration into the gastric mucosa is essential. CCL20 expression was up-regulated in the gastric mucosa, and anti-CCL20 suppressed CD4$^+$ T-cell infiltration into the gastric mucosa. Importantly, anti-IFN-γ suppressed CCL20 expression and infiltration of CD4$^+$ T cells in the gastric mucosa, whereas *in vivo* injection of rIFN-γ up-regulated CCL20 expression in the stomach, suggesting that IFN-γ is critically involved in CD4$^+$ T-cell accumulation into the gastric mucosa through up-regulation of local CCL20 expression.

In contrast, in AIH, neutralization of IFN-γ exacerbated hepatic T-cell infiltration. Because of the loss of anti-proliferative effects by IFN-γ, neutralization of IFN-γ intensified T-cell proliferation in the spleen and liver, resulting in exacerbated T-cell infiltration in the liver. We therefore concluded that although AIG and AIH progress simultaneously and sequentially in NTx–PD-1$^{-/-}$ mice, IFN-γ is involved differently in the development of AIG and AIH.

Using a new AIG model in which gastritis rapidly develops within 4 weeks of age, we found that in the development of AIG, CD4$^+$ T-cell migration into the gastric mucosa is essential for the induction and that the IFN-γ-induced CCL20 expression promotes migration of CD4 T cells into the gastric mucosa. A previous study using mouse models characterized AIG as having a marked infiltration of CD4$^+$ T cells, which produce large amounts of IFN-γ [16]. Mice with depleted CD4$^+$ T cells or administered blocking Abs to IFN-γ show severely impaired development of AIG [15,16]. In mice treated with only a single dose of anti-IFN-γ immediately after thymectomy at 3 days after birth, the incidence of AIG was severely reduced [16]. However, the precise roles of IFN-γ in the induction phase of AIG had not been clear.

Our study demonstrated that in the induction phase of AIG, IFN-γ critically acts on CCL20 up-regulation in the gastric mucosa, resulting in infiltration of CD4$^+$ T cells. The CCR6-CCL20 axis plays an important role in the migration of instructed CD4$^+$ T cells into target tissues, and CCR6 is expressed on Th1 cells, Th17 cells, as well as Treg cells in mice and humans [28–31]. Thus, CCR6 expressing Th1 cells may be critical for the induction of AIG.

In this study, we demonstrated that IFN-γ induces up-regulation of CCL20 expression in the gastric mucosa. In previous studies, *H. pylori* infection in the stomach induces chronic gastritis, and *H. pylori* colonization triggers up-regulation of CCL20 expression in gastric epithelial cells in humans and mice [23,27]. However, the precise roles of up-regulated CCL20 expression in the gastric mucosa in the development of gastritis had not been clear. In this study, we showed that CCL20 up-regulation in the gastric mucosa is essential for the infiltration of CD4$^+$ T cells, but not CD11c DCs in the gastric mucosa. Although precisely how IFN-γ induces CCL20 expression in the gastric mucosa is not clear at present, our data offer insight into the roles of CCL20 in the development of gastritis not only by infection but also by autoimmunity.

Using a new spontaneous AIH model in which hepatitis rapidly and fatally develops within 4 weeks of age, we demonstrated that neutralization of IFN-γ exacerbated T-cell infiltration in the liver and did not reduce hepatic injury. In contrast, IFN-γ is essential for inducing concanavalin A (Con A)-induced acute hepatic injury [32,33].

Con A-induced acute hepatic injury is associated with activation of NKT cells and T cells, and is considered to be an experimental model of human

AIH [25,34]. Con A-induced acute hepatic injury induces rapidly increased serum levels of IFN-γ, and Con A-induced injury of hepatocytes was significantly reduced in neutralization of IFN-γ-mediated signals or deficient IFN-γ [32,33]. In addition, Con A-induced injury of hepatocytes depends on IFN-γ through modulation of signaling by the death receptor Fas [32]. However, neutralization of Fas ligand did not reduce injury of hepatocytes in fatal AIH in NTx–PD-$1^{-/-}$ mice (data not shown).

Because the kinetics of plasma levels of IFN-γ in NTx–PD-$1^{-/-}$ mice differ from those in Con A-induced acute hepatic injury, IFN-γ may clearly act as negative regulator in the development of AIH in NTx–PD-$1^{-/-}$ mice. IFN-γ is undetectable in blood circulation upon injection of Con A into wild-type mice. These cytokines are detectable at 2 h after the single intravenous injection; they reach a maximal level within 6 h and are greatly reduced at 24 h [33,35]. In contrast, high levels of IFN-γ in blood circulation were sustained in AIH-bearing NTx–PD-$1^{-/-}$ mice.

IFN-γ is essential to a regulatory mechanism controlling optimal population expansion of activated $CD4^+$ T cells during an immune response [1–7]. IFN-γ suppresses proliferation of activated $CD4^+$ T cells, whereas IFN-γ induces apoptosis of activated $CD4^+$ T cells [4–7]. In mouse models of *Mycobacterium bovis* Bacille Calmette-Guérin infection, IFN-γ deficiency induces decreased apoptosis of activated $CD4^+$ T cells and a huge expansion of these $CD4^+$ T cells exacerbating inflammation [5].

However, neutralization of IFN-γ in NTx–PD-$1^{-/-}$ mice induced further proliferation of $CD4^+$ and $CD8^+$ T cells but did not reduce apoptosis of these T cells. Because IFN-γ is reported to be critically required for the conversion of $CD4^+CD25^-$ T cells to Treg cells during experimental autoimmune encephalomyelitis [8], converted Treg cells by IFN-γ may suppress proliferation of not only $CD4^+$ T cells but also $CD8^+$ T cells in NTx–PD-$1^{-/-}$ mice.

In contrast, we performed a global quantitative mRNA screening of IFN-γ-related molecules in AIH, finding that inflamed liver tissues of AIH in NTx–PD-$1^{-/-}$ mice produced larger amounts of mRNA for interferon-induced transmembrane protein 1 (IFITM1, data not shown), which negatively regulates cell growth and is key to the anti-proliferative action of IFN-γ in human cell lines [36]. In addition, neutralization of IFN-γ suppressed IFITM1 expression (data not shown). These data suggest that direct and/or indirect anti-proliferative actions that depend on IFN-γ may negatively regulate proliferation of infiltrating T cells in Th1-dependent progression of AIH.

In conclusion, because IFN-γ, regarded as a proinflammatory factor and as one of the signature cytokines of Th1-dominated autoimmunity, can counteract harmful inflammation in autoimmunity, its endogenous production—even during the process of the simultaneous development of AIH and AIG—results in bidirectional immunoregulatory consequences in moderating the pathology of NTx–PD-$1^{-/-}$ mice. Although it is not clear at present whether IFN-γ exerts bidirectional immunoregulatory functions as human organ-specific autoimmune diseases are developing, our data highlight the unique roles of IFN-γ in autoimmunity. Production of IFN-γ induced by an organ-specific autoimmunity may trigger the concurrent development of another organ-specific autoimmune disease.

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■ 特集 小児消化管内視鏡：診断と治療の最前線

小児原発性硬化性胆管炎発症早期の ERCP 所見

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はじめに

原発性硬化性胆管炎（sclerosing cholangitis：PSC）は，病理学的には肝内外の胆管周囲の線維化を特徴とし，胆管造影ではそれを反映し，びまん性に狭窄と拡張がみられる。

診断には Mayo Clinic の診断基準（表 1）[1]が一般的に用いられており，これには病理学的所見の記載はなく，胆道造影所見が重視されていることがわかる。小児ではアルカリフォスファターゼ（ALP）は成長に伴う骨代謝の影響で生理的高値がみられるため，γ-グルタミルトランスペプチダーゼ（γ-GTP）で代用する[2]。胆道造影は近年，Magnetic resonance cholangiography（MRC）で代用されることもあるが[2]，病初期の肝内胆管の微細な変化を描出することは困難であり，筆者らは PSC を疑った場合には積極的に内視鏡的逆行性胆管膵管造影（Endoscopic retrograde cholangiopancreatography：ERCP）を行っている。

また，腹腔鏡を用いた経胆嚢胆管造影では肝外胆管は確実に造影されるが，肝内の病変，とくに末梢の変化は描出できないこともある。

Ⅰ．PSC に特徴的な胆道造影所見

硬化性胆管炎には HIV 感染，免疫不全や Histiocytosis などの基礎疾患がある 2 次性硬化性胆管炎と基礎疾患がなく，高率に炎症性腸疾患を合併する PSC とに大きく分けられる。また，乳児期には胆道閉鎖症（BA）との鑑別が必要となり near-miss BA ともよばれる新生児期発症硬化性胆管炎が知られている。いずれの硬化性胆管炎も図 1 に示すような所見を呈する[3]。肝内胆管の病変を正確に評価するためには末梢胆管まで確実に描出する必要がある。造影剤が十二指腸に漏れ出てしまう場合にはバルーン付きカテーテルを総胆管内に深部挿管し，総胆管内でバルーンを膨らませたのち，十分に肝内胆管に造影剤を満たす（図 2）。

造影剤を末梢胆管まで十分に注入しても，後述するような所見が得られない場合には PSC とは診断できないが，small duct PSC とよばれる概念がある。

Small-duct PSC とは，ERCP で描出されるような比較的太い胆管には病変はないため，ERCP 所見は正常で，肝組織で PSC に特異的な胆管周囲の層状線維化（onion-skin fibrosis）がみられるもの

表 1 Mayo Clinic の PSC の診断基準

胆道造影で典型的な胆道系の異常がみられる。
臨床所見（例：炎症性腸疾患，胆汁うっ滞の既往），血液生化学所見（正常の 3 倍以上のアルカリフォスファターゼ値の上昇が 6 カ月以上みられる）が合致する。
2 次性硬化性胆管炎の原因の除外
- AIDS-胆管炎
- 胆管の悪性新生物（PSC が以前に診断されていない）
- 胆道系の手術，外傷
- 総胆管結石
- 胆道系の先天異常
- 破壊性胆管炎
- 胆管の虚血性狭窄
- floxuridine の動脈内注射に関連する胆管の毒性/狭窄

（露口ら[1]，2001 より引用）

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