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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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Running title: Differential roles of IFN- γ in organ-specific autoimmunity

Keywords

IFN- γ
proinflammatory mediator
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regulatory function
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Abbreviations:

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

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 (PD-1)-deficient mice thymectomized three 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 upregulated 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 upregulated CCL20 expression in the stomach, suggesting that IFN- γ is critically involved in CD4⁺ T-cell accumulation in AIG by upregulating 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.

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 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 autoimmune hepatitis (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 characteristic 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-1^{-/-} mice) and BALB/c mice thymectomized three 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-1^{-/-} BALB/c mice with neonatal thymectomy (NTx-PD-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-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-1^{-/-} mice at three 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-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 upregulated 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-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 three 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 (RT-PCR)

Real-time quantitative RT-PCR was performed as described previously [23]. Liver and gastric tissues were frozen in RNAlater (Qiagen, Hilden, Germany). RNA was extracted using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Single-strand complementary DNA was synthesized with SuperScriptTM II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time quantitative reverse-transcription polymerase chain reaction was performed using SYBR Green I Master (Roche Applied Science, Basel, Switzerland). Real-time quantitative reactions were performed with Light Cycler 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'-CAACTTTGTCAAGCTCATTCC-3' and 5'-GGTCCAGGGTTTCTTACTCC-3'; *Foxp3*: 5'-TCAGGAGCCCACCAGTACA-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'-AGCTTTCCTCCGCATTGACACAG-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'-TCACAGGAAGGGCATTAGC-3'
CCL20: 5'-ATGGCCTGCGGTGGCAAGCGTCTG-3' and
5'-TAGGCTGAGGAGGTTTCACAGCCCT-3';

Enzyme-Linked Immunosorbent Assay (ELISA)

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

In vivo neutralization and injection of cytokines

NTx-PD-1^{-/-} mice at one 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). 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 four weeks age were injected intraperitoneally with 10 µg/kg of recombinant mouse IFN-γ (eBioscience). Before and after 2, 4, 6, or 8 hours 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 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). 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™ II (BD Biosciences). Data were analysed using Cell Quest Pro™ (BD Biosciences).

Statistical analysis

The data are presented as the mean values \pm standard deviations. Statistical analysis was performed by the Student *t* test for pairwise comparisons and analysis of variance with the Tukey-Kramer test for multiple comparisons. *P*-values below .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, two-week-old NTx-PD-1^{-/-} mice showed moderate mononuclear cell infiltrations, predominantly in the portal area of the liver and in the lamia 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 three-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 one to three weeks of age. Previously we showed that severe AIG and AIH-bearing NTx-PD-1^{-/-} mice at three 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 one to two 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 one day after thymectomy and then once a week. After four injections, mice at four weeks of age were sacrificed and the stomach and liver were harvested (Figure 2(A)). Although four-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 2(B) left panel), four injections of anti-IFN-γ suppressed both events (Figure 2(B) right panel). 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 2(C)). 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 2(D) left panel), whereas injection of anti-IFN-γ showed more severe mononuclear cell infiltration in the liver with a massive degeneration of hepatocytes (Figure 2(D) right panel). In addition, injection of anti-IFN-γ revealed significantly increased serum concentrations of AST at four weeks of age (Figure 2(E)).

Because we found the most elevated serum level of IFN-γ at one 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 μ g of anti-IFN- γ or the isotype control at one day before thymectomy. These mice were further injected one day after thymectomy and then once a week with anti-IFN- γ . After five injections, mice at four weeks of age were sacrificed and the stomach and liver were harvested (Figure 3(A)). Five injections of anti-IFN- γ also suppressed the mononuclear cell infiltration and loss of parietal and chief cells in the gastric mucosa (Figure 3(B)). These findings were further confirmed by a gastritis scoring system (Figure 3(C)). 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 four weeks of age (Figure 3(D) 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 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 4(A)).

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 master regulators for T-cell subsets and related cytokines—Foxp3, T-bet, GATA-3, ROR γ t, 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 four weeks of age. Previously we showed that in hepatic CD4⁺ T cells in the progression phase of AIH at three weeks, T-bet and Bcl-6 expressions were upregulated [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 4(B)). However, we could not detect increased expression of any other molecules in the inflamed liver tissues under the neutralization of IFN- γ (Figure 4(B)). 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^{low}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 5(A)). 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 5(A)). These data are further confirmed and more significant in CD4⁺ and CD8⁺ T cells in the spleen (Figure 5(B)). 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 one day after NTx and 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 6(A) 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 infiltrate into the gastric mucosa after the induction of AIG. Next, we monitored immunohistological findings of the stomach from 1 to 3 weeks (Figure 6(C)). 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 lamia propria of the gastric gland (Figure 6(C) upper panels), where two-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 6(C) 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 6(C) lower panels). These data suggest that in NTx-PD-1^{-/-} mice, infiltrating CD4⁺ T cells in the gastric mucosa are essential for the induction of AIG.

Inflamed gastric mucosa upregulates 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 upregulation 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 upregulation 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 upregulation of CCL20 gene expression (Figure 7(A)). In addition, neutralization of IFN- γ suppressed upregulation of CCL20 gene expression in the stomach (Figure 7(A)), suggesting that in the development of AIG, IFN- γ may be involved in upregulating 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 upregulated expression of CCL20 in the stomach *in vivo*, four-week-old PD-1^{-/-} mice were injected intraperitoneally with 10 μ g/kg of recombinant mouse IFN- γ . Four hours later, we found upregulated mRNA expression of CCL20 in the stomach but not liver (Figure 7(B)). These data confirmed that IFN- γ can induce upregulated 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 four weeks of age showed that both neutralization of IFN- γ and CCL-20 suppressed the infiltration of CD4⁺ cells but not

CD11c⁺ cells into the gastric mucosa (Figure 7(C-E)). Our results, taken together, indicate that increased production of IFN- γ induces upregulation 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 upregulated 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 upregulated CCL20 expression in the stomach, suggesting that IFN- γ is critically involved in CD4⁺ T-cell accumulation into the gastric mucosa thorough upregulation 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 four 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 upregulation 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 upregulation of CCL20 expression in the gastric mucosa. In previous studies, *H. pylori* infection in the stomach induces chronic gastritis, and *H. pylori* colonization triggers upregulation of CCL20 expression in gastric epithelial cells in humans and mice [23, 27]. However, the precise roles of upregulated CCL20 expression in the gastric mucosa in the development of gastritis had not been clear. In this study, we showed that CCL20 upregulation 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 autoimmunity.

Using a new spontaneous AIH model in which hepatitis rapidly and fatally develops within four 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 (BCG)-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, then 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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Figure legends

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 one to three 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.

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 one day after thymectomy were injected intraperitoneally (ip) every week with 100 μ g of neutralizing anti-mouse IFN- γ (n=5) or the isotype control mAb (n=10). After four injections, mice at four 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 hematoxylin and eosin. (C) Gastritis score of NTx–PD-1^{-/-} mice at four 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 hematoxylin and eosin. (E) Serum levels of the liver transaminase, AST and ALT, and total bilirubin. Sera from NTx–PD-1^{-/-} mice at four 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 μ m.

Figure 3. Neutralization of IFN- γ from one 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) one day before thymectomy and every week from one day after thymectomy with 100 μ g 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 Fig. 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 μ m.

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 Fig. 2. After four injections, mice at 4 weeks of age were sacrificed, and the livers were harvested. (A) Staining of the livers for hematoxylin and eosin (HE), CD4 and CD8. Scale bars, 100 μ m. (B) Real-time quantitative RT-PCR analyses for mRNA expressions of indicated molecules in the liver. Data are shown as the mean of at least three mice. *Error bars* represent SD. *, P < 0.05.

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) x (number of viable cells). Data are shown as the mean of at least three mice. *Error bars* represent SD. *; P < 0.05.

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 one 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 four 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 hematoxylin and eosin. 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 hematoxylin and eosin (HE), CD11c and CD4 in NTx-PD-1^{-/-} mice at indicated age in weeks. *Scale bars*, 50µm (left panels) and 100µm (middle and right panels).

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 Fig. 2. After four injections, mice at four weeks of age were sacrificed, and the stomachs were harvested. Staining of the stomachs for hematoxylin and eosin (HE), CD11c and CD4. *Scale bars*, 100 μ m. (E) Gastritis score in NTx-PD-1^{-/-} mice at four 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 1

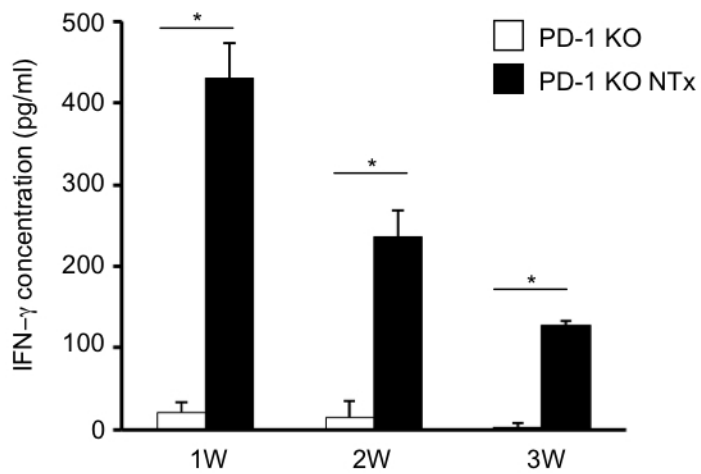


Figure 2

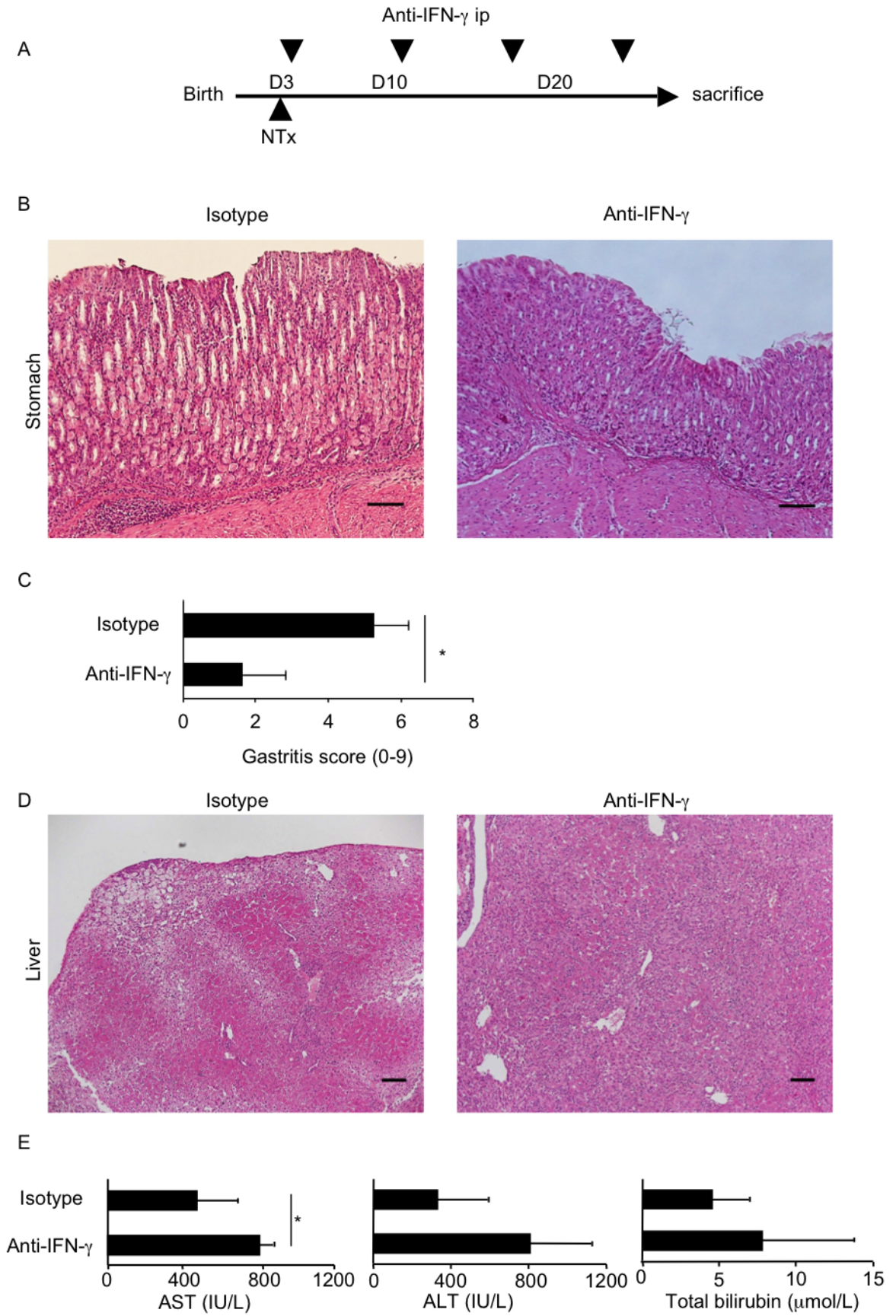


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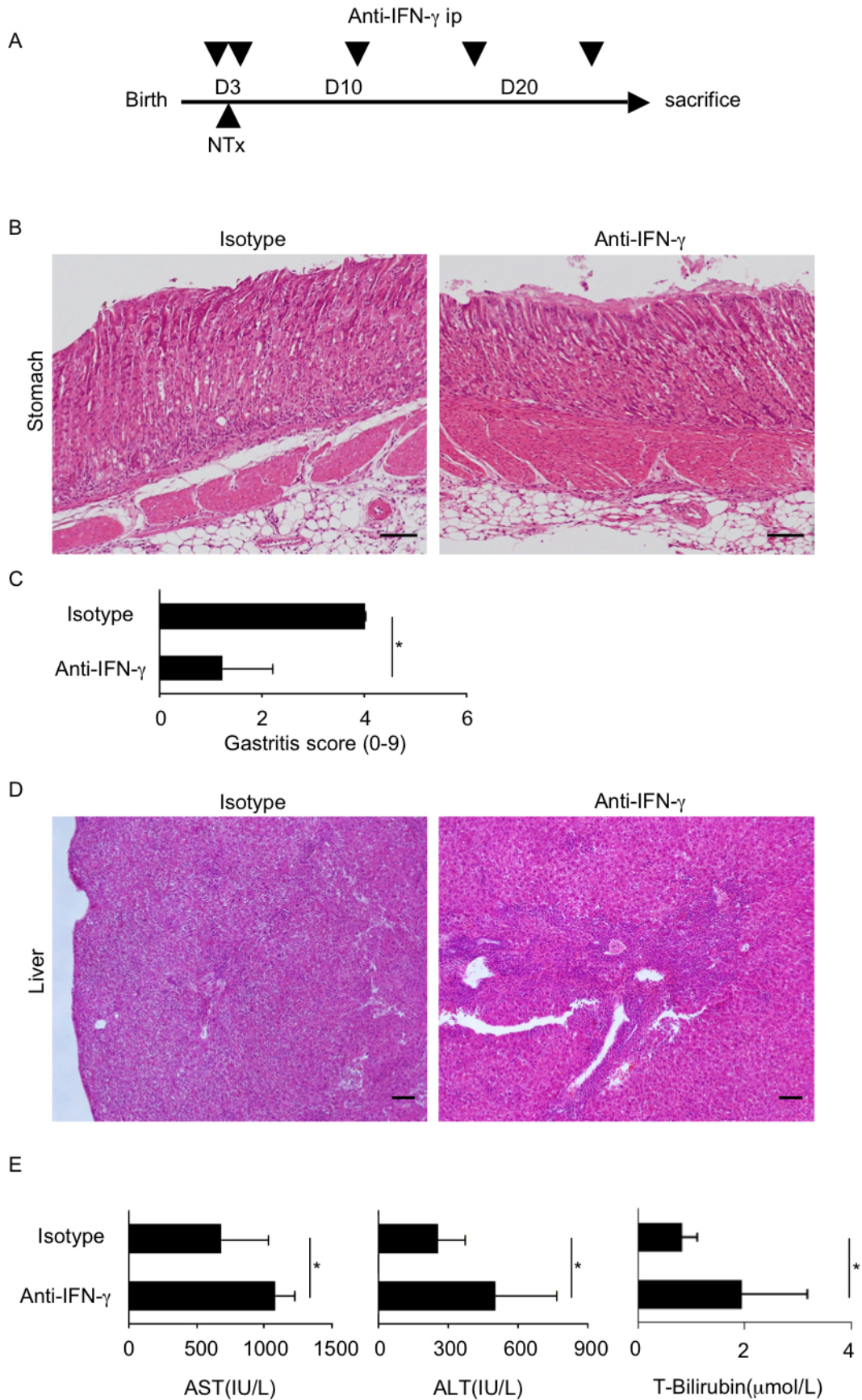
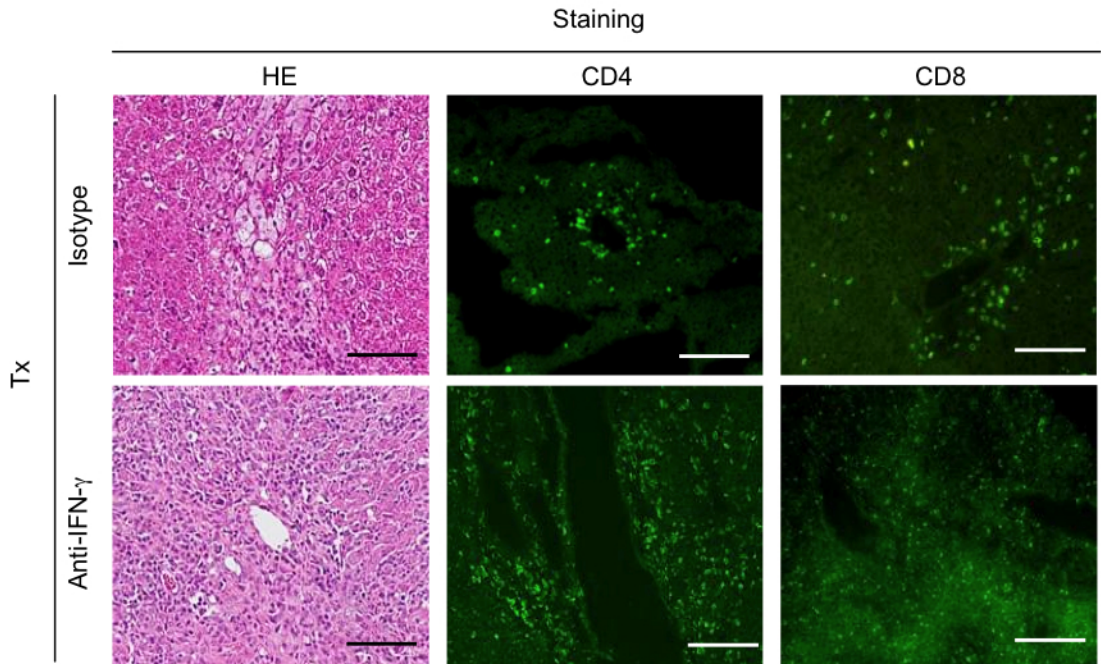


Figure 4

A



B

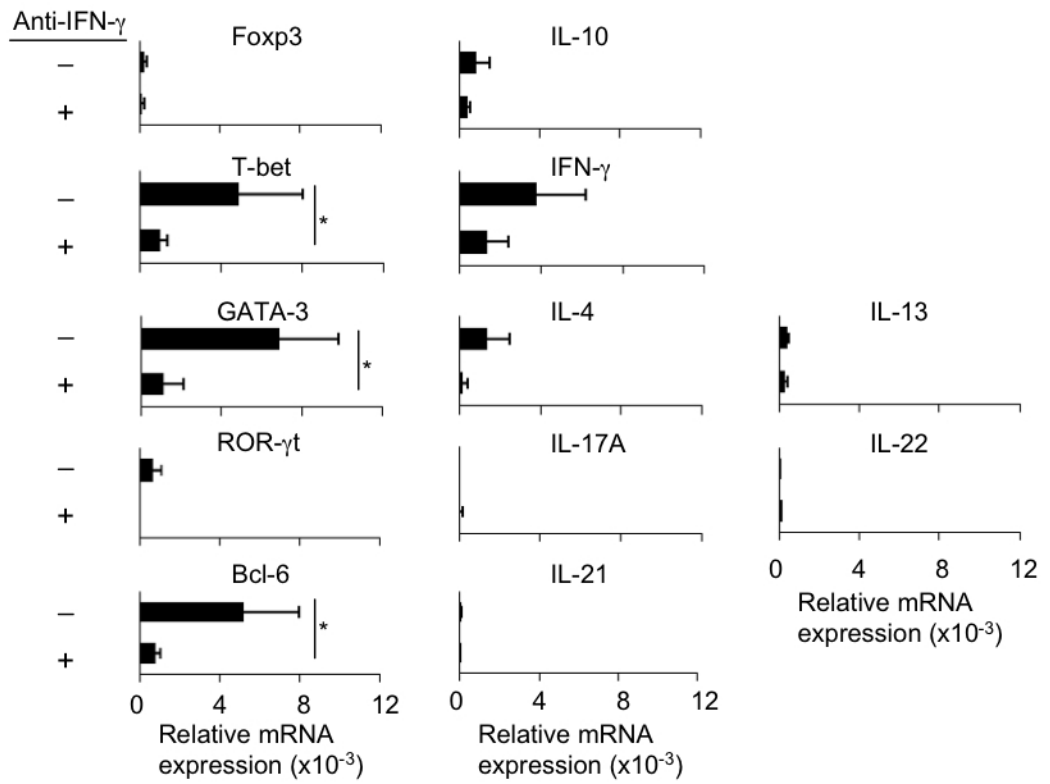
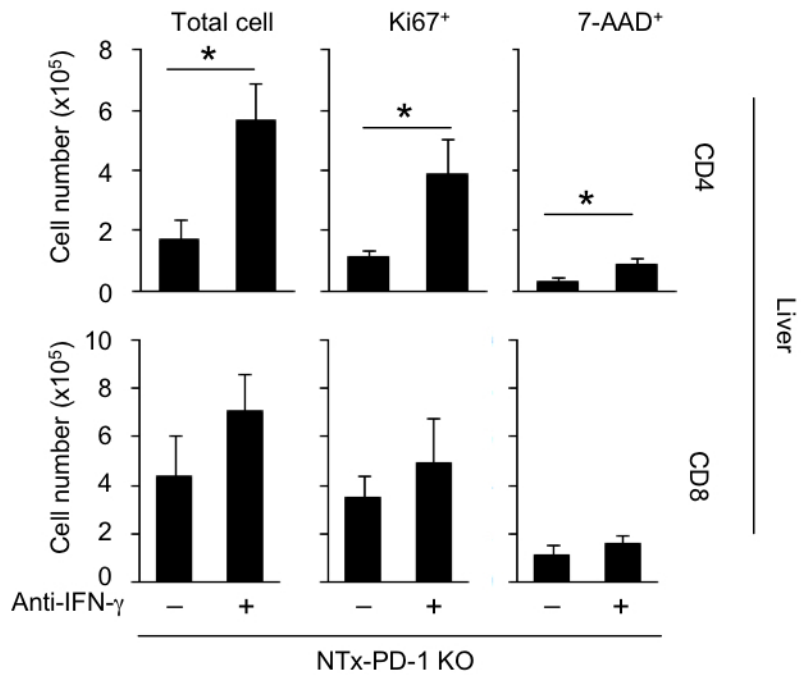


Figure 5

A



B

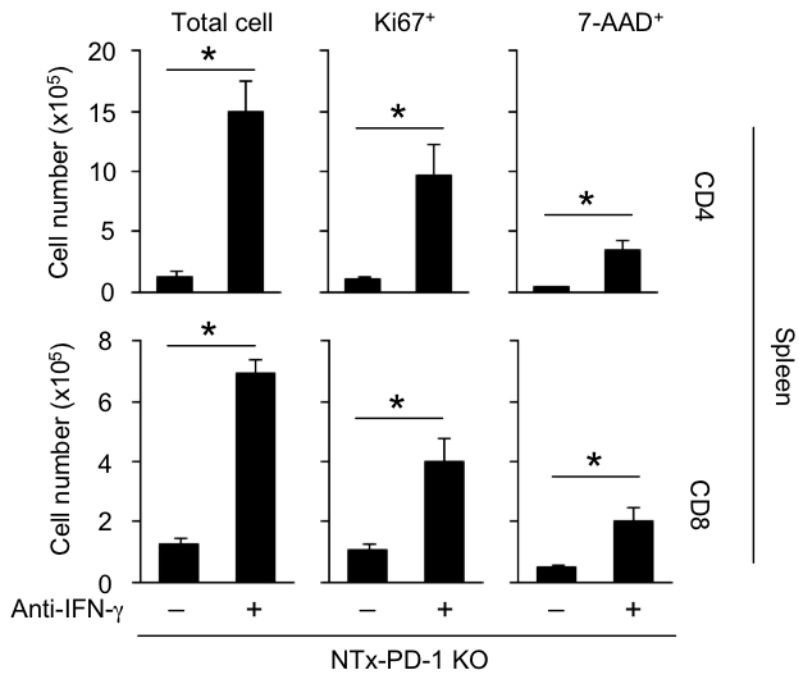


Figure 6

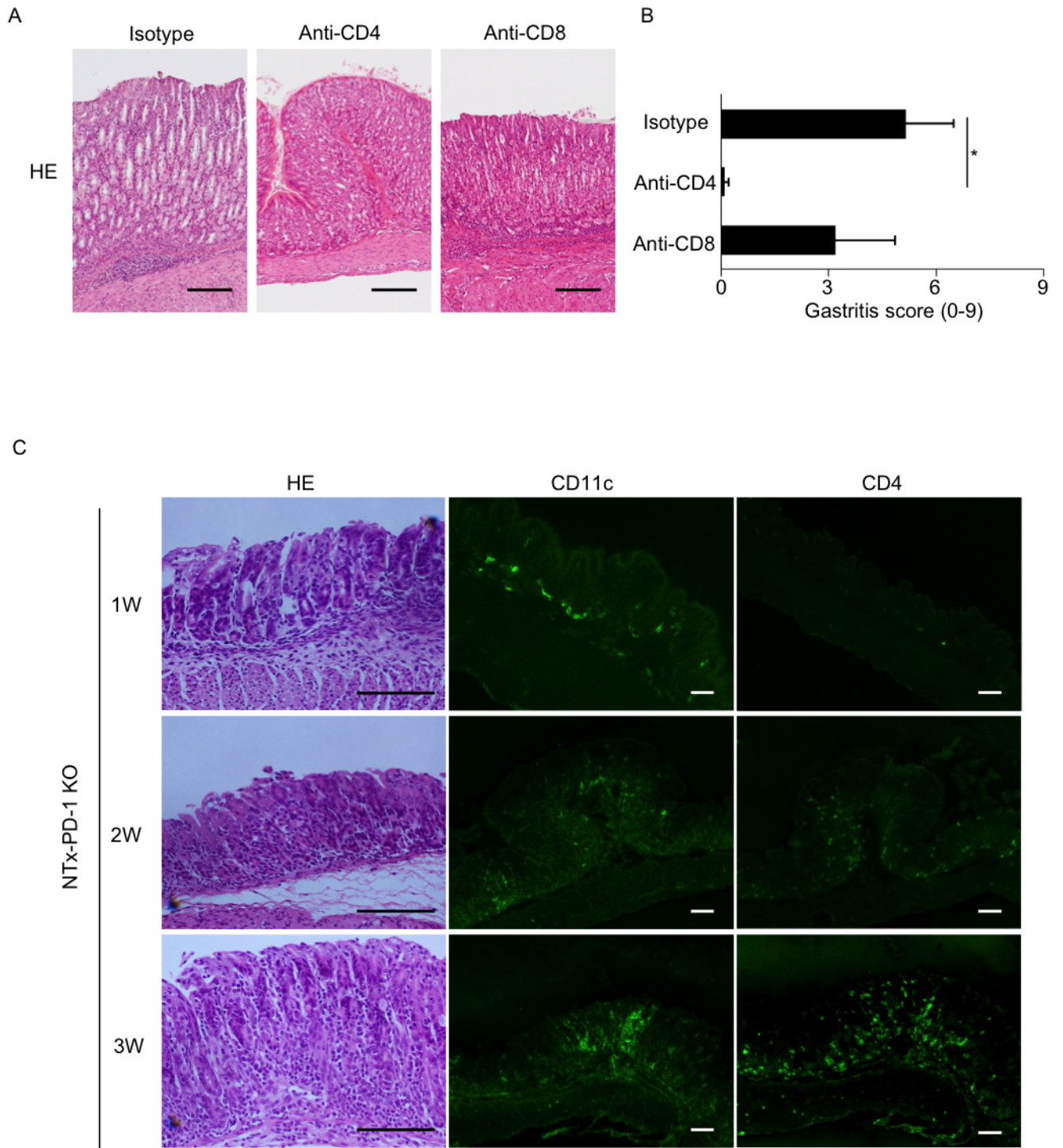


Figure 7

