

TNF- α is essential in the induction of fatal autoimmune hepatitis in mice through upregulation of hepatic CCL20 expression

Satoru Iwamoto,^{1,2} Masahiro Kido,^{1,2} Nobuhiro Aoki,^{1,2} Hisayo Nishiura,^{1,2} Ryutaro Maruoka,^{1,2} Aki Ikeda,^{1,2} Taku Okazaki,³ Tsutomu Chiba,² and Norihiko Watanabe^{1,2}

¹Center for Innovation in Immunoregulative Technology and Therapeutics, and ²Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan, ³Division of Immune Regulation, Institute for Genome Research, University of Tokushima, Tokushima 770-8503, Japan

Correspondence: Norihiko Watanabe
Tel: +81-75-751-4319
Fax: +81-75-751-4303
e-mail: norihiko@kuhp.kyoto-u.ac.jp

Abbreviations:

AIH, autoimmune hepatitis;
Con A, concanavalin A;
GC, germinal center;
IFN, interferon
mAb, monoclonal antibody;
NTx, neonatal thymectomy;
NTx mice, mice thymectomized three days after birth;
PD-1, programmed cell death 1;
TNF, tumor necrosis factor;
T_{FH}, follicular helper T

Keywords: mouse model; autoimmune hepatitis; TNF- α ; CCL20; TNF- α antagonist

Abstract

It is unclear what roles TNF- α has in the development of autoimmune hepatitis (AIH) and whether AIH is responsive to anti-TNF- α . We recently developed a mouse model of fatal AIH that develops in PD-1-deficient mice thymectomized three days after birth, finding that CCR6-CCL20 axis-dependent migration of dysregulated splenic T cells is crucial to induce AIH. In this study, we show the indispensable role of TNF- α in the development of AIH. Administering anti-TNF- α prevented the induction, but treatment by anti-TNF- α after the induction did not suppress progression. Administering anti-TNF- α did not prevent splenic T-cell activation, but did suppress hepatic CCL20 expression. In contrast, administering anti-CCL20 suppressed AIH but not elevated serum TNF- α levels. TNF- α stimulation enhanced CCL20 expression in hepatocytes. These findings suggest that TNF- α is essential in the induction of AIH through upregulation of hepatic CCL20 expression, which allows migration of dysregulated splenic T cells.

1. Introduction

Dysregulated production of tumor necrosis factor (TNF)- α is involved in the pathology of various immune-mediated inflammatory diseases, including inflammatory bowel diseases and rheumatoid arthritis in humans [1-4]. In the liver, TNF- α directly and indirectly induces cell death of hepatocytes, whereas it can mediate production of inflammatory mediators, hepatocyte proliferation, and liver regeneration [5]. In human diseases, TNF- α is involved in the pathophysiology of viral hepatitis, alcoholic liver disease, nonalcoholic fatty liver disease, and ischemia-reperfusion injury [5]. In addition, serum TNF- α levels were elevated in untreated children with type 1 autoimmune hepatitis (AIH) in comparison with those in healthy controls [6]. A polymorphism at position 308 in a promoter region of TNF- α has been reported to be associated with severity of AIH type 1 in Europe and North America [7,8]. However, it is unclear whether TNF- α is critical in the development of AIH or whether TNF antagonists have therapeutic efficacies for AIH in humans.

Several mouse models of AIH have demonstrated the roles of cytokines, costimulatory molecules, T-cell subsets, and autoantigens in the development of AIH. Gorham et al. reported that BALB/c background TGF- β 1 deficient mice develop fatal hepatitis characterized by massive CD4⁺ T cell infiltration [9]. In this model, interferon (IFN)- γ produced by CD4⁺ T cells drove lethal hepatic damage [10]. Mice deficient in B and T lymphocyte attenuator (BTLA), negative costimulatory molecules expressed on lymphocytes, developed AIH-like disease with hypergammaglobulinemia and production of anti-nuclear antibodies [11]. In addition, mice immunizing human liver autoantigens and mice infected with adenovirus Ad5 expressing human cytochrome P450 2D6 developed persistent immune-mediated hepatitis [12]. Mice

vaccinated with dendritic cells loaded with liver antigens and subsequently administered IL-12 also developed persistent immune mediated hepatitis [13]. Furthermore, influenza virus hemagglutinin-specific T-cell receptor transgenic mice further expressing the hemagglutinin specifically in the liver spontaneously developed chronic autoimmune-mediated hepatitis [14]. However, in these models, the roles of TNF- α in the development of hepatitis are not clear.

Concanavalin A (Con A)-induced acute hepatic injury, associated with activation of NKT cells and T cells, is considered to be an experimental model of human AIH [15]. A single intravenous injection of Con A into mice rapidly induces injury of hepatocytes together with increased serum levels of TNF- α [16]. Although one report demonstrated that neutralization of TNF- α by anti-TNF- α significantly suppressed Con A-induced hepatic injury, Tagawa et al. showed that hepatocytes in TNF- α -deficient mice were severely injured by Con A to levels similar to wild-type mice [17]. Thus, an essential role of TNF- α in inducing Con A-induced acute hepatic injury is still controversial.

To clarify mechanisms involved in the development of AIH, we recently developed a new mouse model of fatal AIH [18,19]. Neither programmed cell death 1-deficient mice (PD-1^{-/-} mice) nor BALB/c mice thymectomized three days after birth (NTx mice), with severely reduced numbers of naturally arising Foxp3⁺ regulatory T cells in periphery, developed inflammation of the liver. However, PD-1^{-/-} BALB/c mice with neonatal thymectomy (NTx-PD-1^{-/-} mice) develop fatal AIH characterized by CD4⁺ and CD8⁺ T-cell infiltration, with massive lobular necrosis. Because of the massive destruction of the parenchyma of the liver, these mice start to die as early as two weeks of age, with most dying by four weeks. Notably, the hepatitis in NTx-PD-1^{-/-}

mice was characterized by hyper-gammaglobulinemia and huge production of anti-nuclear antibody, both diagnostic hallmarks of AIH patients [18,19].

In this mouse model, both CD4⁺ and CD8⁺ T cells are indispensable for the development of fatal AIH [18,19]. CD8⁺ T cells are crucially involved in progression to fatal hepatic damage, whereas CD4⁺ T cells are responsible inducing fatal AIH. Initial activation of CD4⁺ T cells occurs in the spleen. In the induction phase of AIH, splenic CD4⁺ T cells were localized in B-cell follicles with huge germinal centers (GCs) and showed the Bcl6⁺ICOS⁺IL-21⁺IL-21R⁺ follicular helper T (T_{FH}) cell phenotype [19]. IL-21 produced by T_{FH} cells has been shown to drive CD8⁺ T-cell activation [19]. Splenic T_{FH} cells and activated CD8⁺ T cells expressed CCR6, and CCL20 expression was elevated in the liver. CCR6-CCL20 axis-dependent migration of splenic T cells is crucial to induce AIH in NTx-PD-1^{-/-} mice [19]. However, it is not known how hepatic CCL20 expression is upregulated in the induction phase of AIH.

AIH-bearing NTx-PD-1^{-/-} mice at three weeks old showed markedly increased levels of TNF- α in the serum [18]. However, it is not clear whether TNF- α is essential in the development of fatal AIH in this mouse model. This study aimed to identify the roles of TNF- α in the development of fatal AIH. We found that serum levels of TNF- α were markedly elevated from the induction phase and that administration of anti-TNF- α prevented induction of AIH, but treatment by anti-TNF- α after the induction did not significantly suppress the progression of fatal hepatic inflammation. Administering anti-TNF- α did not prevent splenic T cell activation in the induction phase of AIH, but suppressed CCL20 expression in the hepatocytes. In contrast, administering anti-CCL20 suppressed fatal AIH but not elevated serum levels of TNF- α . In addition, we found that stimulation by recombinant TNF- α (rTNF- α) upregulated CCL20 expression in

hepatocytes *in vivo* and *ex vivo*. These findings suggest that TNF- α is critically involved in the induction of fatal AIH in mice through upregulation of hepatic CCL20 expression.

2. Materials and methods

2.1. 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 [20]. These mice were bred and housed under specific pathogen-free conditions. Thymectomy and splenectomy of the mice three days after birth were performed as described previously [18, 19]. All mouse protocols were approved by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

2.2. *In vivo* neutralization of cytokines and depletion of T cell subsets, and injection of cytokines

NTx-PD-1^{-/-} mice were injected intraperitoneally every week from day 3 or day 17 after birth with 100 µg of neutralizing monoclonal antibodies (mAbs) to mouse TNF-α (eBioscience, San Diego, CA), or mouse CCL20 (R&D Systems, Minneapolis, MN), and isotype control Abs. After injections, mice at indicated weeks of age were sacrificed, and the livers, spleens, and sera were harvested. For depletion of CD4⁺ T cells *in vivo*, anti-mouse CD4 from eBioscience was used. After two injections of anti-CD4, flow cytometric analysis was performed. Viable CD4⁺ T cell numbers were calculated as follows: (the percentage of CD3⁺CD4⁺ cells in viable cells) x (the number of viable cells). Numbers of CD4⁺ T cells in the spleen were reduced less than 5% compared with those in mice without treatment. On the other hand, PD-1^{-/-} mice at four weeks age were injected intraperitoneally with 10 µg/kg of mouse rTNF-α (eBioscience) or PBS. Before and after 1, 2, 3, 6, or 9 hours following injections, mice

were sacrificed, and their livers and sera were harvested. For immunostaining with anti-CCL20, four-week-old PD-1^{-/-} mice were injected with rTNF- α or PBS four times every three hours. Three hours after the last injection, mice were sacrificed.

2.3. Histological and Immunohistological 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 [18, 19], using FITC-conjugated anti-CD4, and anti-CD8 α (eBioscience), peanut agglutinin (PNA, Vector Laboratories, Burlingame, CA), biotin-labeled anti-B220 (BD Biosciences, San Jose, CA) followed by Texas red-conjugated avidin (Vector Laboratories). For CCL20 staining, rabbit polyclonal antibodies to CCL20/MIP3- α (Abcam, Cambridge, UK) were used, followed by FITC-conjugated goat anti-rabbit immunoglobulin (BD Biosciences). The diameter of GC and the number of CD4⁺ T cells within GC of the spleen were determined in several high-power fields of the spleen in at least 3 sections from each mouse.

2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

Concentrations of TNF- α in sera and CCL20 in culture supernatants were measured by using mouse TNF- α ELISA set (eBioscience) and CCL20/MIP-3 α set (R&D Systems) according to the manufacturer's protocols, respectively.

2.5. Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

Liver tissues were frozen in RNAlater (Qiagen, Hilden, Germany). Real-time quantitative RT-PCR was performed as described previously [19]. Values are expressed as arbitrary units relative to glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The following primers were used: *GAPDH*: 5'-CAACTTTGTCAAGCTCATTTC-3' and 5'-GGTCCAGGGTTTCTTACTCC-3'; *CCL20*: 5'-ATGGCCTGCGGTGGCAAGCGTCTG-3' and 5'-TAGGCTGAGGAGGTTACAGCCCT-3'; *CCL25*: 5'-GAGTGCCACCCTAGGTCATC-3' and 5'-CCAGCTGGTGCTTACTCTGA-3'

2.6. Flow cytometry analysis

Single-cell suspensions of mononuclear cells from spleens were prepared as described previously [18, 19]. For intracellular cytokine staining, cells were restimulated with 50 ng/ml phorbol myristate acetate (Sigma, St. Louis, MO) + 2 µg/ml ionomycin (Sigma) at a concentration of 1×10^6 cells/ml in RPMI Medium 1640 (Invitrogen, Eugene, OR), supplemented with 10% fetal bovine serum (Sigma), penicillin G, and streptomycin (Invitrogen), and 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan). After 2 h culture with 10 µg/ml brefeldin A (Sigma), cells were collected and stained for cell-surface molecules using PE-anti-CD3 ϵ and APC-Cy7-anti-CD4 (eBioscience) or APC-anti-CD8 α . Cells were fixed and permeabilized, using a Fix and Perm Cell PermeabilizationTM Kit (Caltag Laboratories, An Der Grub, Austria), and stained with FITC-anti-IFN- γ (eBioscience). For Ki-67 antigen staining, a FITC-conjugated Ab set (BD Bioscience) was used with PE-anti-CD3 ϵ and APC-Cy7-anti-CD4 or APC-anti-CD8 α . For CD44 staining, cells were stained with FITC-anti-CD4 or

-anti-CD8 α and PE-anti-CD44, and PerCP-Cy5.5-conjugated Anti-CD3 ϵ (all from eBioscience). Stained cells were analyzed with a FACSCanto™ II (BD Biosciences). Data were analyzed using Cell Quest Pro™ (BD Biosciences). Dead cells were excluded on the basis of side- and forward-scatter characteristics.

2.7. Primary Hepatocyte culture

Primary hepatocytes were obtained from adult PD-1^{-/-} mice using the two-step collagenase perfusion method, followed by centrifugation to collect the mature hepatocytes and cultured as described previously [21, 22]. Hepatocytes were resuspended in D-MEM/Ham's F-12 supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The cells seeded on a 24-well microplate with collagen type 1 coating at a density of 1×10^5 cells/cm² and cultured for 72 hours with or without 10 or 20 ng/ml of rTNF- α .

2.8. Statistical analysis

The data are presented as the mean values \pm standard deviations. Statistical analysis was performed by the Student *t* test for unpairwise comparisons. Survival rates were estimated by the Kaplan-Meier method and compared with the log-rank test. *P*-values below .05 were considered significant.

3. Results

3.1. Production of TNF- α increases in the induction phase of AIH, and neutralizing TNF- α suppresses the development of AIH in NTx-PD-1^{-/-} mice

Previously, we reported that in three-week-old NTx-PD-1^{-/-} mice with severe AIH, serum levels of TNF- α significantly increased [18]. In this study, we performed a time-course study on serum levels of TNF- α from one to three weeks of age. In 1.5-week-old NTx-PD-1^{-/-} mice, the serum level of TNF- α was significantly higher than in PD-1^{-/-} mice (Fig. 1A). The elevated serum level of TNF- α further increased in two-week-old NTx-PD-1^{-/-} mice, at a level similar to NTx-PD-1^{-/-} mice with severe AIH at three weeks old. These data suggest that TNF- α may play important roles in the process of AIH development.

Next, we examined whether neutralization of TNF- α can suppress the development of fatal AIH. NTx-PD-1^{-/-} mice at one day after thymectomy were injected intraperitoneally every week with 100 μ g of neutralizing mAb to mouse TNF- α or the isotype control. We found that neutralization of TNF- α showed a significantly increased survival rate (Fig. 1B). After four injections, mice at four weeks of age were sacrificed, and their livers and sera were harvested. Four-week-old NTx-PD-1^{-/-} mice injected with the isotype control developed severe mononuclear cell infiltration in the liver and a massive degeneration of hepatocytes (Fig. 1C left). However, neutralization of TNF- α suppressed mononuclear cell infiltration in the liver and degeneration of hepatocytes (Fig. 1C right). In addition, neutralization of TNF- α showed significantly decreased serum concentrations of aspartate aminotransferase and alanine aminotransferase, and total bilirubin at four weeks of age (Fig. 1D). These data

indicate that neutralization of TNF- α suppressed the development of fatal AIH in NTx-PD-1^{-/-} mice, suggesting an essential role for TNF- α in the development of AIH.

3.2. Neutralization of TNF- α inhibits the infiltration of T cells into the liver in the induction phase of AIH, whereas it does not suppress the progression of fatal AIH after the development of hepatitis

Next, we examined whether neutralizing TNF- α can suppress the infiltration of CD4⁺ and CD8⁺ T cells in two-week-old NTx-PD-1^{-/-} mice at the induction phase of AIH. After two injections of neutralizing anti-TNF- α , NTx-PD-1^{-/-} mice at two weeks of age were sacrificed, and their livers were harvested. Two-week-old NTx-PD-1^{-/-} mice injected with anti-TNF- α did not show any mononuclear cell infiltrations in the portal area or an increased number of CD4⁺ and CD8⁺ T cells in the liver, suggesting an essential role for TNF- α in the induction of AIH (Fig. 2A).

In NTx-PD-1^{-/-} mice from two to three weeks of age, mononuclear cell infiltrations in the liver rapidly progressed and were followed by massive destruction of the parenchyma of the liver [18]. To investigate whether neutralization of TNF- α can suppress progression of fatal AIH even after the development of AIH, NTx-PD-1^{-/-} mice at 14 days after thymectomy were injected every week with anti-TNF- α . We found that the neutralizing TNF- α did not significantly increase the survival rate (Fig. 2B). After the second injection, mice at four weeks of age were sacrificed, and we confirmed that the neutralizing TNF- α did not reduce a massive degeneration of hepatocytes or severe CD4⁺ and CD8⁺ cell infiltration in the liver (Fig. 2C). Thus, neutralization of TNF- α after the development of hepatitis did not significantly

suppress progression of fatal AIH.

3.3. Increased TNF- α production in the induction phase of AIH depends on activation of CD4⁺ T cells in the spleen

Previously, we reported that in NTx-PD-1^{-/-} mice, CD4⁺ T cells are responsible for induction of fatal AIH and initial activation of responsible CD4⁺ T cells occurs in the spleen [18,19]. Indeed, neonatal splenectomy suppressed elevated serum levels of TNF- α in two-week-old NTx-PD-1^{-/-} mice (Fig. 3A). In addition, *in vivo* depletion of CD4⁺ T cells suppressed elevated serum levels of TNF- α in two-week-old mice (Fig. 3B). These data indicate that increased TNF- α production in the induction phase of AIH depends on activation of CD4⁺ T cells in the spleen.

3.4. Neither T_{FH}-cell differentiation nor activation of CD8⁺ T cells in the spleen is suppressed by neutralization of TNF- α in the induction phase of fatal AIH

In this mouse model, initially activated CD4⁺ T cells showing T_{FH} cell phenotype were localized in B-cell follicles with huge GCs in the spleen [19]. In this study, we looked at CD4⁺ and CD8⁺ T cells in the spleen of two-week-old NTx-PD-1^{-/-} mice treated with anti-TNF- α , finding that splenic CD4⁺ and CD8⁺ T cells treated with anti-TNF- α showed Ki-67^{high} activated T-cell phenotype with high proliferation potential at levels similar to those in mice treated with isotype (Fig. 3C left). In addition, expression levels of IFN- γ in these splenic T cells of two-week-old NTx-PD-1^{-/-} mice treated with anti-TNF- α were similar to those in mice treated with isotype (Fig. 3C right). When we looked at expression levels of CD44 in T cells,

CD44^{high} cells in the CD4⁺ T-cell populations in mice treated with anti-TNF- α were similar to those in mice treated with isotype (Fig. 3D). However, CD44^{high} cells in the CD8⁺ T-cell populations had increased, suggesting further accumulation of activated CD8⁺ T cells in the spleen. Moreover, when we looked at B-cell follicles in the spleen of four-week-old NTx-PD-1^{-/-} mice injected with or without neutralizing anti-TNF- α , anti-TNF- α did not alter the number of CD4⁺ T cells within B220⁺ follicles or the size of PNA⁺ GC in B220⁺ follicles (Supplementary Figure 1A and 1B). These data suggest that neither T_{FH}-cell differentiation nor activation of CD8⁺ T cells in the spleen was suppressed by neutralization of TNF- α in NTx-PD-1^{-/-} mice, implying that neutralization of TNF- α predominantly affects the migration step but not the activation of those T cells in the induction phase of fatal AIH.

3.5. Neutralization of TNF- α suppresses upregulated expression of hepatic CCL20

Previously, we reported that CCR6-CCL20 axis-dependent migration of splenic T cells is crucial to induce AIH in NTx-PD-1^{-/-} mice [19]. In this study, we showed that serum levels of TNF- α were elevated from 1.5 to three weeks of age in NTx-PD-1^{-/-} mice (Fig. 1A). In parallel with this observation, expressions of hepatic CCL20 were upregulated at 1.5 to three weeks of age (Fig. 4A). Therefore, we examined whether TNF- α is essential in the upregulated expression of hepatic CCL20 in NTx-PD-1^{-/-} mice. After two injections of anti-TNF- α into NTx-PD-1^{-/-} mice at two weeks of age at the induction phase of AIH, neutralizing TNF- α significantly suppressed mRNA expression of CCL20 in the liver (Fig. 4B left). After four

injections of anti-TNF- α , decreased hepatic CCL20 expression was sustained in four-week-old NTx-PD-1^{-/-} mice (Fig. 4B right). These data were further confirmed by immunohistology. Hepatocytes in four-week-old NTx-PD-1^{-/-} mice with injection of the isotype control revealed CCL20 staining, whereas after four injections of anti-TNF- α , continuous neutralization of TNF- α suppressed CCL20 staining in hepatocytes (Fig. 4C). Thus, neutralization of TNF- α suppressed upregulated expression of hepatic CCL20.

3.6. Administering anti-CCL20 suppressed the development of fatal AIH but did not alter elevated serum levels of TNF- α

Conversely, we examine whether administration of anti-CCL20 alters elevated serum levels of TNF- α . Administration of anti-CCL20 suppressed mononuclear cell infiltration and destruction of organ structure in the liver at four weeks of age and showed a significantly increased survival rate in NTx-PD-1^{-/-} mice (Fig. 4D and 4E). 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 $\mu\text{g}/\text{kg}$ 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, two hours 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, two hours 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 $\mu\text{g}/\text{kg}$ of rTNF- α four times every 3 hours into four-week-old PD-1^{-/-} mice, hepatocytes from these mice at 3 hours 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 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.

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- κ B 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- κ B 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 *Propionibacterium acnes* priming plus LPS challenge [25]. In addition, we showed that rTNF- α injection induced CCL20 expression in the liver 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 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 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 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 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 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, 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 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.

Acknowledgments

We thank Dr. Dovie Wylie for assistance in preparation of the manuscript; Ms. Chigusa Tanaka for excellent technical assistance; Drs. Tasuku Honjo, Shuh Narumiya, Nagahiro Minato, Shimon Sakaguchi, Takeshi Watanabe, and Ichiro Aramori for critical discussion and suggestions. *Funding:* The Center for Innovation in Immunoregulative Technology and Therapeutics is supported in part by the Special Coordination Funds for Promoting Science and Technology of the Japanese Government and in part by Astellas Pharma Inc. in the Formation of Innovation Center for Fusion of Advanced Technologies Program. This work is partially supported by Grants-in-aid for Scientific Research 21229009 and 23590973 from Japan Society for the Promotion of Science (JSPS), a Health and Labour Sciences Research Grant for Research on Intractable Diseases, and Research on Hepatitis from the Ministry of Health, Labour and Welfare, Japan, Grants-in-Aid for Research by The Kato Memorial Trust for Nambyo Research, and The Waksman Foundation of Japan.

References

- [1] CF. Ware, Network communications: lymphotoxins, LIGHT, and TNF, *Annu. Rev. Immunol.* 23 (2005) 787-819.
- [2] M. Wong, D. Ziring, Y. Korin, S. Desai, S. Kim, J. Lin, D. Gjertson, J. Braun, E. Reed, R.R. Singh, TNF alpha blockade in human diseases: mechanisms and future directions, *Clin. Immunol.* 126 (2008) 121-136.
- [3] J. Lin, D. Ziring, S. Desai, S. Kim, M. Wong, Y. Korin, J. Braun, E. Reed, D.Gjertson, R.R. Singh, TNF alpha blockade in human diseases: an overview of efficacy and safety, *Clin. Immunol.* 126 (2008) 13-30.
- [4] D. Tracey, L. Klareskog, E.H. Sasso, J.G. Salfeld, P.P. Tak, Tumor necrosis factor antagonist mechanisms of action: a comprehensive review, *Pharmacol. Ther.* 117 (2008) 244-279.
- [5] R.F. Schwabe, D.A. Brenner, Mechanisms of Liver Injury. I. TNF- α -induced liver injury: role of IKK, JNK, and ROS pathways, *Am. J. Physiol. Gastrointest. Liver Physiol.* 290 (2006) G583-589.
- [6] G. Maggiore, F. De Benedetti, M. Massa, P. Pignatti, A. Martini, Circulating levels of interleukin-6, interleukin-8, and tumor necrosis factor-alpha in children with autoimmune hepatitis, *J. Pediatr. Gastroenterol. Nutr.* 20 (1995) 23-27.
- [7] A.J. Czaja, S. Cookson, P.K. Constantini, M. Clare, J.A. Underhill, P.T. Donaldson, Cytokine polymorphisms associated with clinical features and treatment outcome in type 1 autoimmunehepatitis, *Gastroenterology* 117 (1999) 645–652.

- [8] S. Cookson, P.K. Constantini, M. Clare, J.A. Underhill, W. Bernal, A.J. Czaja, P.T. Donaldson, Frequency and nature of cytokine gene polymorphisms in type 1 autoimmune hepatitis, *Hepatology* 30 (1999) 851–856.
- [9] J.D. Gorham, J.T. Lin, J.L. Sung, L.A. Rudner, M.A. French, Genetic regulation of autoimmune disease: BALB/c background TGF-beta 1-deficient mice develop necroinflammatory IFN-gamma-dependent hepatitis, *J. Immunol.* 166 (2001) 6413-6422.
- [10] R.T. Robinson, J. Wang, J.G. Cripps, M.W. Milks, K.A. English, T.A. Pearson, J.D. Gorham, End-organ damage in a mouse model of fulminant liver inflammation requires CD4⁺ T cell production of IFN- γ but is independent of Fas, *J. Immunol.* 182 (2009) 3278-3284.
- [11] Y. Oya, N. Watanabe, T. Owada, M. Oki, K. Hirose, A. Suto, S. Kagami, H. Nakajima, T. Kishimoto, I. Iwamoto, T.L. Murphy, K.M. Murphy, Y. Saito, Development of autoimmune hepatitis-like disease and production of autoantibodies to nuclear antigens in mice lacking B and T lymphocyte attenuator, *Arthritis Rheum.* 58 (2008) 2498-2510.
- [12] M. Holdener, E. Hintermann, M. Bayer, A. Rhode, E. Rodrigo, G. Hintereder, E.F. Johnson, F.J. Gonzalez, J. Pfeilschifter, M.P. Manns, M.G. Herrath, U. Christen, Breaking tolerance to the natural human liver autoantigen cytochrome P450 2D6 by virus infection, *J. Exp. Med.* 205 (2008) 1409-1422.
- [13] S. Tamaki, S. Homma, Y. Enomoto, H. Komita, M.Zeniya, T. Ohno, G. Toda, Autoimmune hepatic inflammation by vaccination of mice with dendritic cells loaded with well-differentiated hepatocellular carcinoma cells and administration of interleukin-12, *Clin. Immunol.* 117 (2005) 280-293.

- [14] M. Zierden, E. Kühnen, M. Odenthal, H.P. Dienes, Effects and regulation of autoreactive CD8⁺ T cells in a transgenic mouse model of autoimmune hepatitis, *Gastroenterology* 139 (2010) 975-986.
- [15] G. Tiegs, J. Hentschel, A. Wendel, A T cell-dependent experimental liver injury in mice inducible by concanavalin A, *J. Clin. Invest.* 90 (1992) 196-203.
- [16] H. Mizuhara, E. O'Neill, N. Seki, T. Ogawa, C. Kusunoki, K. Otsuka, S. Satoh, M. Niwa, H. Senoh, H. Fujiwara, T cell activation-associated hepatic injury: mediation by tumor necrosis factors and protection by interleukin 6, *J. Exp. Med.* 179 (1994) 1529–1537.
- [17] Y. Tagawa, K. Sekikawa, Y. Iwakura, Suppression of concanavalin A-induced hepatitis in IFN- γ (-/-) mice, but not in TNF- α (-/-) mice: role for IFN- γ in activating apoptosis of hepatocytes, *J. Immunol.* 159 (1997) 1418-1428.
- [18] M. Kido, N. Watanabe, T. Okazaki, T. Akamatsu, J. Tanaka, K. Saga, A. Nishio, T. Honjo, T. Chiba, Fatal autoimmune hepatitis induced by concurrent loss of naturally arising regulatory T cells and PD-1-mediated signaling, *Gastroenterology* 135 (2008) 1333-1343.
- [19] N. Aoki, M. Kido, S. Iwamoto, H. Nishiura, R. Maruoka, J. Tanaka, T. Watanabe, Y. Tanaka, T. Okazaki, T. Chiba, N. Watanabe, Dysregulated generation of follicular helper T cells in the spleen triggers fatal autoimmune hepatitis in mice, *Gastroenterology* 140 (2011) 1322-1333.
- [20] H. Nishimura, T. Okazaki, Y. Tanaka, K. Nakatani, M. Hara, A. Matsumori, S. Sasayama, A. Mizoguchi, H. Hiai, N. Minato, T. Honjo, Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice, *Science* 291 (2001) 319-322.

- [21] P.O. Seglen, Preparation of isolated rat liver cells, *Methods. Cell Biol.* 13 (1976) 29-83.
- [22] M. Fujiwara, H. Marusawa, H.Q. Wang, A. Iwai, K. Ikeuchi, Y. Imai, A. Kataoka, N. Nukina, R. Takahashi, T. Chiba, Parkin as a tumor suppressor gene for hepatocellular carcinoma, *Oncogene* 27 (2008) 6002-6011.
- [23] D.L. Rossi, A.P. Vicari, K. Franz-Bacon, T.K. McClanahan, A. Zlotnik, Identification through bioinformatics of two new macrophage proinflammatory human chemokines: MIP-3 α and MIP-3 β , *J. Immunol.* 158 (1997) 1033-1036.
- [24] A. Reboldi, C. Coisne, D. Baumjohann, F. Benvenuto, D. Bottinelli, S. Lira, A. Uccelli, A. Lanzavecchia, B. Engelhardt, F. Sallusto, C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE, *Nat. Immunol.* 10 (2009) 514-523.
- [25] S. Sugita, T. Kohno, K. Yamamoto, Y. Imaizumi, H. Nakajima, T. Ishimaru, T. Matsuyama, Induction of macrophage-inflammatory protein-3 α gene expression by TNF-dependent NF-kappaB activation, *J. Immunol.* 168 (2002) 5621-5628.
- [26] Y. Shimizu, H. Murata, Y. Kashii, K. Hirano, H. Kunitani, K. Higuchi, A. Watanabe, CC-chemokine receptor 6 and its ligand macrophage inflammatory protein 3 α might be involved in the amplification of local necroinflammatory response in the liver, *Hepatology.* 34 (2001) 311-319.
- [27] E. Roth, H. Pircher, IFN- γ promotes Fas ligand- and Perforin- mediated liver cell destruction by cytotoxic CD8 T cells, *J. Immunol.* 172 (2004) 1588-1594.
- [28] I.A. Rowe, K. Webb, B.K. Gunson, N. Mehta, S. Hague, J. Neuberger, The impact of disease recurrence on graft survival following liver transplantation: a

- single centre experience, *Transpl. Int.* 21 (2008) 459-465.
- [29] C. Schramm, M. Bubenheim, R. Adam, V. Karam, J. Buckels, J.G. O'Grady, N. Jamieson, S. Pollard, P. Neuhaus, M.M. Manns, R. Porte, D. Castaing, A. Paul, O. Traynor, J. Garden, S. Friman, B.G. Ericzon, L. Fischer, S. Vitko, M. Krawczyk, H.J. Metselaar, A. Foss, M. Kilic, K. Rolles, P. Burra, X. Rogiers, A.W. Lohse, Primary liver transplantation for autoimmune hepatitis: a comparative analysis of the European Liver Transplant Registry, *Liver. Transpl.* 16 (2010) 461-469.
- [30] C. Weiler-Normann, C. Wiegard, C. Schramm, A.W. Lohse, A case of difficult-to-treat autoimmune hepatitis successfully managed by TNF-alpha blockade, *Am. J. Gastroenterol.* 104 (2009) 2877-2878.
- [31] M. Ramos-Casals, Roberto-Perez-Alvarez, C. Diaz-Lagares, M.J. Cuadrado, M.A. Khamashta; BIOGEAS Study Group, Autoimmune diseases induced by biological agents A double-edged sword?, *Autoimmun Rev.* 9 (2010) 188-193.
- [32] M. Cravo, R. Silva, M. Serrano, Autoimmune hepatitis induced by infliximab in a patient with Crohn's disease with no relapse after switching to adalimumab, *BioDrugs.* 24 (2010) Suppl 1:25-27.
- [33] C.J. Del Prete, N.S. Cohen, A Case of Rituximab-induced hepatitis, *Cancer Biother Radiopharm.* 25 (2010) 747-748.

Figure legends

Figure 1 Production of TNF- α increases in the induction phase of fatal AIH, and neutralization of TNF- α suppresses its development in NTx-PD-1^{-/-} mice. (A) Serum TNF- α levels of indicated mice were measured by ELISA. Data are shown as the mean of at least five mice. (B-D) NTx-PD-1^{-/-} mice at one day after thymectomy were injected intraperitoneally every week with 100 μ g 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 control (dotted line, n=10) at six injections *; $P < 0.05$ (B). After four injections, mice at four weeks of age were sacrificed, and the liver and serum were harvested. Staining of the liver for hematoxylin and eosin. All *scale bars*, 100 μ m (C). Serum levels of the liver transaminases (AST and ALT) and total bilirubin. Sera from indicated mice were measured. Data are shown as the mean of at least four mice. Error bars represent SD. *; $P < 0.05$ (D).

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 μ g 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 μ m. (B and C) NTx-PD-1^{-/-} mice at 14 days after thymectomy were injected intraperitoneally every week with 100 μ g 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 μ m (C).

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) NTx-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 μ g 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 μ m (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 μ m (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 $\mu\text{g}/\text{kg}$ 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 two hours 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 three hours after the last injection. All scale bars, 50 μm (C). Hepatocytes were isolated from PD-1^{-/-} mice and cultured for 72 hours 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).

Figure 1

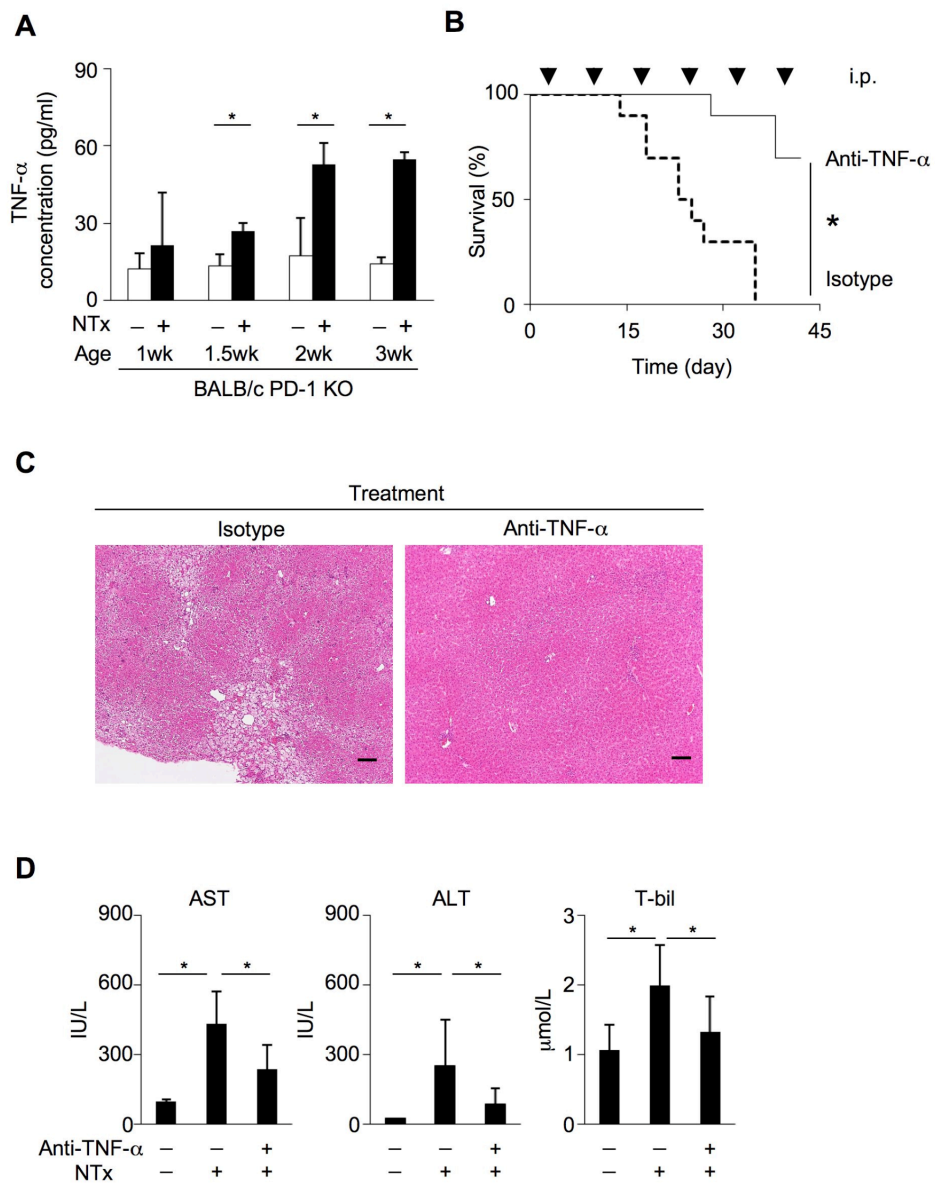


Figure 2

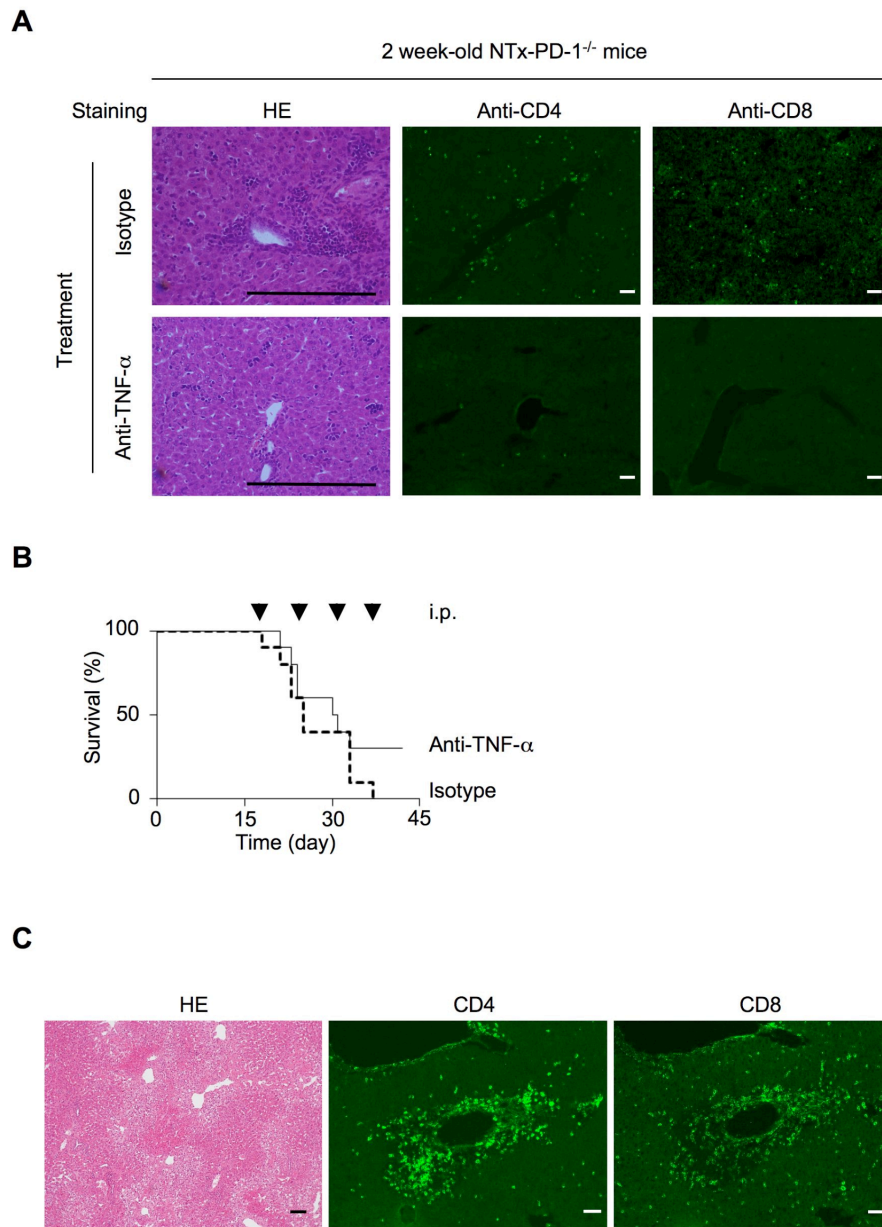


Figure 3

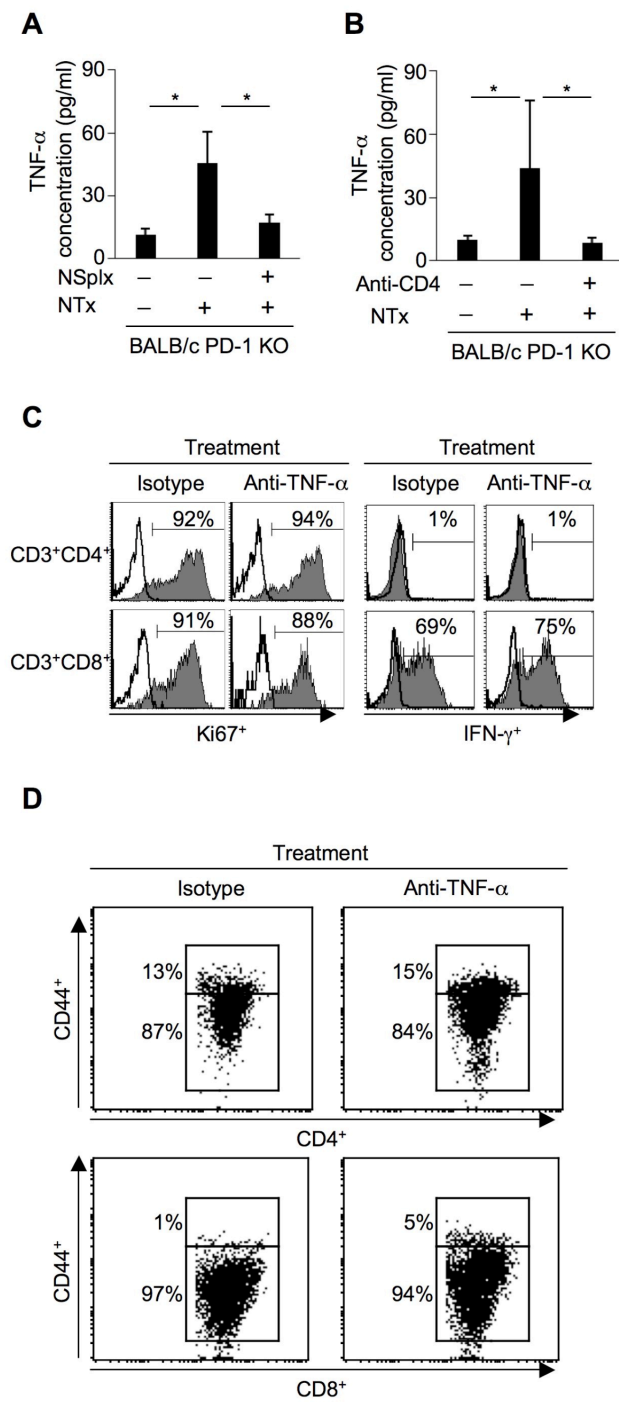


Figure 4

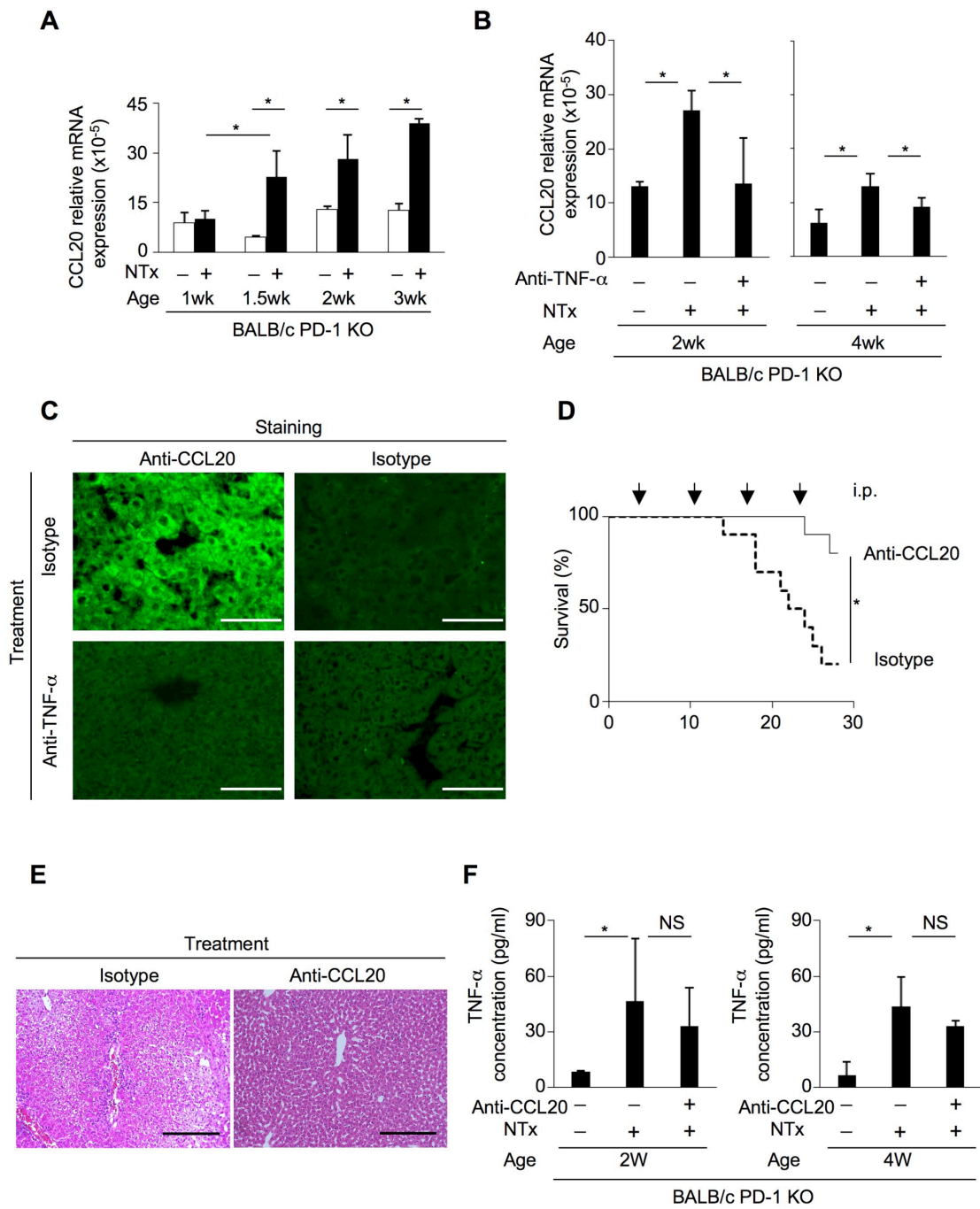
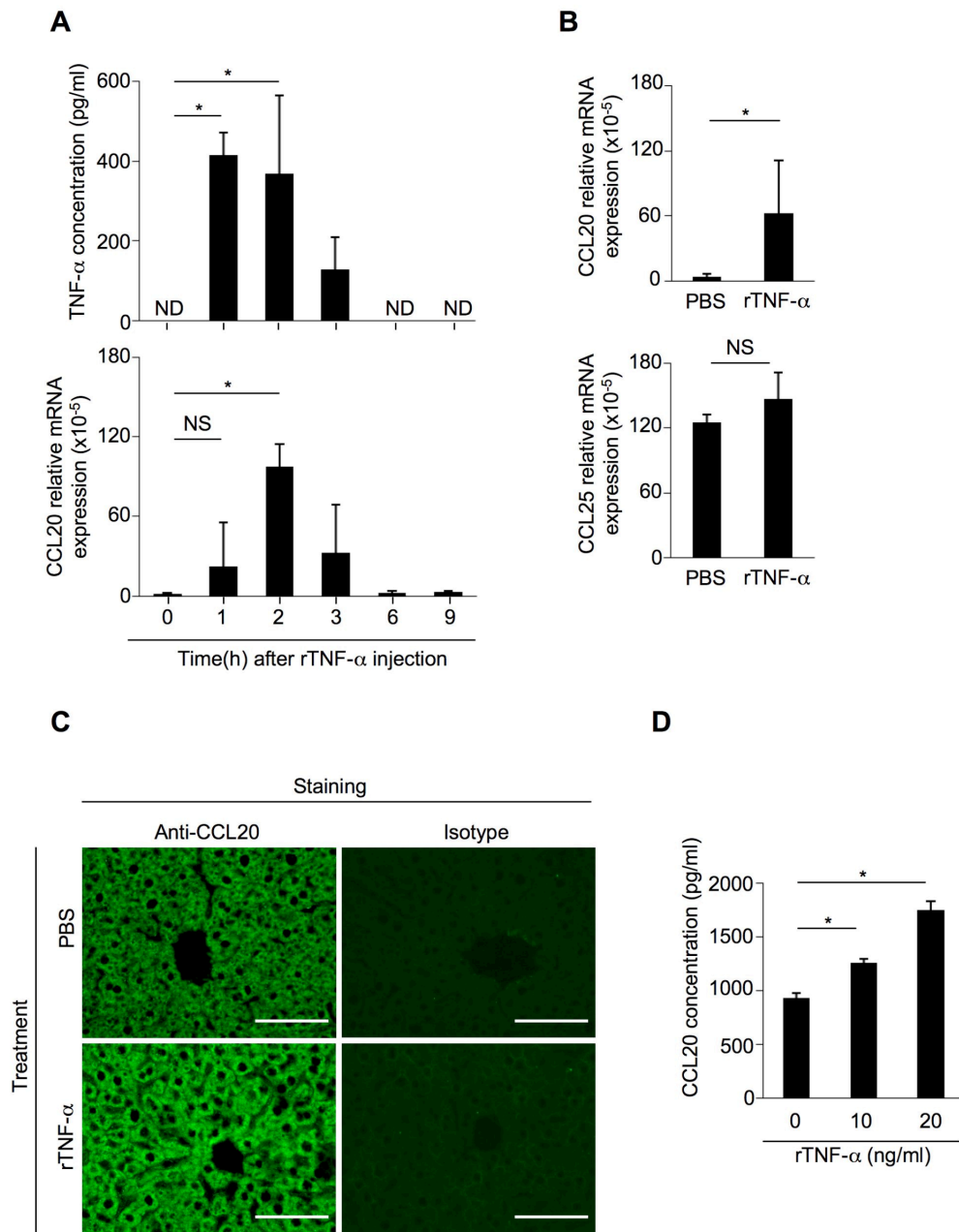
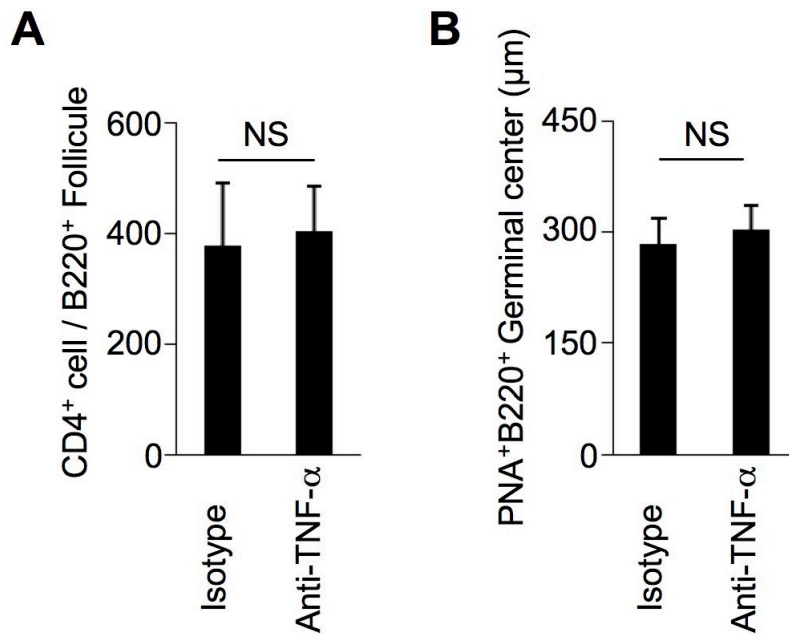


Figure 5



Supplemental Materials:**Supplementary Figure 1**

Anti-TNF- α does not alter the number of CD4⁺ T cells within B220⁺ follicles or the size of PNA⁺ GC in B220⁺ follicles. The spleens from indicated mice were stained with FITC-conjugated anti-CD4, or PNA and biotin-labeled anti-B220 followed by Texas red-conjugated avidin. CD4⁺ T cell numbers (A) and diameters of PNA⁺ GC (B) in the B220⁺ follicles of the spleen were determined in several high-power fields in at least 3 sections of each mouse. Bars indicate the mean of each group. Horizontal short bars indicate the SD.

