

# Progression of Autoimmune Hepatitis is Mediated by IL-18-Producing Dendritic Cells and Hepatic CXCL9 Expression in Mice

Aki Ikeda<sup>1,2,#</sup>, Nobuhiro Aoki<sup>1,2,#</sup>, Masahiro Kido<sup>1,2,#</sup>, Satoru Iwamoto<sup>1,2</sup>, Hisayo Nishiura<sup>1,2</sup>, Ryutaro Maruoka<sup>1,2</sup>, Tsutomu Chiba<sup>2</sup>, and Norihiko Watanabe<sup>1,2,\*</sup>

<sup>1</sup>Center for Innovation in Immunoregulative Technology and Therapeutics, and <sup>2</sup>Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan

# These authors contributed equally to this work.

Keywords: autoimmune hepatitis; fatal progression; IL-18; dendritic cell; CXCR3-CXCL9 axis

\* Correspondence: Norihiko Watanabe, Address: Yoshidakonoe-cho Sakyo-ku Kyoto city, Kyoto 606-8501, Japan, E-mail: norihiko@kuhp.kyoto-u.ac.jp, Tel: +81-75-751-4319, Fax: +81-75-751-4303

Abbreviations: AIH, autoimmune hepatitis; ANA, anti-nuclear antibody; DCs, dendritic cells; GC, germinal center; ICOS, inducible costimulator; IL-21R, IL-21 receptor; NTx, neonatal thymectomy; NTx-PD-1<sup>-/-</sup> mice, PD-1-deficient BALB/c mice thymectomized three days after birth; PD-1, programmed cell death 1; PNA, peanut agglutinin; T<sub>FH</sub>, follicular helper T; Tregs, regulatory T cells

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## Abstract

Clinical manifestations of autoimmune hepatitis (AIH) range from mild chronic to acute, sometimes fulminant hepatitis. However, it is unknown how the progression to fatal hepatitis occurs. We developed a mouse model of fatal AIH by inducing a concurrent loss of Foxp3<sup>+</sup> regulatory T cells and PD-1-mediated signaling. In this model, dysregulated follicular helper T cells in the spleen are responsible for the induction, and the CCR6-CCL20 axis is crucial for the migration of these T cells into the liver. Using this fatal AIH model, we aimed to clarify key molecules triggering fatal AIH progression. During progression, T-bet together with IFN- $\gamma$  and CXCR3 were highly expressed in the inflamed liver, suggesting Th1-type inflammation. T cells that dominantly expanded in the spleen and the inflamed liver were CXCR3-expressing CD8<sup>+</sup> T cells; depletion of these CD8<sup>+</sup> T cells suppressed AIH progression. Expression of one CXCR3 ligand, CXCL9, was elevated in the liver. CXCL9 expressing macrophages/Kupffer cells were co-localized with infiltrating T cells, and *in vivo* administration of anti-CXCL9 suppressed AIH progression. In addition, serum levels of IL-18 but not IL-1 $\beta$  were elevated during progression, and dendritic cells in the spleen and liver highly produced IL-18. *In vivo* administration of anti-IL-18R suppressed the increase of splenic CXCR3<sup>+</sup> T cells and the progression to fatal AIH. Moreover, TNF- $\alpha$  but not IFN- $\gamma$  was involved in upregulating CXCL9 in the liver and for increased serum levels of IL-18. *Conclusion:* These data suggest that in our mouse model, fatal progression of AIH is mediated by IL-18-dependent differentiation of T cells into Th1 cells and effector T cells, respectively, and that CXCR3-CXCL9 axis-dependent migration of those T cells is crucial for fatal progression.

Human autoimmune hepatitis (AIH) typically presents as asymptomatic or mild chronic hepatitis. However, presentation as acute severe hepatitis also occurs, and some of these AIH patients manifest liver failure at initial presentation.<sup>1,2</sup> Untreated patients with severe AIH rapidly decline, with a mortality rate of up to 50% from 3 to 5 years following diagnosis.<sup>3</sup> Patients progressing to acute liver failure respond poorly to corticosteroid treatment, some of them needing liver transplantation.<sup>4, 5</sup> In addition, approximately 20 to 30% of patients undergoing liver transplantation for AIH develop features of recurrent disease; in some, recurrent AIH behaves more aggressively, with progression to cirrhosis and graft failure.<sup>6</sup> However, it is unknown how this progression to fatal hepatic damages occurs.

Recently, we developed a mouse model of spontaneous fatal AIH.<sup>7-11</sup> Neither *programmed cell death 1*-deficient mice (*PD-1*<sup>-/-</sup> mice) nor BALB/c mice thymectomized three days after birth (NTx mice) developed any inflammation of the liver. In *PD-1*<sup>-/-</sup> BALB/c mice with neonatal thymectomy (NTx-*PD-1*<sup>-/-</sup> mice), however, immune dysregulation by a concurrent loss of naturally arising Foxp3<sup>+</sup> regulatory T cells (Tregs) and PD-1-mediated signaling induced fatal AIH. Massive destruction of the parenchyma of the liver resulted in most mice dying by four weeks. Fatal AIH in NTx-*PD-1*<sup>-/-</sup> mice was characterized by CD4<sup>+</sup> and CD8<sup>+</sup> T-cell infiltration with massive lobular necrosis in the liver and by hyper-gammaglobulinemia and production of anti-nuclear antibodies (ANA).<sup>7, 8</sup>

In our mouse model of fatal AIH, we identified induction sites, responsible T cell subsets, and key molecules for induction of AIH.<sup>8</sup> The spleen is an induction site for fatal AIH, and splenic CD4<sup>+</sup> T cells were autonomously differentiated into follicular helper T (T<sub>FH</sub>) cells in two-week-old NTx-*PD-1*<sup>-/-</sup> mice. T<sub>FH</sub> cells expressing Bcl6, interleukin (IL)-21, IL-21 receptor, inducible costimulator (ICOS), and CXCR5 comprise a newly defined effector T cell subset that powerfully assists B cells in forming germinal centers (GCs).<sup>12</sup> Indeed, in NTx-*PD-1*<sup>-/-</sup> mice, the dysregulated T<sub>FH</sub> cells promoted hyper-gammaglobulinemia and ANA production. In addition, these T<sub>FH</sub> cells in the spleen directly migrated into the liver via the CCR6-CCL20 axis, triggering induction of fatal AIH.<sup>8</sup>

On the other hand, in the progression phase of AIH in three-week-old NTx-*PD-1*<sup>-/-</sup> mice, infiltrated CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the liver produced large amounts of

inflammatory cytokines, such as IFN- $\gamma$  and TNF- $\alpha$ .<sup>7,8</sup> Therefore, dysregulated T<sub>FH</sub> cells in the induction and Th1 cells with effector CD8<sup>+</sup> T cells in the progression may play their roles at different time points in the development of fatal AIH. In this study, using our mouse model, we examined mechanisms in the progression process to identify key molecules triggering fatal AIH progression. We found that in the progression, CXCR3-expressing Th1 cells and CD8<sup>+</sup> effector T cells infiltrated in the liver, CD8<sup>+</sup> effector T cells triggering the fatal destruction of the liver; that hepatic macrophages/Kupffer cells producing CXCL9 is critical for migration of these T cells; and that DC-derived IL-18 is critical for differentiation of Th1 cells and CD8<sup>+</sup> effector T cells. These data suggest that in this mouse model of AIH, IL-18 and the CXCR3-CXCL9 axis are critical for T-cell differentiation and migration in fatal progression of AIH.

## Materials and Methods

BALB/c mice were purchased from Japan SLC (Shizuoka, Japan), and *PD-1*<sup>-/-</sup> on a BALB/c background were generated as described previously.<sup>13</sup> These mice were bred and housed under specific pathogen-free conditions. Thymectomies were performed as described.<sup>7-11</sup> All mouse protocols were approved by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

All other protocols for histological and immunohistological analysis, real-time quantitative reverse-transcription polymerase chain reactions (RT-PCR), flow cytometry analysis and isolation of single cells, administration of Abs *in vivo*, enzyme-linked immunosorbent assay (ELISA), *in vivo* injection of cytokines, DC coculture, and statistical analysis are detailed in the supplemental materials and methods.

## Results

***T-bet, IFN- $\gamma$ , and CXCR3 Are Highly Upregulated in Inflamed Livers of Three-Week-Old NTx-PD-1<sup>-/-</sup> Mice.*** In our mouse model, AIH induction was started as early as two weeks of age by dysregulated T<sub>FH</sub> cells in the spleen.<sup>7,8</sup> As shown in Fig. 1A, livers in two-week-old NTx-PD-1<sup>-/-</sup> mice showed mononuclear cell infiltrations, predominantly in the portal area, as described.<sup>7</sup> Within seven days of induction, these mononuclear cell infiltrations rapidly progressed and were followed by massive destruction of the parenchyma of the liver (Fig. 1A). To investigate whether cytokines contributed to the severely inflamed livers of these three-week-old NTx-PD-1<sup>-/-</sup> mice, we performed real-time quantitative RT-PCR analysis to measure the expression levels of mRNA encoding T cell lineage-specific transcription factors and various related cytokines. In contrast to expression of Th2 or Th17-related molecules, expression of Th1 lineage-specific transcription factor T-bet, together with IFN- $\gamma$  and TNF- $\alpha$ , were upregulated in inflamed liver tissues of these mice (Fig. 1B). These data suggest that inflammatory cytokines related to Th1-type inflammation may be involved in the fatal progression of AIH. Notably, we found that in the inflamed livers of these mice, mRNA expressions of CXCR3 were highly upregulated along with Th1-related molecules (Fig. 1C). Although AIH induction was mediated by dysregulated T<sub>FH</sub> cells in the spleen, in the progression phase of AIH, Th1-type responses were predominant.

***T Cells Dominantly Expanded in the Inflamed Liver Were CXCR3-Expressing CD8<sup>+</sup> T Cells.*** Next, we monitored T cell numbers of the liver, spleen, and mesenteric lymph nodes in NTx-PD-1<sup>-/-</sup> mice from 1 to 3 weeks old (Fig. 1D). In the AIH progression phase in three-week-old mice, we found that CD8<sup>+</sup> T cells, and to a lesser extent CD4<sup>+</sup> T cells, extensively increased in the liver, as described previously.<sup>7</sup> Notably, the predominant increase of CD8<sup>+</sup> T cells at three weeks was observed only in the liver but not in the spleen or mesenteric lymph nodes (Fig. 1D), implying that CD8<sup>+</sup> T cells had accumulated in the severely inflamed liver. In addition, we analyzed splenic and hepatic CD8<sup>+</sup> T-cell expression of the chemokine receptors CCR6, CCR9, and CXCR3 by flow cytometry. As with CD4<sup>+</sup> T cells in the spleen and liver,<sup>8</sup> splenic and hepatic CD8<sup>+</sup> T cells mainly expressed CXCR3 in three-week-old NTx-PD-1<sup>-/-</sup> mice (Fig. 1E).

***CD8<sup>+</sup> T Cells During the Progression of AIH Were Indispensable for Fatal Destruction of the Liver.*** In a previous study, we showed that in the induction of fatal AIH, CD4<sup>+</sup> T cells are indispensable for recruiting CD8<sup>+</sup> T cells in the liver and that CD8<sup>+</sup> T cells may be major effector T cells, fatally destroying the liver in AIH progression.<sup>8</sup> To examine whether depletion of CD8<sup>+</sup> T cells in the progression is sufficient to suppress fatal liver destruction, AIH-developed NTx-*PD-I*<sup>-/-</sup> mice were injected intraperitoneally at fourteen days after NTx and then once a week with anti-CD8 mAbs *in vivo* (Fig. 2A). After two injections of anti-CD8, the number of CD8<sup>+</sup> T cells in the spleen was greatly reduced (Fig. 2B). Although depleting CD8<sup>+</sup> T cells did not completely suppress hepatic infiltrations of mononuclear cells, the infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was diminished, and fatal progression of AIH was suppressed by the treatment (Fig. 2C-2E). These data suggest that CXCR3-expressing CD8<sup>+</sup> T cells extensively infiltrating the liver are indispensable for fatal progression.

***Production of a CXCR3 Ligand, CXCL9, Was Elevated in the Fatal Progression of AIH.*** CXCR3-expressing T cells can be guided by three ligands — CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC — and expression of these CXCR3 ligands in the inflamed tissues determines inflamed-tissue-specific infiltration of CXCR3-expressing T cells in various immunoinflammatory settings, including autoimmune diseases.<sup>14-17</sup> We performed real-time quantitative RT-PCR analysis to measure the expression levels of mRNA encoding these three CXCR3 ligands. In contrast to non-inflamed livers in the control mice, severely inflamed livers of 3-week-old NTx-*PD-I*<sup>-/-</sup> mice showed markedly elevated gene expression of CXCL9 but not of CXCL10 and CXCL11 (Fig. 3A). In contrast to inflamed livers, no organs except those with inflamed gastric tissues showed a significantly increased level of mRNA expression of CXCL9 (Fig. 3B).

In addition, we confirmed elevated protein expression of CXCL9 only in the inflamed liver but not the stomach by immunohistochemistry (Fig. 3C and supplementary Fig. 1). Furthermore, when we looked at serum concentrations of CXCL9 and CXCL10 at one to four weeks of age, the serum level of CXCL9 but not CXCL10, at three to four weeks of age, was significantly higher than controls (Fig. 3D). These data suggest that CXCL9 plays a key role in the progression of AIH.

***In Fatal Progression of AIH, the CXCR3-CXCL9 Axis Was Crucial for T-cell***

**Migration into the Liver.** To determine whether the axis formed by CXCR3 and its ligands contributes to T-cell migration leading to fatal progression of AIH, NTx-*PD-I*<sup>-/-</sup> mice were injected intraperitoneally at one day after NTx and then once a week with anti-CXCL9 and/or anti-CXCL10 mAbs *in vivo*. After four injections, in contrast to anti-CXCL10 injections, anti-CXCL9 injections induced a significantly higher survival rate (Fig. 4A and 4B). Administering anti-CXCL9 and a combination with anti-CXCL9 and anti-CXCL10, but not anti-CXCL10 alone, greatly reduced infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells into the liver and liver destruction at four weeks (Fig. 4C). These data suggest that in the progression phase of fatal AIH, the CXCR3-CXCL9 axis is crucial for migration of Th1 cells and effector CD8<sup>+</sup> T cells into the liver.

**The Main Cellular Source of CXCL9 Was Hepatic Macrophages/Kupffer Cells in the Progression of AIH.** Next, we examined which cell types express CXCL9 in the inflamed liver by immunohistochemistry. We found that the majority of CXCL9-expressing cells in the inflamed liver were F4/80 antigen positive macrophages/Kupffer cells and that CD4<sup>+</sup> and CD8<sup>+</sup> T cells were co-localized with CXCL9-expressing cells in the inflamed liver (Fig. 5A).

In AIH progression, mRNA expression of IFN- $\gamma$  and TNF- $\alpha$  in the inflamed liver as well as serum levels of these cytokines were markedly elevated<sup>7-10</sup>, and IFN- $\gamma$  mediated the induction of all three CXCR3 ligands: CXCL9, CXCL10, and CXCL11.<sup>14,15</sup> When we injected intraperitoneally with 10 mg/kg of IFN- $\gamma$  and TNF- $\alpha$  in four-week-old *PD-I*<sup>-/-</sup> mice, after two hours, IFN- $\gamma$  and TNF- $\alpha$  significantly upregulated mRNA expression of both CXCL9 and CXCL10 in the liver. Interestingly, we found sustained CXCL9 upregulation by TNF- $\alpha$  (Fig. 5B). Indeed, neutralization of TNF- $\alpha$  but not IFN- $\gamma$  suppressed hepatic CXCL9-expression in four-week-old NTx-*PD-I*<sup>-/-</sup> mice (Fig. 5C).

In NTx-*PD-I*<sup>-/-</sup> mice, TNF- $\alpha$  is essential in the induction of AIH by upregulating hepatic CCL20 expression, allowing TNF- $\alpha$ -producing activated T cells to migrate from the spleen into the liver.<sup>10</sup> In AIH progression, immunohistochemistry for TNF- $\alpha$  revealed TNF- $\alpha$  production in several infiltrating cell types (Fig. 5D, left panels), suggesting that TNF- $\alpha$ -dependent upregulation of CXCL9 expression may be induced by hepatic macrophages/Kupffer cells in autocrine fashion and/or by activated T cells in paracrine fashion. However, after migration of TNF- $\alpha$ -producing activated T cells into



the liver, neutralizing serum levels of TNF- $\alpha$  could not suppress CXCL9 expression in the liver and serum levels of CXCL9 (Fig. 5D right panels and 5E). These data suggest that TNF- $\alpha$  secretion in autocrine and/or paracrine fashion may induce uncontrollable CXCL9 expression in the progression of AIH, resulting in unsuccessful anti-TNF $\alpha$  monotherapy as described.<sup>10</sup>

***Serum Levels of IL-18 Were Elevated in AIH Progression, and In Vivo Administration of Blocking Abs for IL-18R Signaling Suppressed the Development of Fatal AIH.*** IL-12 is decisive in the development of Th1 subsets. A recent study showed that IL-12 can trigger naïve T cells to transitionally differentiate into T cells with features of T<sub>FH</sub> and Th1 cells,<sup>18, 19</sup> However, neutralizing IL-12p40 did not suppress hepatic inflammation as described previously<sup>8</sup> (supplementary Fig. 2). In addition, although IFN- $\gamma$  has been shown to be essential for IL-12 induced Th1 differentiation,<sup>20</sup> neutralizing it did not suppress the development of AIH.<sup>9</sup> These data suggest that IL-12 is not exclusively involved in differentiation into T cells with features of Th1 cells in the progression of fatal AIH.

Serum levels of IL-18 are increased in patients with AIH and fatal hepatitis.<sup>21, 22</sup> IL-18 is critical for liver injury in mice sequentially treated with *P. acnes* and LPS, and for acute hepatic injury induced by concanavalin A (Con A).<sup>23, 24</sup> When we looked at serum levels of IL-18 at one to three weeks of age, those of IL-18 but not IL-1 $\beta$  were elevated, and IL-18 elevation gradually increased through the progression of AIH (Fig. 6A and 6B). IL-18 signals through the IL-18 receptor complex (IL-18R), and IL-18R contains the heterodimer IL-18R $\alpha$  and IL-18R $\beta$  subunits. The IL-18R $\alpha$  subunit is responsible for extracellular binding of IL-18, whereas the IL-18R $\beta$  subunit is nonbinding but confers high affinity binding for the ligand and is responsible for biological signals.<sup>25, 26</sup> Therefore, to examine the roles of IL-18 in AIH development, NTx-*PD-1*<sup>-/-</sup> mice at one day after thymectomy were injected with IL-18R $\beta$  mAb, which can neutralize the IL-18-mediated biological function in IL-18R-expressing cells. Administering anti-IL-18R $\beta$  but not anti-IL-1 $\beta$  suppressed mononuclear cell infiltration, including CD4<sup>+</sup> and CD8<sup>+</sup> T cells, in the liver (Fig. 6C and 6D), resulting in decreased serum concentrations of aspartate aminotransferase and alanine aminotransferase and a significantly increased survival rate at four weeks of age (Fig. 6E and 6F). These data indicate that IL-18-mediated signaling is critical for the development of fatal AIH in

NTx-*PD-I*<sup>-/-</sup> mice.

***IL-18 Is Mainly Produced by DCs in the Spleen and Liver of NTx-*PD-I*<sup>-/-</sup> Mice.*** Next, we investigated how IL-18 mediates fatal AIH progression in NTx-*PD-I*<sup>-/-</sup> mice. We isolated mononuclear cells from the liver and spleen of 2.5-week-old NTx-*PD-I*<sup>-/-</sup> mice, and purified them to CD3<sup>+</sup>CD4<sup>+</sup> T cells, CD3<sup>+</sup>CD8<sup>+</sup> T cells, B220<sup>+</sup> B cells, CD11b<sup>+</sup>CD11c<sup>-</sup> macrophages, CD11c<sup>+</sup> DCs, and CD3<sup>-</sup>DX5<sup>+</sup> NK cells, then measuring mRNA expression of IL-18. We found that isolated splenic and hepatic DCs increased IL-18 mRNA expression, together with upregulated expression of NACHT, LRR, and pyrin domain-containing protein-3 (NALP3) and, to a lesser extent, IL-1 $\beta$  (Fig. 7A and 7B). In contrast, when we cultured isolated splenic DCs, IL-18 but not IL-1 $\beta$  was secreted from DCs from NTx-*PD-I*<sup>-/-</sup> mice but not from *PD-I*<sup>-/-</sup> mice (Fig. 7C and data not shown). These data suggest that in NTx-*PD-I*<sup>-/-</sup> mice, DCs non-canonically secrete IL-18 by activating inflammasome and promoting further differentiation of CD4<sup>+</sup> T cells into Th1 cells and CD8<sup>+</sup> T cells into effector T cells, respectively.

***DCs as well as CD4<sup>+</sup> and CD8<sup>+</sup> T Cells in the Spleen and Liver Expressed IL-18R in NTx-*PD-I*<sup>-/-</sup> Mice.*** To evaluate whether DCs secreting IL-18 directly or indirectly modulate differentiation of T cells in NTx-*PD-I*<sup>-/-</sup> mice, we next examined mRNA expression of IL-18R $\alpha$  on CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen and liver. We isolated these cells in the spleen and liver of 2.5-week-old NTx-*PD-I*<sup>-/-</sup> mice and measured mRNA expression of IL-18R $\alpha$ . We found that isolated splenic and hepatic CD4<sup>+</sup> and CD8<sup>+</sup> T cells increased IL-18R $\alpha$  mRNA expression, suggesting that IL-18 can directly affect differentiation of these cells (Fig. 7D). Interestingly, isolated DCs in the spleen and liver of 2.5-week-old mice expressed upregulated expression of IL-18R $\alpha$  mRNA (Fig. 7E). In addition, four-week-old NTx-*PD-I*<sup>-/-</sup> mice injected with anti-IL-18R $\beta$  mAb showed decreased serum levels of IL-18 (Fig. 7F), suggesting that IL-18 may act as an autocrine for differentiation and/or function of proinflammatory IL-18R-expressing DCs. In AIH progression, mRNA expression of IFN- $\gamma$  and TNF- $\alpha$  in the inflamed liver as well as serum levels of these cytokines were markedly elevated<sup>7-10</sup>, so TNF- $\alpha$  could be involved in the maturation of DCs. Indeed, neutralization of TNF- $\alpha$  but not IFN- $\gamma$  reduced serum levels of IL-18 (Fig. 7F), implying that TNF- $\alpha$  is also directly/indirectly involved in differentiation and/or function of proinflammatory IL-18R-expressing DCs.

***Neutralization of IL-18R Signaling Altered Splenic T Cell Function and Ab Production in NTx-PD-I<sup>-/-</sup> Mice.*** We found that DCs and T cells, not only in inflamed liver but also in the spleen, expressed IL18R $\alpha$  in AIH progression (Fig. 7D and 7E). In addition, CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen predominantly expressed CXCR3 (Fig. 1E).<sup>8</sup> We next examined whether IL-18 is involved in differentiation of splenic T cells in NTx-PD-I<sup>-/-</sup> mice. We found that injecting anti-IL-18R $\beta$  significantly reduced the number of CXCR3<sup>+</sup> cells in CD4<sup>+</sup> T cells as well as in CD8<sup>+</sup> T cells of the spleen in 2.5-week-old NTx-PD-I<sup>-/-</sup> mice (Fig. 8A). In addition, we found that neutralizing IL-18-mediated signaling suppressed expression of T-bet, IFN- $\gamma$ , TNF- $\alpha$ , and IL-18R $\alpha$  and upregulated expression of GATA3 in splenic CD4<sup>+</sup> T cells (Fig. 8B). Moreover, although production of total immunoglobulin and ANA increased in NTx-PD-I<sup>-/-</sup> mice, injecting anti-IL-18R $\beta$  reduced total immunoglobulin and ANA in the Th1-dependent IgG2a subclass (supplementary Fig.3). In this mouse model, splenic CD4<sup>+</sup> T cells showing the T<sub>FH</sub> cell phenotype were localized in B-cell follicles with huge GCs.<sup>8</sup> Although injections of anti-IL-12p40 did not significantly reduce the size of GCs in the spleen at four weeks, injecting anti-IL-18R $\beta$  mAb induced enlargement of PNA<sup>+</sup> GC in B220<sup>+</sup> follicles (supplementary Fig. 4A and 4B). Taken together, these data suggest that DC-derived IL-18 is involved in the differentiation of CD4<sup>+</sup>T cells into Th1 cells and CD8<sup>+</sup> T cells into effector T cells, respectively, in the spleen of NTx-PD-I<sup>-/-</sup> mice.

## Discussion

In this study, using our fatal AIH model, we examined molecules key to the triggering of fatal progression of AIH. We found that in the progression, CXCR3 expressing Th1 cells and CD8<sup>+</sup> effector T cells infiltrated the liver, CD8<sup>+</sup> effector T cells triggering the fatal destruction of the liver; that hepatic macrophages/Kupffer cells producing CXCL9 is critical for migration of these T cells; and that DC-derived IL-18 is critical for differentiation of Th1 cells and CD8<sup>+</sup> effector T cells (Fig. 8C).

We previously reported that in the induction phase of AIH in two-week-old NTx-*PD-I*<sup>-/-</sup> mice, IL-21-producing splenic T<sub>FH</sub> cells directly migrated into the liver via the CCR6-CCL20 axis, triggering AIH.<sup>8</sup> In contrast, we showed here that in severely inflamed livers in three-week-old NTx-*PD-I*<sup>-/-</sup> mice, DC-derived IL-18 mediates the differentiation of Th1 cells and CD8<sup>+</sup> effector T cells, and CXCR3-CXCL9-axis triggers the migration of these T cells, resulting in fatal AIH progression. Therefore, in the development of fatal AIH in our model, different types of T cells are critically involved at different time points in the induction and fatal progression of AIH. This involvement has also been reported in experimental autoimmune encephalomyelitis (EAE), a CD4<sup>+</sup> T cell-mediated disease of the central nervous system.<sup>27</sup> In EAE, Th17 cells migrate via the CCR6-CCL20 axis, triggering inflammation in the induction phase, whereas Th1 cells are mainly involved in inflamed lesions in the central nervous system during active progression.<sup>27</sup> In addition, a recent study reported that T<sub>FH</sub>-like cells were transiently generated during IL-12-mediating Th1 cell differentiation. In mice infected with *Toxoplasma gondii*, an obligate intracellular parasite, T<sub>FH</sub>-like cells were generated 7 days after infection, the proportion of T<sub>FH</sub>-like cells declined, and IFN- $\gamma$  producing Th1 cells increased at day 15.<sup>19</sup>

In this study, we showed that DC-derived IL-18 is critical for differentiation of Th1 cells and CD8<sup>+</sup> effector T cells in AIH progression. IL-18 is known to be produced by various types of immune cells and epithelial cells.<sup>25, 26</sup> In humans, IL-18 produced by DCs promotes Th1 induction.<sup>28</sup> IL-18 stimulates Th1-mediated immune responses and activates Th1 cells, which highly express functional IL-18 receptor, producing large amounts of IFN- $\gamma$ .<sup>25, 26</sup> In addition, in an atopic dermatitis mouse model, IL-18 could induce differentiation of Th1-like cells that expressed IFN- $\gamma$  and CXCR3.<sup>29</sup> In humans, IL-18 has been shown to be involved in disease processes associated with excessive Th1 responses in several inflammatory diseases, including autoimmune diseases.<sup>30-32</sup> Patients

with acute hepatitis, chronic liver disease, fulminant hepatitis, primary biliary cirrhosis, or AIH all show elevated serum levels of IL-18<sup>21, 22</sup>, which correlates with disease severity.<sup>33,34</sup>

We found that splenic and hepatic DCs increased IL-18 mRNA expression, together with upregulated expression of NALP3 and, to a lesser extent, IL-1 $\beta$ . However, DCs only secreted IL-18 and induced elevation of serum levels of IL-18. Indeed, administering anti-IL-18R $\beta$  but not anti-IL-1 $\beta$  suppressed fatal AIH. After inflammasome activation via NALP3 occurs in the cells, inactive pro-caspase-1 is activated into active caspase-1. Following cleavage by active caspase-1, mature IL-18 as well as IL-1 $\beta$  can be secreted from the cells<sup>35</sup>. These canonical IL-1 $\beta$  and IL-18 secretions via inflammasome activation are involved in acetaminophen-induced liver injury<sup>36</sup>. However, several recent studies suggest that secretion of IL-18, but not IL-1 $\beta$ , via the activation of inflammasome and caspase-1 can be orchestrated by several distinct regulatory mechanisms<sup>37-39</sup>. Thus, in NTx-*PD-1*<sup>-/-</sup> mice, distinct licensing of IL-1 $\beta$  and IL-18 secretion may be involved in the non-canonical secretion of IL-18 via the activation of inflammasome.

Because TNF- $\alpha$  can directly induce the maturation of DCs, TNF- $\alpha$  and IL-18 may directly induce inflammasome upregulation and skew toward IL-18 production via repression of IL-1 $\beta$  transcript but upregulation of IL-18 transcript. On the other hand, TNF- $\alpha$  directly and indirectly induces cell death of hepatocytes<sup>40</sup> and free DNA released from apoptotic hepatocytes can activate Tlr9, triggering a signaling cascade to induce pro-IL-1 $\beta$  and pro-IL-18<sup>36</sup>. Therefore, TNF- $\alpha$  may induce apoptosis of hepatocytes, triggering canonical IL-18 production initially. However, IL-18 may act as an autocrine for skewing prolonged IL-18 secretion in DCs.

Although first described as IFN- $\gamma$ -inducing factor, IL-18 may not make a major contribution to elevated serum levels of IFN- $\gamma$  in AIH progression. In contrast to IL-18, serum levels of IFN- $\gamma$  reached the maximal level at one week of age before AIH development; the elevated serum level of IFN- $\gamma$  gradually decreased during AIH progression.<sup>9</sup> Indeed, IFN- $\gamma$  was dispensable for upregulating CXCL9 in the liver. Neutralizing IFN- $\gamma$  did not prevent the development of AIH and induced increased T-cell proliferation in the spleen and liver, resulting in exacerbated T-cell infiltration in AIH<sup>9</sup>. So although IFN- $\gamma$  generally acts as a critical proinflammatory mediator, it exerts regulatory functions to limit tissue damage associated with inflammation of AIH in

progression.

We showed here that the migration of exclusively CXCR3-expressing T cells was triggered by hepatic macrophages/Kupffer cells producing one CXCR3 ligand, CXCL9. Although CXCL9, CXCL10, and CXCL11 can bind to the common receptor CXCR3, differences have been reported in the kinetics and the tissue/cell type expression patterns of these three chemokine genes and their proteins during infection or inflammatory responses.<sup>41-44</sup> Studies using CXCL9- or CXCL10-deficient mice have shown the non-redundant function of these chemokines in various immunoinflammatory settings, including a hepatitis B virus (HBV) transgenic mouse model and a liver injury model.<sup>41-44</sup>

In this study, we showed that CXCL9-expressing cells are macrophages/Kupffer cells in AIH progression. Although rIFN- $\gamma$  and rTNF- $\alpha$  upregulated hepatic CXCL9 expression, anti-IFN- $\gamma$  did not suppress hepatic CXCL9 upregulation. In NTx-*PD-1*<sup>-/-</sup> mice, cell types responsible for secreting CXCR3 ligands in various organs may exhibit a refractory response to constitutively elevated serum IFN- $\gamma$ . In addition, TNF- $\alpha$  secreted in autocrine and in paracrine fashion by activated T cells may induce uncontrollable CXCL9 expression in AIH progression. Therefore, anti-TNF- $\alpha$  monotherapy may not significantly prevent fatal AIH in mice.

In conclusion, we have identified the pivotal role of the IL-18 and CXCR3-CXCL9 axis in fatal progression of AIH, implying that blocking these systems may have clinical potential for protecting against fatal progression of this disease.

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## Figure Legends

**Fig. 1.** Histological and immunological analysis of AIH-bearing NTx-*PD-I*<sup>-/-</sup> mice. (A) Histological findings of the liver in NTx-*PD-I*<sup>-/-</sup> mice at indicated ages in weeks. All scale bars, 100 μm. (B and C) Livers from 3-week-old *PD-I*<sup>-/-</sup> mice with or without NTx were used for real-time quantitative RT-PCR analyses for mRNA expressions of lineage-specific transcription factors such as T-bet, GATA-3, or ROR-γt, and various cytokines (B) and Th1-cell-expressing chemokine receptor CXCR3 (C). (D) Cell numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen, liver, and mesenteric lymph nodes (MLN) of *PD-I*<sup>-/-</sup> mice with or without NTx at the indicated age. Isolated cells were stained with FITC-anti-CD3e and APC-Cy7-anti-CD4 or APC-anti-CD8. (E) Cell numbers of splenic and hepatic CD8<sup>+</sup> T cells expressing indicated chemokine receptors. Isolated cells were stained with FITC-anti-CD3e, APC-anti-CD8 and PE-anti-CCR6, -anti-CCR9, or -anti-CXCR3. Flow cytometric analyses were carried out as described in Supplementary Materials and Methods. Numbers of indicated T cell populations were calculated by (percentage of the cells in viable cells) x (no. of viable cells). Data are shown as the mean of at least three mice. *Error bars* represent SD. *Asterisks* indicate P < 0.05. n.s., not significant. ND, not detected.

**Fig. 2.** Immunological and histological analysis for NTx-*PD-I*<sup>-/-</sup> mice injected with anti-CD8 in the progression phase of AIH. (A) NTx-*PD-I*<sup>-/-</sup> mice at 14 days after thymectomy were injected intraperitoneally (i.p.) every week with 100 μg of depletion antibody to CD8 (n=6) or the isotype control mAb (n=6). After two injections, mice at four weeks of age were sacrificed. (B) Cell numbers of CD8<sup>+</sup> T cells in the spleen of NTx-*PD-I*<sup>-/-</sup> mice injected with indicated Abs. (C) Survival rates at four weeks of age. (D) Representative stainings of the liver for hematoxylin and eosin are shown. (E) Cell numbers of infiltrating T cells in the liver of NTx-*PD-I*<sup>-/-</sup> mice injected with indicated Abs. Isolated cells were stained with FITC-anti-CD3e and APC-Cy7-anti-CD4 or APC-anti-CD8. *Error bars* represent SD. *Asterisks* indicate P < 0.05.

**Fig. 3.** Expression levels of CXCR3 ligands in NTx-*PD-I*<sup>-/-</sup> mice. (A) Livers from 3-week-old *PD-I*<sup>-/-</sup> and *PD-I*<sup>+/+</sup> mice with or without NTx were used for real-time quantitative RT-PCR analyses for mRNA expressions of CXCR3 ligands, CXCL9, CXCL10, and CXCL11. (B) CXCL9 mRNA expression in various organs. The stomach,

heart, lung, intestine, pancreas and kidney were from 3-week-old *PD-1*<sup>-/-</sup> mice with or without NTx. (C) Immunostaining with anti-CXCL9, CXCL10, or isotype controls. The livers from three-week-old *PD-1*<sup>-/-</sup> and *PD-1*<sup>+/+</sup> mice with or without NTx were used. (D) Serum levels of CXCL9 and CXCL10 were measured by ELISA. Data are shown of sera from indicated aged *PD-1*<sup>-/-</sup> and *PD-1*<sup>+/+</sup> mice with or without NTx. Data are shown as the mean of at least three mice. *Error bars* represent SD. *Asterisks* indicate  $P < 0.05$ . n.s., not significant. Scale bars, 100  $\mu\text{m}$ .

**Fig. 4.** Survival rate and histological analysis of the liver in NTx-*PD-1*<sup>-/-</sup> mice injected with neutralizing Abs for CXCR3 ligands. (A and B) NTx-*PD-1*<sup>-/-</sup> mice at one day after thymectomy were injected intraperitoneally every week with 100  $\mu\text{g}$  of neutralizing anti-CXCL9 (n=5), anti-CXCL10 (n=5) or the isotype control mAbs (n=5). Survival rates at four weeks of age. (C) After four injections of anti-CXCL9, anti-CXCL10 or a combination with anti-CXCL9 and anti-CXCL10 (n=5), mice at four weeks of age were sacrificed, and the livers were harvested. Representative stainings of the liver for hematoxylin and eosin (HE), CD4, and CD8 are shown. Upper panels are control stainings of the liver in *PD-1*<sup>-/-</sup> mice with or without NTx. *Asterisks* indicate  $P < 0.05$ . n.s., not significant. All *scale bars*, 100  $\mu\text{m}$ .

**Fig. 5.** The cellular source of CXCL9 and the role of cytokines in inducing CXCR3 ligands in NTx-*PD-1*<sup>-/-</sup> mice. (A) Immunostaining with anti-CXCL9, F4/80, CD4, and CD8. The livers from 3-week-old NTx-*PD-1*<sup>-/-</sup> mice were used. Scale bars, 20 $\mu\text{m}$ . (B) Four-week-old *PD-1*<sup>-/-</sup> mice were injected intraperitoneally (i.p.) with 10  $\mu\text{g}/\text{kg}$  of mouse rIFN $\gamma$  or rTNF $\alpha$ . The livers at the indicated time after injection were subjected to real-time quantitative RT-PCR analyses for mRNA expressions of CXCL9 and CXCL10. (C) NTx-*PD-1*<sup>-/-</sup> mice at one day after thymectomy were injected intraperitoneally every week with 100  $\mu\text{g}$  of neutralizing anti-IFN $\gamma$ , anti-TNF $\alpha$ , or isotype controls. After four injections, mice at four weeks of age were sacrificed. *PD-1*<sup>-/-</sup> mice without NTx at the same age were used for controls. The livers from these mice were used for real-time quantitative RT-PCR analyses for mRNA expressions of CXCL9. (D, E) NTx-*PD-1*<sup>-/-</sup> mice at fourteen day after thymectomy were injected intraperitoneally every week with 100  $\mu\text{g}$  of neutralizing anti-TNF $\alpha$  or isotype control. After two injections, mice at four weeks of age were sacrificed. Liver stainings are shown for hematoxylin and eosin, and

immunostaining with anti-TNF $\alpha$ . Scale bars, 50  $\mu$ m (upper four panels). Immunostaining with anti-TNF $\alpha$  and anti-CXCL9. Scale bars, 20  $\mu$ m (lower four panels) (D). Serum levels of CXCL9 were measured by ELISA (E). Data are shown as the mean of at least three mice. *Error bars* represent SD. *Asterisks* indicate  $P < 0.05$ . n.s., not significant.

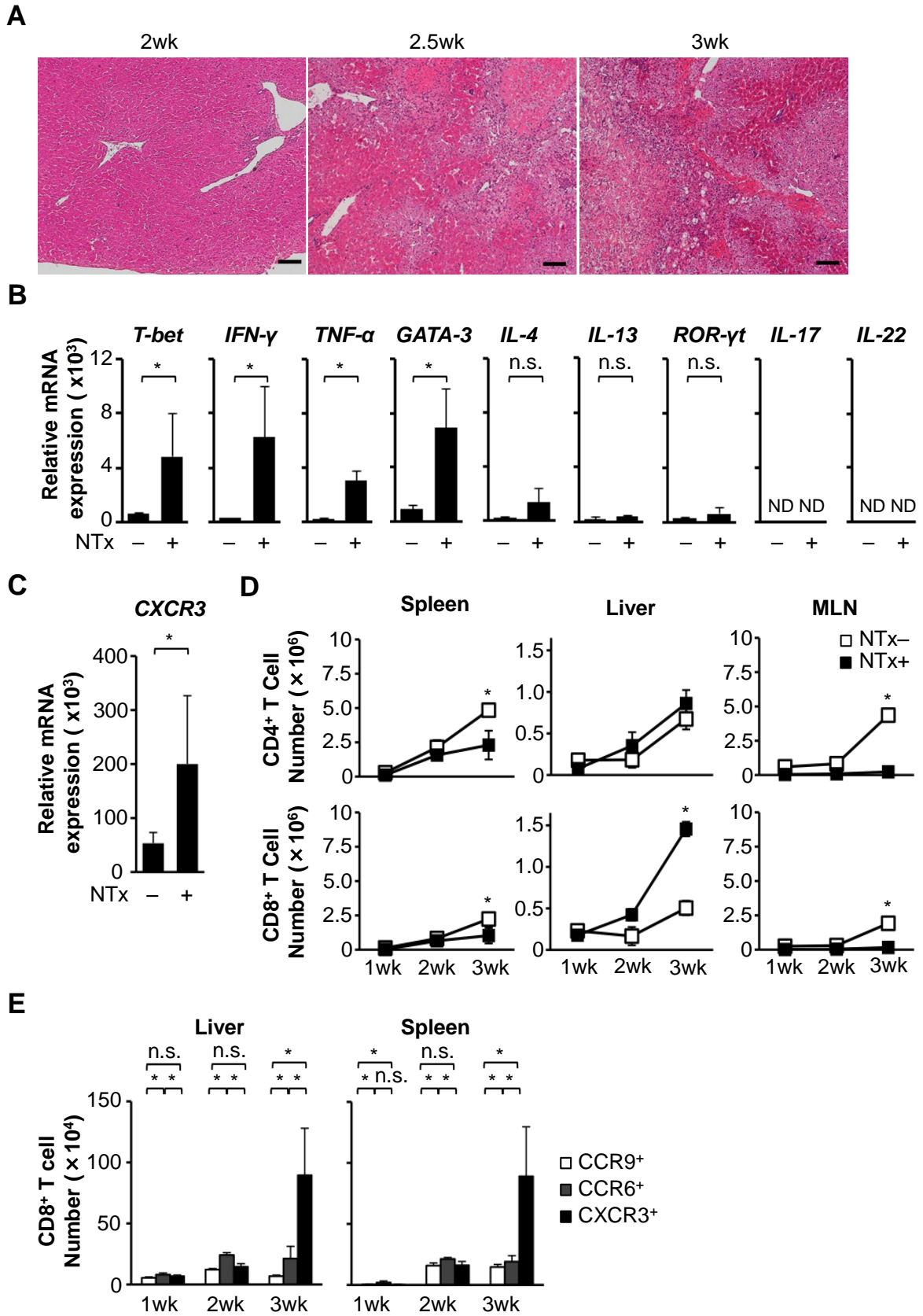
**Fig. 6.** Serum levels of IL-18 and IL-1 $\beta$ , and analysis for NTx-*PD-1*<sup>-/-</sup> mice injected with blocking Abs for IL-18R signaling and neutralizing Abs for IL-1 $\beta$ . (A, B) Serum levels of IL-18 at indicated ages and IL-1 $\beta$  at 3 weeks of age of *PD-1*<sup>-/-</sup> mice with or without NTx were measured by ELISA. (C, E, F) NTx-*PD-1*<sup>-/-</sup> mice at one day after thymectomy were injected intraperitoneally every week with 100  $\mu$ g of IL-18R $\beta$  mAb (n=10), or the isotype control mAb (n=10). (D) NTx-*PD-1*<sup>-/-</sup> mice were injected with 100  $\mu$ g of IL-1 $\beta$  mAb (n=3), or the isotype control mAb (n=3) as described above. After four injections, mice at four weeks of age were sacrificed. Stainings of the liver for hematoxylin and eosin (HE), CD4, and CD8 (C, D), serum levels of AST and ALT (E) and survival rates at four weeks of age (F) are shown. Data are shown as the mean of at least three mice. *Error bars* represent SD. *Asterisks* indicate  $P < 0.05$ . n.s., not significant. Scale bars, 100  $\mu$ m.

**Fig. 7.** Expression levels of IL-18, IL-1 $\beta$ , NALP3, and IL-18R $\alpha$  in AIH-bearing 2.5-week-old NTx-*PD-1*<sup>-/-</sup> mice. (A) Expression levels of mRNA encoding IL-18 of CD3<sup>+</sup>CD4<sup>+</sup> T cells, CD3<sup>+</sup>CD8<sup>+</sup> T cells, B220<sup>+</sup> B cells, CD11b<sup>+</sup>CD11c<sup>-</sup> macrophages, CD11c<sup>+</sup> DCs and CD3<sup>+</sup>DX5<sup>+</sup> NK cells in the spleen and liver of 2.5-week-old NTx-*PD-1*<sup>-/-</sup> mice. Data represent 1 of 3 independent experiments. (B) Expression levels of mRNA encoding IL-1 $\beta$  and NALP3 of CD11c<sup>+</sup> DCs in the spleen of 2.5-week-old NTx-*PD-1*<sup>-/-</sup> mice. (C) Concentration of IL-18 in DC-culture supernatants measured by ELISA. CD11c<sup>+</sup> DCs were isolated from the spleen in *PD-1*<sup>-/-</sup> mice with or without NTx and DCs were cultured for 24 h. Data are shown as the mean of triplicates. (D and E) Expression levels of mRNA encoding IL-18R $\alpha$  of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells (D), and CD11c<sup>+</sup> DCs (E) in the spleen and liver of 2.5-week-old NTx-*PD-1*<sup>-/-</sup> mice. (F) Serum levels of IL-18 were measured by ELISA. NTx-*PD-1*<sup>-/-</sup> mice at one day after thymectomy were injected intraperitoneally every week with 100  $\mu$ g of neutralizing anti-IL-18R $\beta$ , anti-IFN, or anti-TNF $\alpha$ . After four injections, mice at four weeks of age

were sacrificed. Data is shown of sera from *PD-I*<sup>-/-</sup> mice of 4-week-old with indicated condition. Data are shown as the mean of at least three mice. *Error bars* represent SD. *Asterisks* indicate  $P < 0.05$ . n.s., not significant. ND, not detected.

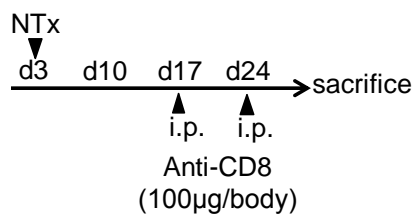
**Fig. 8.** Immunological and RT-PCR analysis for NTx-*PD-I*<sup>-/-</sup> mice injected with blocking Abs for IL-18R signaling, and the model of pathological mechanisms in the progression phase of AIH in NTx-*PD-I*<sup>-/-</sup> mice. (A-B) NTx-*PD-I*<sup>-/-</sup> mice at one day after thymectomy were injected with IL-18R $\beta$  mAb. (A) After four injections, mice at four weeks of age were sacrificed as described in Fig 6. Cell numbers of CXCR3<sup>+</sup> cells in CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the spleen. Data are shown as the mean of at least three mice. (B) After three injections, mice at three weeks of age were sacrificed, and CD4<sup>+</sup> T cells were isolated from the spleens. Expression levels of mRNA encoding T-bet, GATA-3, ROR- $\gamma$ t, IFN $\gamma$ , TNF $\alpha$ , and IL-18R $\alpha$  were measured. Data are shown as the mean of triplicates. *Error bars* represent SD. *Asterisks* indicate  $P < 0.05$ . n.s., not significant. (C) The model of mechanistic links of cytokines and chemokines in the progression phase of NTx-*PD-I*<sup>-/-</sup> mice. In the progression, DC-derived IL-18 is critical for differentiation of CXCR3-expressing Th1 cells (Th1) and CD8<sup>+</sup> effector T cells (T<sub>E</sub>). CXCL9 production by hepatic macrophages/Kupffer cells triggers migration of these T cells into the liver. CXCR3-expressing T<sub>E</sub> and, to a lesser extent, Th1 infiltrate the liver and T<sub>E</sub> trigger the fatal destruction of the liver.

**Fig. 1.**

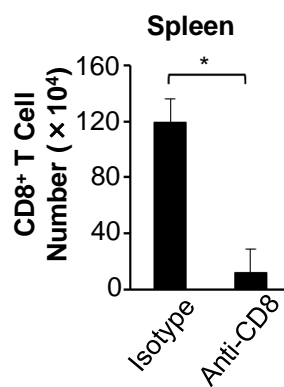


**Fig. 2.**

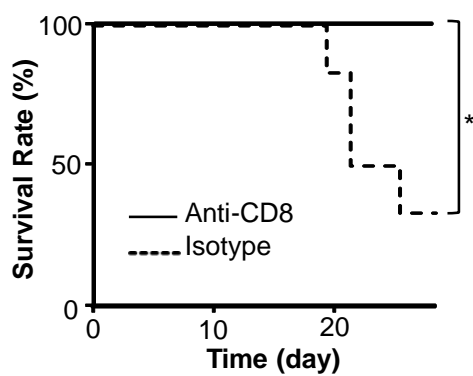
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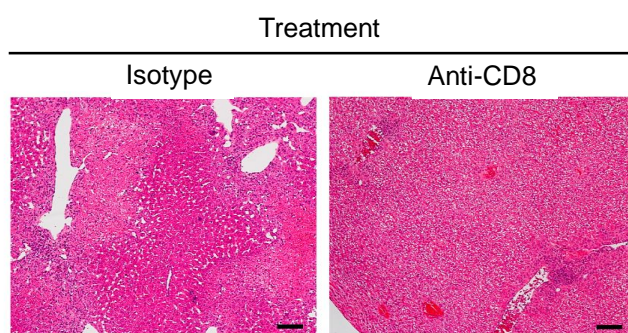
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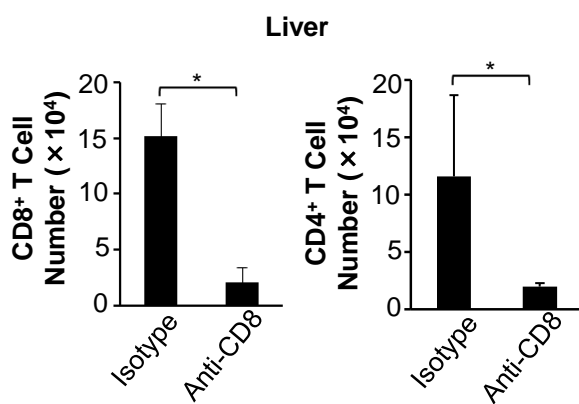
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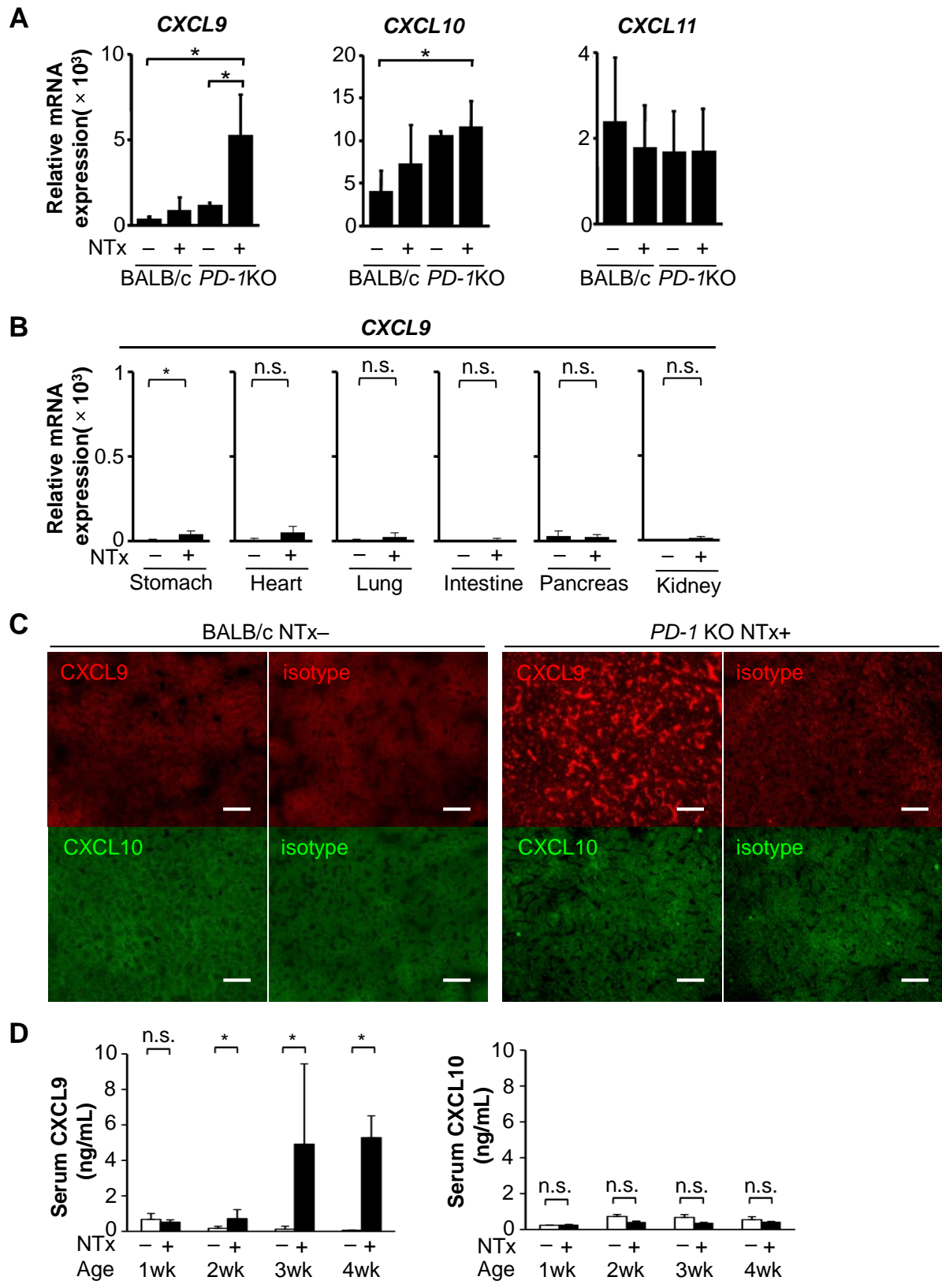


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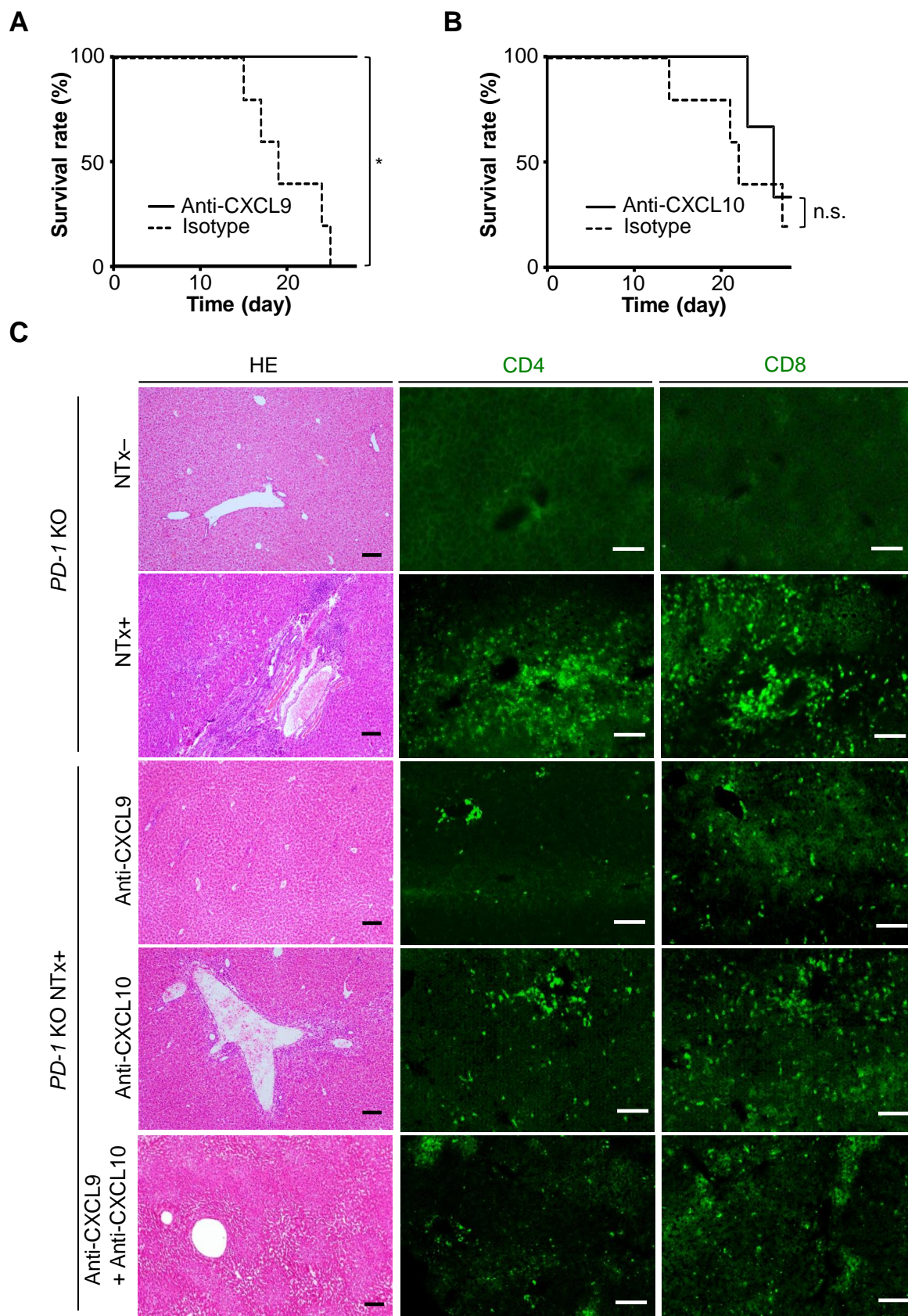




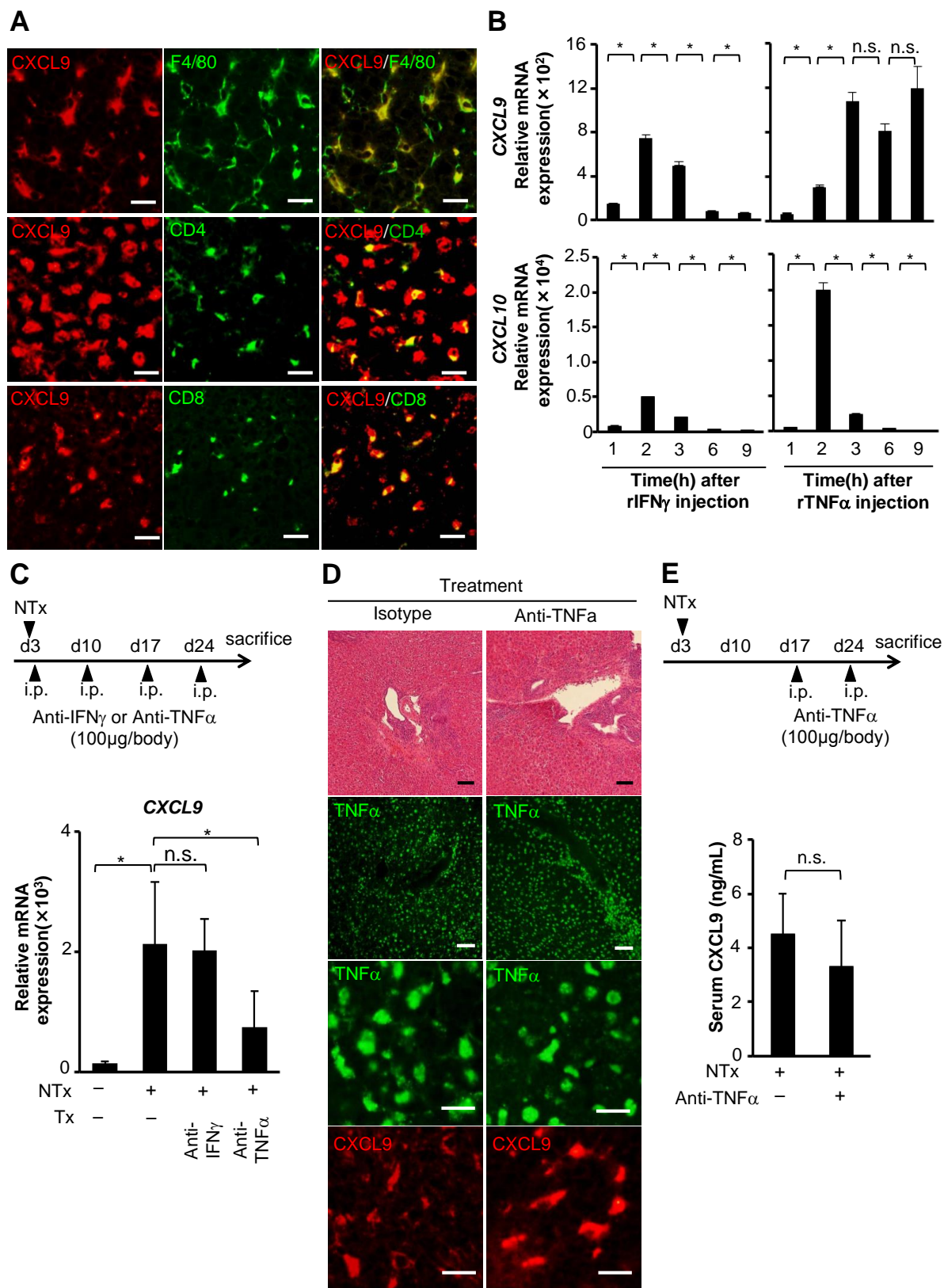
**Fig. 3.**



**Fig. 4.**

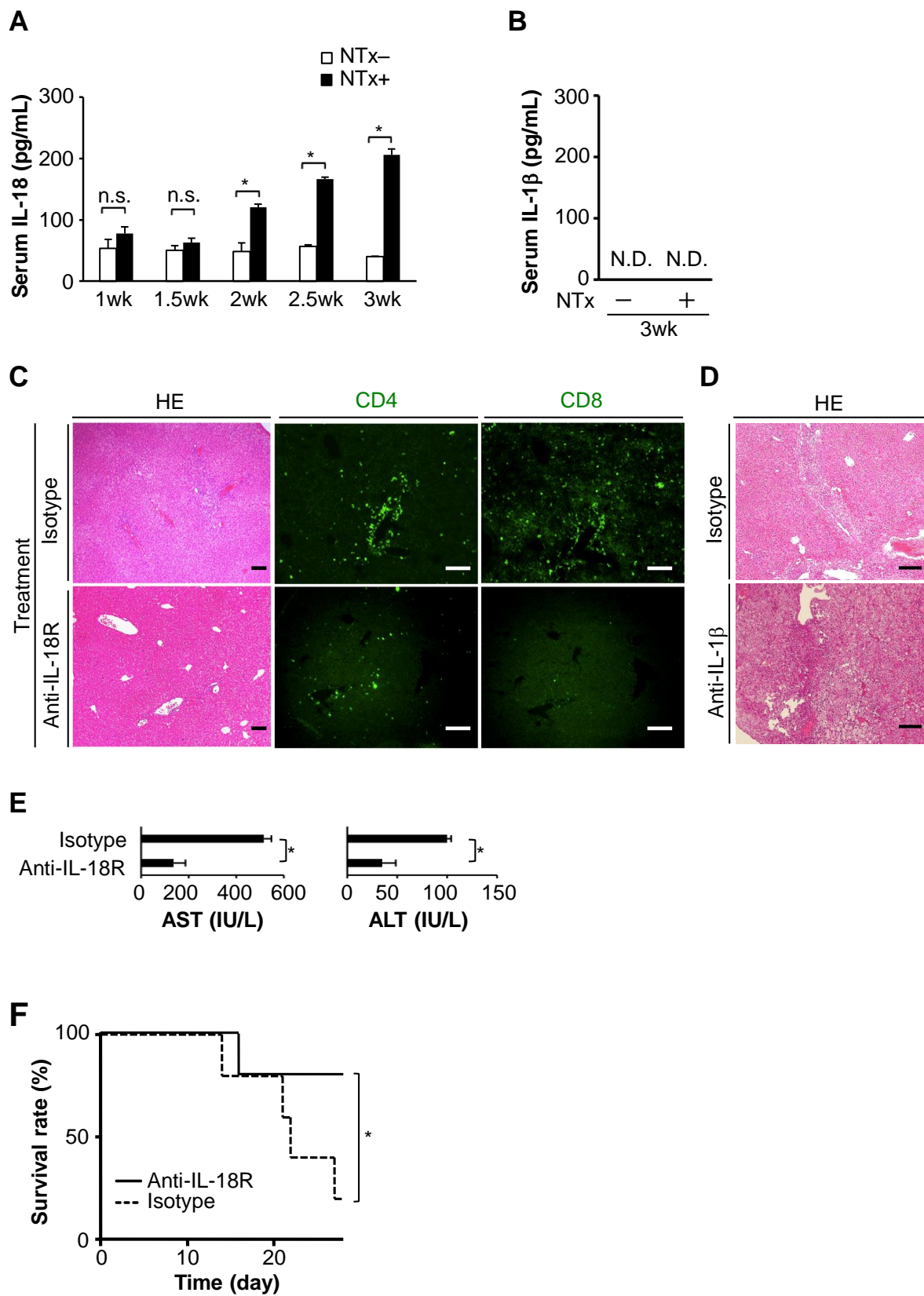


**Fig. 5.**

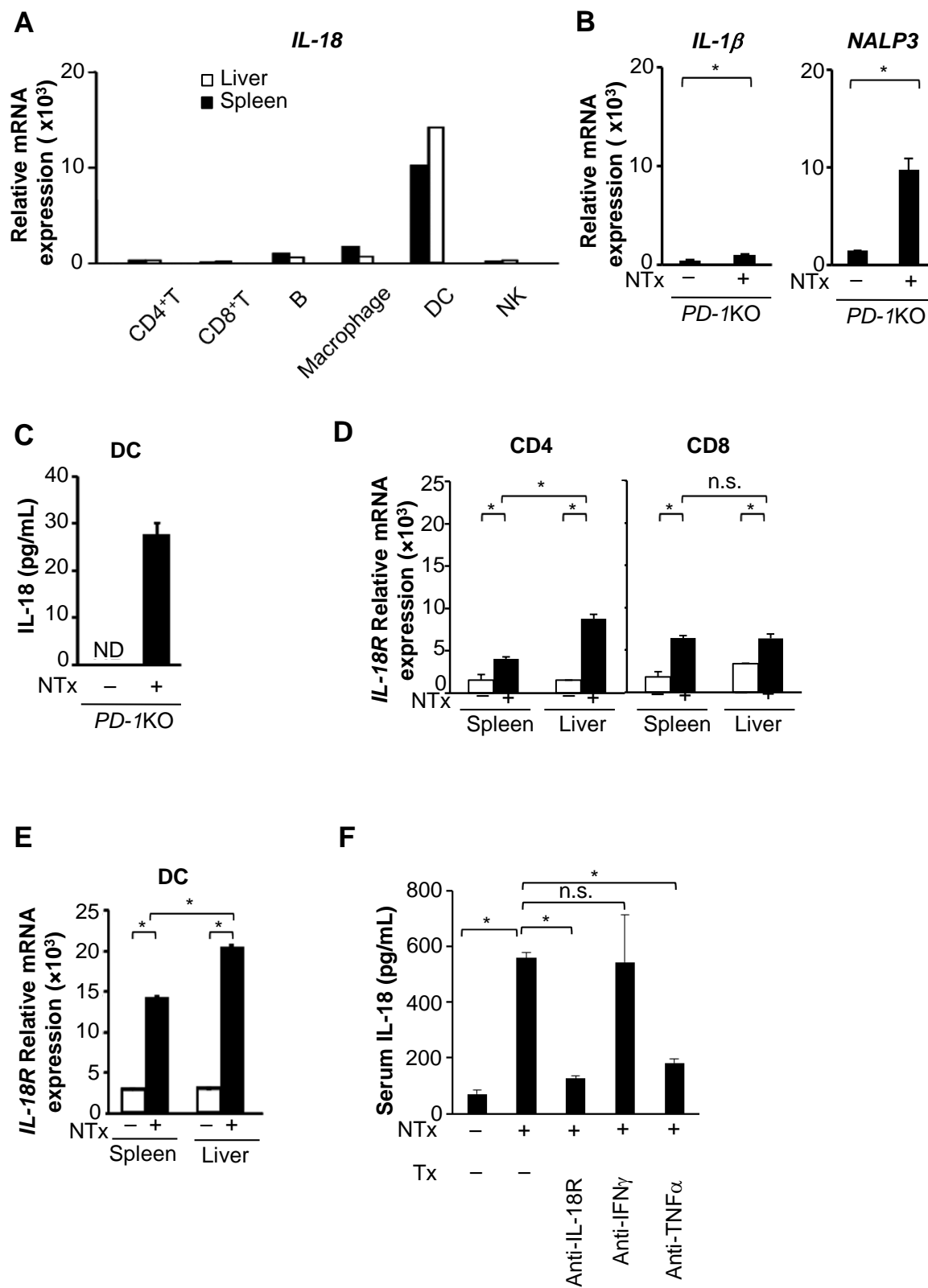




**Fig. 6.**

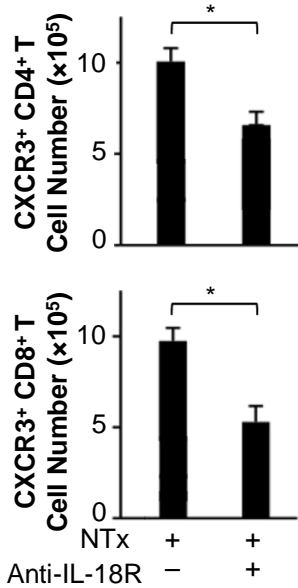


**Fig. 7.**

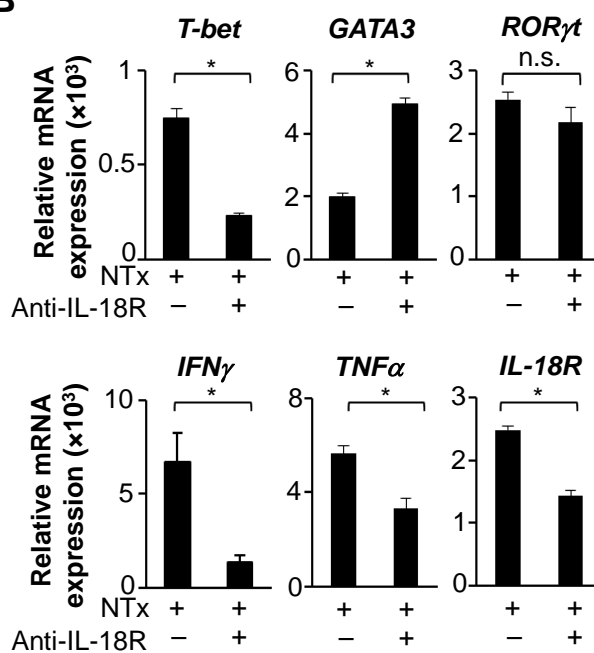


**Fig. 8.**

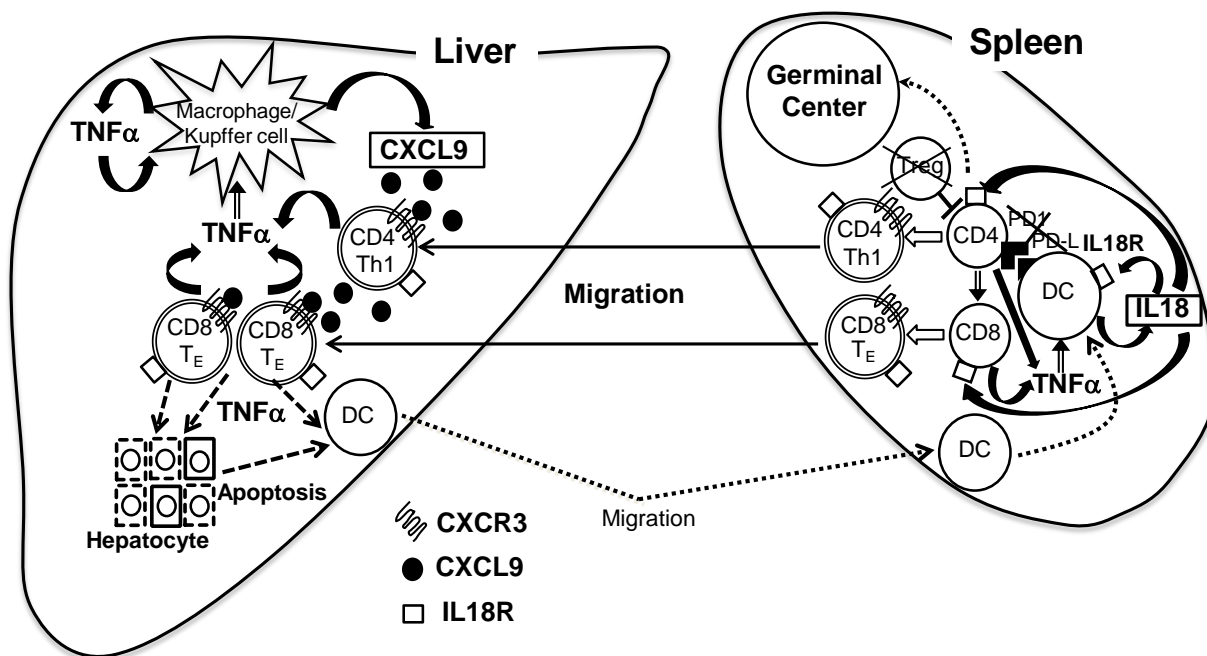
**A**



**B**



**C**



## <Supplementary Information>

### Supplemental Materials and Methods

**Histological and Immunohistological Analysis.** Organs were fixed in neutral buffered formalin, embedded in paraffin wax, and cut into sections 4  $\mu\text{m}$  thick. These sections were stained with hematoxylin and eosin for histopathology. Fluorescence immunohistology was performed on frozen sections using FITC-conjugated anti-CD4 or anti-CD8a (eBioscience, San Diego, CA), peanut agglutinin (PNA, Vector Laboratories, Burlingame, CA), biotin-labeled anti-B220 (BD Biosciences, San Jose, CA) followed by Texas red-conjugated avidin (Vector Laboratories) as described previously.<sup>1-5</sup> To stain CXCR3 ligands, rabbit polyclonal Abs to CXCL9/MIG (sc-50302, Santa Cruz Biotechnology, Santa Cruz, CA) and rat monoclonal Ab (mAb) to CXCL10/IP-10 (R&D Systems, Minneapolis, MN) were used, followed by Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) and FITC-conjugated goat anti-rat IgG (BD Biosciences), respectively. For staining of macrophages/kupffer cells, rat mAb to F4/80 (eBioscience) was used, followed by Alexa Fluor 488 goat anti-rat IgG (Invitrogen). As for the staining of TNF $\alpha$ , rabbit polyclonal Ab to TNF $\alpha$  (Abcam, Cambridge, UK) was used followed by Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen). Germinal center (GC) diameters were measured in several high-power fields in at least three sections of each mouse, as described previously.<sup>2</sup>

**Real-time Quantitative Reverse-transcription Polymerase Chain Reactions. (RT-PCR)** Real-time quantitative RT-PCR was performed as described previously.<sup>1-4</sup> Spleen and liver tissues or isolated cells from the spleen and liver were frozen in RNAlater. RNA was prepared with an RNeasy mini kit (Qiagen, Hilden, Germany), and single-strand complementary DNA was synthesized with SuperScript<sup>TM</sup> II reverse transcriptase (Invitrogen). Real-time quantitative RT-PCR was performed using SYBR Green I Master (Roche Applied Science, Basel, Switzerland). The real-time quantitative reactions were performed using a Light Cycler 480 (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'-CAACTTTGTCAAGCTCATTTC-3' and 5'-GGTCCAGGGTTTCTTACTCC-3'; *T-bet*: 5'-TCAACCAGCACCAGACAGAG-3' and 5'-AAACATCCTGTAATGGCTTGTG-3';

*GATA-3*: 5'-TTATCAAGCCAAGCGAAG-3' and  
 5'-TGGTGGTGGTCTGACAGTTC-3';

*ROR- $\gamma$* : 5'-CCGCTGAGAGGGCTTCAC-3' and  
 5'-TGCAGGAGTAGGCCACATTACA-3';

*IFN- $\gamma$* : 5'-GGATGCATTCATGAGTATTGC-3' and  
 5'-CCTTTTCCGCTTCCTGAGG-3'; *TNF- $\alpha$* :  
 5'-CCCTCACACTCAGATCATCTTCT-3' and 5'-GCTACGACGTGGGCTACAG-3';  
*IL-1 $\beta$* : 5'-TGTAATGAAAGACGGCACACC-3' and  
 5'-TCTTCTTTGGGTATTGCTTGG-3'; *IL-4*: 5'-TCATCGGCATTTTGAACGAG-3'  
 and 5'-CGTTTGGCACATCCATCTCC-3'; *IL-13*:  
 5'-TGGGTGACTGCAGTCCTGGCT-3' and 5'-GTTGCTTTGTGTAGCTGAGCA-3';  
*IL-17A*: 5'-CTCCAGAAGGCCCTCAGACTAC-3' and  
 5'-AGCTTTCCTCCGCATTGACACAG-3'; *IL-18*:  
 5'-GACAACACGCTTTACTTTATACCTGA-3' and  
 5'-GTGAAGTCGGCCAAAGTTGT-3'; *IL-18R $\alpha$* :  
 5'-GGAACACAACACGGACCAT-3' and  
 5'-CGAGAAGGATGTATACAAACACCA-3'; *IL-22*:  
 5'-AGAAGGCTGAAGGAGACAGT-3' and  
 5'-GACATAAACAGCAGGTCCAGTT-3'; *CXCR3*:  
 5'-GGCATCTAGCACTTGACGTTC-3' and 5'-AGGCAGCACGAGACCTGA-3';  
*CXCL9*: 5'-GCATCGTGCATTTCCTTATCA-3' and  
 5'-CTTTTCCTCTTGGGCATCAT-3'; *CXCL10*: 5'-TCTCACTGGCCCGTCATC-3'  
 and 5'-GCTGCCGTCATTTTCTGC-3'; *CXCL11*:  
 5'-CCCTGTTTGAACATAAGGAAGC-3' and  
 5'-GCTGCTGAGATGAACAGGAA-3'; *NALP3*:  
 5'-TGCCTGTTCTTCCAGACTGGTGA-3'; 5'-CACAGCACCTCATGCCCGG-3'.

**Administration of Abs in vivo.** NTx-*PD-1*<sup>-/-</sup> mice were intraperitoneally injected every week from day3 with 100 $\mu$ g of mAbs to CXCL9, CXCL10, IL-12p40 and IL1 $\beta$  (R&D Systems), IFN $\gamma$  and TNF $\alpha$  (eBioscience), and IL-18R $\beta$  (BD Bioscience), and from day17 with mAbs to TNF $\alpha$ . For depletion of CD8<sup>+</sup> T cells in vivo, anti-mouse CD8 mAbs (eBioscience) were used at day17 and day24. All isotypes were from



eBioscience or R&D Systems. After injections, mice at indicated ages were sacrificed, and their livers, spleens, and sera were harvested. We did not detect any serious adverse events.

**Flow Cytometry Analysis and Isolation of Single Cells.** Single cells from the livers and spleens were prepared, and flow cytometric analysis was performed as described previously.<sup>1-5</sup> Cells were stained with FITC-conjugated anti-CD3e (BD Bioscience), and either APC-Cy7-conjugated anti-CD4 (BD Bioscience) or APC-conjugated anti-CD8a (eBioscience). For chemokine receptors, cells were stained with FITC-conjugated anti-CD3e (145-2C11), APC-conjugated anti-CD8a (eBioscience), and PE-conjugated anti-CCR6, anti-CCR9 or anti-CXCR3 (R&D Systems). Stained cells were analyzed with FACSCanto™ II (BD Biosciences). Data were analyzed using Cell Quest Pro™ (BD Biosciences). Dead cells were excluded based on side- and forward-scatter characteristics. The number of viable indicated cells was calculated as follows: (the percentage of cells in the cell type) x (the number of viable cells). For isolation of cells in Figure 7A, the following mAbs were used for surface staining: FITC-conjugated anti-CD3e, anti-DX5, PE-conjugated anti-CD3e, anti-B220, APC-conjugated anti-CD8a, and PE-streptavidin purchased from eBioscience, FITC-conjugated anti-CD11c, biotinylated anti-CD11b, and APC-Cy7-conjugated anti-CD4 from BD Bioscience. CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>-</sup>B220<sup>+</sup>, CD11b<sup>+</sup>CD11c<sup>-</sup>, CD11b<sup>or-</sup>CD11c<sup>+</sup>, CD3<sup>-</sup>DX5<sup>+</sup> cells from the spleen or liver were obtained by a FACS Aria™ (BD Biosciences) to reach >99% purity, as described.<sup>1,2</sup> For CD4<sup>+</sup> T cell isolation in Figure 8B, NTx-*PD-I*<sup>-/-</sup> mice were injected intraperitoneally with anti-IL-18Rβ or isotype controls at one day after thymectomy and then once a week with the mAbs *in vivo*. After three mAbs injections, CD4<sup>+</sup> T cells were isolated from the spleens of three-week-old mice by positive selection using mouse CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. CD4<sup>+</sup> T cells reached >94% purity, as assessed by flow cytometry.

**Enzyme-Linked Immunosorbent Assay (ELISA).** Concentrations of CXCL9 and CXCL10 in serum were measured by using mouse CXCL9 and CXCL10 ELISA kits (Abcam, Cambridge, UK) according to the manufacturer's protocols. To detect IL-18 in serum and culture supernatants, a mouse IL-18 ELISA kit (Medical & Biological

Laboratories, Nagoya, Japan) was used. Serum levels of IL-1 $\beta$  were measured using a mouse IL-1 $\beta$  ELISA kit (eBioscience). Serum Ig levels were determined by ELISA as described,<sup>6</sup> and Ab sets for detection of mouse IgG1 and IgG2a from BD Biosciences (San Jose, CA) and anti-mouse IgM from AbD Serotec (Oxford, UK) were used. To detect serum ANAs, microtiter plates (Nunc, Roskilde, Denmark) were incubated with 10  $\mu$ g/ml antigens, and the nuclear fraction was prepared from normal liver.<sup>7</sup> Ab sets for detection of mouse ANA subclasses were the same as above.

***In vivo Injection of Cytokines.*** *PDI*<sup>-/-</sup> mice at four weeks of age were injected intraperitoneally with 10 $\mu$ g/kg of mouse recombinant IFN $\gamma$ , TNF $\alpha$  (eBioscience), or PBS. After 1, 2, 3, 6 or 9 hours following injections, mice were sacrificed, and their livers were harvested. We did not detect any serious adverse events.

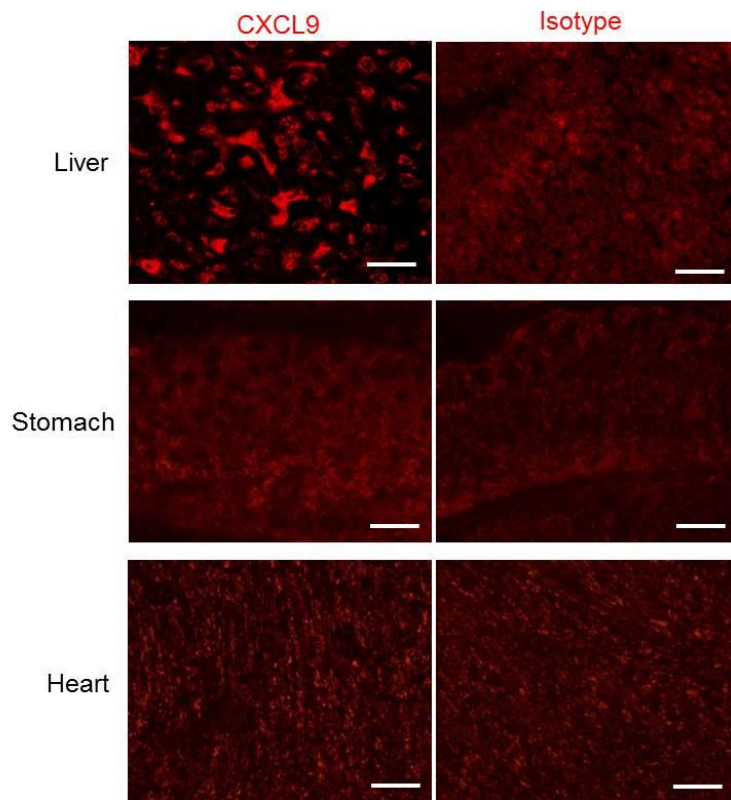
***Dendritic Cell (DC) Coculture.*** CD11b<sup>+/-</sup>CD11c<sup>+</sup> DCs were isolated from the spleen of 2.5-week-old NTx-*PD-I*<sup>-/-</sup> mice using a FACS Aria<sup>TM</sup>II (BD Biosciences) to reach >99% purity, as described.<sup>1,2</sup> Isolated DCs ( $5 \times 10^5$ ) were cultured for 24 h in round-bottomed 96-well culture plates in D-MEM supplemented with 10% fetal bovine serum, 50  $\mu$ mol/L 2-mercaptoethanol, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin.

***Statistical Analysis.*** The data are presented as the mean values  $\pm$  SD. Statistical analysis was performed by the Student's *t*-test for unpaired data to compare the values between two groups; variance was analyzed with the Tukey-Kramer test for multiple comparisons. Survival rates were estimated by the Kaplan-Meier method and compared with the log-rank test. *P*-values below .05 were considered significant.

## References

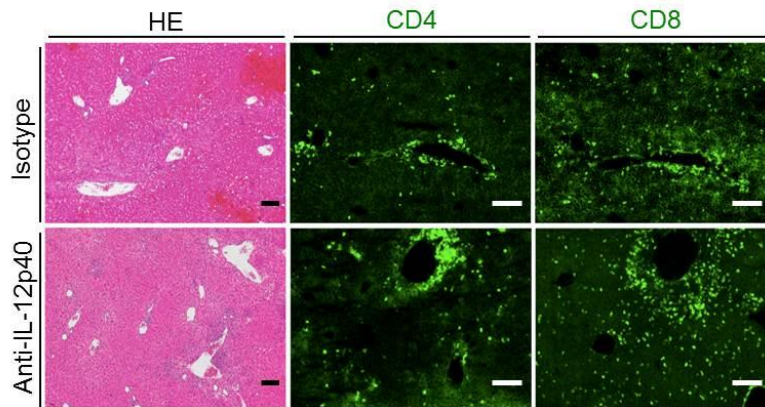
1. Kido M, Watanabe N, Okazaki T, Akamatsu T, Tanaka J, Saga K, et al. Fatal autoimmune hepatitis induced by concurrent loss of naturally arising regulatory T cells and PD-1-mediated signaling. *Gastroenterology* 2008;135:1333-43.
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7. Blobel G, Potter VR. Nuclei from rat liver: isolation method that combines purity with high yield. *Science* 1966;154:1662-5.

**Supplementary Fig. 1.**



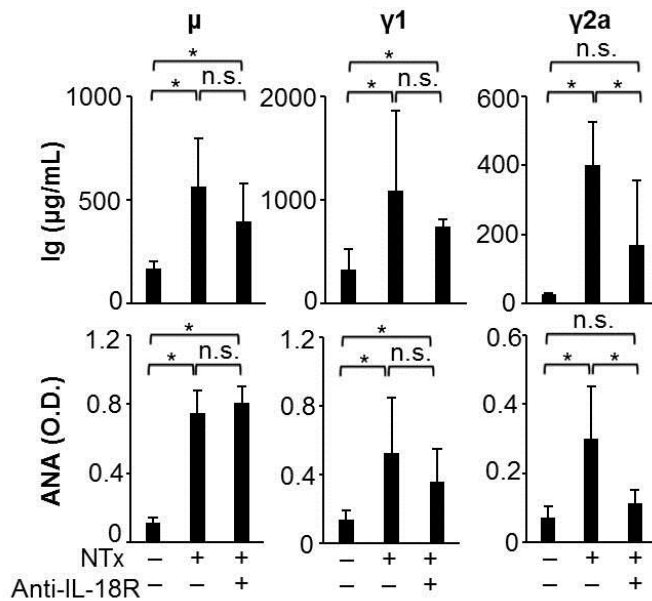
**Supplementary Fig. 1.** Immunohistological analysis of the liver, stomach and heart in  $NTx-PD-1^{-/-}$  mice. Immunostaining with anti-CXCL9 or isotype control. The livers from three-week-old  $NTx-PD-1^{-/-}$  mice were used. Scale bars, 50  $\mu m$ .

**Supplementary Fig. 2.**



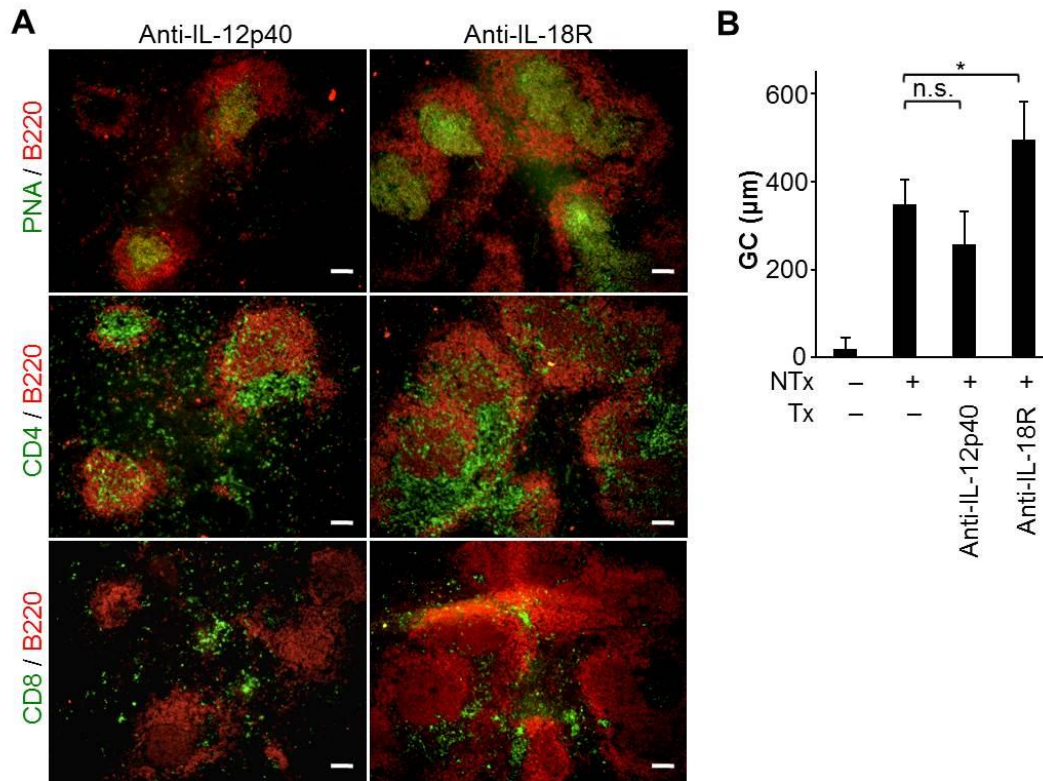
**Supplementary Fig 2.** Histological and immunohistological analysis for the liver in NTx-*PD-I*<sup>-/-</sup> mice injected with neutralizing Abs for IL-12p40. (A) NTx-*PD-I*<sup>-/-</sup> mice at one day after thymectomy were injected intraperitoneally every week with 100  $\mu$ g of neutralizing anti-IL-12p40 (n=5) or the isotype control mAb (n=5). After four injections, mice at four weeks of age were sacrificed, and the livers were harvested. Representative stainings of the liver for hematoxylin and eosin (HE), CD4, and CD8 are shown. All scale bars, 100  $\mu$ m.

**Supplementary Fig. 3.**



**Supplementary Fig. 3.** Immunological analysis for NTx-*PD-I*<sup>-/-</sup> mice injected with blocking Abs for IL-18R signaling. NTx-*PD-I*<sup>-/-</sup> mice at one day after thymectomy were injected intraperitoneally every week with 100 µg of IL-18Rβ mAb (n=10), or the isotype control mAb (n=10). After four injections, mice at four weeks of age were sacrificed. The serum levels of total immunoglobulin (Ig) and ANAs subclasses in IgM, IgG1, and IgG2a in indicated mice were determined by ELISA. *Error bars* represent SD. *Asterisks* indicate P < 0.05. n.s., not significant.

**Supplementary Fig. 4.**



**Supplementary Fig. 4.** Immunohistological analysis for NTx-*PD-1*<sup>-/-</sup> mice injected with blocking Abs for IL-18R signaling and with neutralizing Abs for IL-12p40. NTx-*PD-1*<sup>-/-</sup> mice at one day after thymectomy were injected with anti-IL-12p40 or IL-18Rβ mAb. After four injections, mice at four weeks of age were sacrificed as described in Supplementary Fig. 2 and Fig. 3. Immunohistological analysis of the spleens from mice with indicated treatments (A) and diameters of germinal center (GC) in the follicles of the spleen. Spleens were stained with FITC-conjugated anti-CD4, anti-CD8, or PNA (green) and biotin-labeled anti-B220 followed by Texas red-conjugated avidin (red). Diameters of PNA<sup>+</sup> GC in the B220<sup>+</sup> follicles were determined in several high-power fields. Data are shown as the mean of at least three mice. *Error bars* represent SD. *Asterisks* indicate P < 0.05. n.s., not significant. Scale bars, 100 µm.