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Reprint requests

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Conflicts of interest

The authors disclose no conflicts. Q1

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Supplementary Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from Japan SLC (Shizuoka, Japan), and *PD-1*$^{-/-}$ and *RAG-2*$^{-/-}$ mice on a C57BL/6 or BALB/c background were generated as described.[1-3] All of these mice were bred and housed under specific pathogen-free conditions. Thymectomy and splenectomy of the mice were performed as described.[4-6] The sham splenectomy was performed by cutting the peritoneum without removing the spleen. All mouse protocols were approved by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

Administration of DEX In Vivo

For the preventive protocol, starting 1 day after thymectomy, BALB/c-NTx-*PD-1*$^{-/-}$ mice were intraperitoneally injected every other day with 1.0 mg/kg DEX (Sigma-Aldrich, St Louis, MO) diluted in PBS or PBS alone. After 13 injections, mice at 4 weeks of age were killed, and the livers, spleens, and sera were harvested. For the therapeutic protocol, starting 14 days after thymectomy, BALB/c-NTx-*PD-1*$^{-/-}$ mice were intraperitoneally injected every other day with 1.0 mg/kg DEX diluted in PBS or PBS alone. In C57BL/6-NTx-*PD-1*$^{-/-}$ mice, therapeutic injections of 0.5 mg/kg DEX every other day were started at 4 weeks of age. After the indicated injections, the mice were killed.

Histological and Immunohistological Analysis

Organs were fixed in neutral buffered formalin and embedded in paraffin wax. Sections were stained with H&E or Masson's trichrome for histopathology. Fluorescence immunohistology was performed on frozen sections as described previously[4,5] using fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (RM4-5), anti-CD8a (Ly-2) (eBioscience, San Diego, CA), peanut agglutinin (PNA; Vector Laboratories, Burlingame, CA), and biotin-labeled anti-B220 (RA3-6B2; BD Biosciences, San Jose, CA) followed by Texas Red–conjugated avidin (Vector Laboratories). To detect autoantibodies, livers were collected from wild-type BALB/c and C57BL/6 mice. Sections were stained with 100× to 3200× diluted sera from indicated mice, followed by FITC-conjugated anti-mouse IgG (Cappel, Chester, PA).[4]

Flow Cytometry Analysis and Isolation of Lymphocytes

Single cells from the livers and spleens were prepared as described.[4,5] The following monoclonal antibodies were used for surface staining: FITC-conjugated anti-CD4, anti-CXCR5 (2G8), anti-GL7, anti-CD11c (HL3) (BD Biosciences), and anti-DX5 (eBioscience); PE-conjugated anti-CD3e (145-2C11), anti-ICOS, and anti–Gr-1 (RB6-8C5) (eBioscience); anti-CD95/Fas (Jo2) (BD Biosciences); APC-Cy7–conjugated anti-CD4 (GK1.5) and biotin-labeled B220 (BD Biosciences); and APC-conjugated streptavidin, anti-CD8a, anti-CD25 (PC61.5), and anti-CD11b (M1/70) (eBioscience). For flow cytometric analysis of splenic CD4$^+$ T cells in Figure 2*C* and Supplementary Figure 6*A*, spleen cells were stained with FITC/anti-CXCR5, PE/anti-ICOS, and APC-Cy7/anti-CD4. To analyze splenic B220$^+$ B cells in Figure 4*B*, cells were stained with FITC/anti-GL7, PE/anti-CD95/Fas, and biotin-labeled B220 followed by APC-conjugated streptavidin. Data of flow cytometric analysis in Figure 5*E* and *F* represent cell numbers of the following cell subsets in the liver: CD11b$^+$CD11c$^-$ macrophages, CD11c$^+$ dendritic cells, CD11b$^+$Gr-1$^+$ myeloid cells, CD3$^-$DX5$^+$ natural killer cells, CD3$^+$DX5$^+$ natural killer T cells, CD3$^+$DX5$^-$ T cells, B220$^+$ B cells, CD3$^+$CD4$^+$ T cells, and CD3$^+$CD8$^+$ T cells. For the analysis of TCR V_β use in Figure 6*F*, cells were stained with FITC/anti-V_β2 (B20.6), anti-V_β3 (KJ25), anti-V_β4 (KT4), anti-V_β5.1 and V_β5.2 (MR9-4), anti-V_β6 (RR4-7), anti-V_β7 (TR310), anti-V_β8.1 and V_β8.2 (MR5-2), anti-V_β8.3 (IB3.3), anti-V_β9 (MR10-2), anti-$V_\beta10^b$ (B21.5), anti-V_β11 (RR3-5), anti-V_β12 (MR11-1), anti-V_β13 (MR12-3), anti-V_β14 (14-2), anti-$V_\beta17^a$ (KJ23), PE/anti-CD3e, and APC-Cy7/anti-CD4 for CD4$^+$ T cells using the Mouse V_β TCR Screening Panel (BD Biosciences). Stained cells were analyzed with a FACSCanto II (BD Biosciences). Data were analyzed using CellQuest Pro (BD Biosciences). Dead cells were excluded on the basis of side- and forward-scatter characteristics, and viable cell numbers were calculated as follows: (Percentage of Cells in the Cell Type) × (Number of Viable Cells). For 7-AAD staining in Figure 2*D*, single cells were isolated from the spleens of 3-week-old BALB/c-NTx-*PD-1*$^{-/-}$ mice injected with DEX therapeutically. Isolated spleen cells (1×10^6) were cultured with 10–1000 ng/mL DEX under plate-bound anti-CD3 (1 μg/mL) and soluble anti-CD28 (5 μg/mL). Round-bottomed 96-well culture plates were used with Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 50 mmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. Three days after the culture, cells were harvested and stained with FITC-conjugated anti-CD3e (eBioscience) and 7-AAD (BD Biosciences) and either PE/Texas Red–conjugated anti-CD4 (Abcam, Cambridge, England) or PE/Texas Red–conjugated anti-CD8 (Abcam). In Foxp3 staining, cells were fixed and permeabilized using Foxp3 staining buffer (eBioscience) and stained with PE/anti-Foxp3 (eBioscience).

ELISA

Serum Ig levels were determined by ELISA as described,[7] and antibody sets to detect mouse IgG1, IgG2a, IgG2b, and IgG3 (BD Biosciences) and anti-mouse IgM (AbD Serotec, Oxford, England) were used. To detect serum ANAs, microtiter plates (Nunc, Roskilde, Denmark) were incubated with 10 μg/mL antigen, and the nuclear fraction was prepared from normal liver.[8] Antibody sets to detect mouse ANA subclasses were the same as previously described. Serum concentrations of TNF-α and interferon gamma were measured with mouse cytokine ELISA sets (eBioscience) according to the manufacturer's protocols.

Isolation of Lymphocytes and Adoptive Transfer

For transfer of total spleen cells, BALB/c-NTx-*PD-1*$^{-/-}$ mice at 14 days after thymectomy were intraperitoneally injected every other day with or without 1.0 mg/kg DEX diluted in PBS. Single cells were isolated from the spleens of 3-week-old mice. Isolated spleen cells (1×10^7) were intravenously injected into *RAG2*$^{-/-}$ recipient mice on a BALB/c background at 4 to 6 weeks of age. To transfer CD4$^+$ T cells or CD4$^+$ T cell–depleted spleen cells, 3-week-old BALB/c-NTx-*PD-1*$^{-/-}$ mice were therapeutically injected with DEX. Cells were prepared using mouse CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocols. Purity was assessed by flow cytometry. CD4$^+$ T cells were purified to reach >90% purity, and CD4$^-$ splenocytes reached >99% purity. Isolated cells (1×10^6) were intravenously injected into BALB/c-*RAG2*$^{-/-}$ mice at 4–6 weeks of age.

For transfer of CD4$^+$ T cells from C57BL/6-*PD-1*$^{-/-}$ mice, CD3$^+$CD4$^+$ T cells were prepared from the spleens of 8-week-old C57BL/6-*PD-1*$^{-/-}$ mice with or without NTx obtained using a FACSAria II. Isolated CD3$^+$CD4$^+$ T cells (1×10^6) were intravenously injected into C57BL/6-*RAG2*$^{-/-}$ recipient mice at 4 to 6 weeks of age. For transfer, CD4$^+$CD25$^+$ Tregs were purified from the spleens of 8-week-old C57BL/6-*PD-1*$^{-/-}$ mice. Tregs were obtained using a FACSAria II and isolated with a CD4$^+$CD25$^+$ T cell to reach >99% purity. Tregs (1×10^6) were intravenously injected into C57BL/6-NTx-*PD-1*$^{-/-}$ mice at 4 weeks of age. Four weeks after transfer, the recipient mice at 8 weeks of age were killed.

HAI Score

Histological activity of chronic active hepatitis was assessed according to a semiquantitative scoring system, as described previously for humans.[9] For histological assessment, all specimens were reviewed in a blinded manner by at least 2 hepatologists. Using Knodell's HAI scoring system, liver specimens were graded in 4 categories. Category I, periportal and/or bridging hepatocellular necrosis, was graded from 0 to 10, where 0 = none, 1 = mild piecemeal necrosis, 3 = moderate piecemeal necrosis (involves less than 50% of the circumference of most portal tracts), 4 = marked piecemeal necrosis (involves more than 50% of the circumference of most portal tracts), 5 = moderate piecemeal necrosis plus bridging necrosis, 6 = marked piecemeal necrosis plus bridging necrosis, and 10 = multilobular necrosis. Category II, intralobular degeneration and focal hepatocellular necrosis, was graded from 0 to 4, where 0 = none, 1 = mild (acidophilic bodies, ballooning degeneration, and/or scattered foci of hepatocellular necrosis in less than one-third of lobules or nodules), 3 = moderate (involvement of one-third to two-thirds of lobules or nodules), and 4 = marked (involvement of more than two-thirds of lobules or nodules). Category III, portal inflammation, was graded from 0 to 4, where 0 = no portal inflammation, 1 = mild (sprinkling of inflammatory cells in less than one-third of portal tracts), 3 = moderate (increased inflammatory cells in one-third to two-thirds of portal tracts), and 4 = marked (dense packing of inflammatory cells in more than two-thirds of portal tracts). Category IV, fibrosis, was graded from 0 to 4, where 0 = no fibrosis, 1 = fibrous portal expansion, 3 = bridging fibrosis (portal-portal or portal-central linkage), and 4 = cirrhosis.

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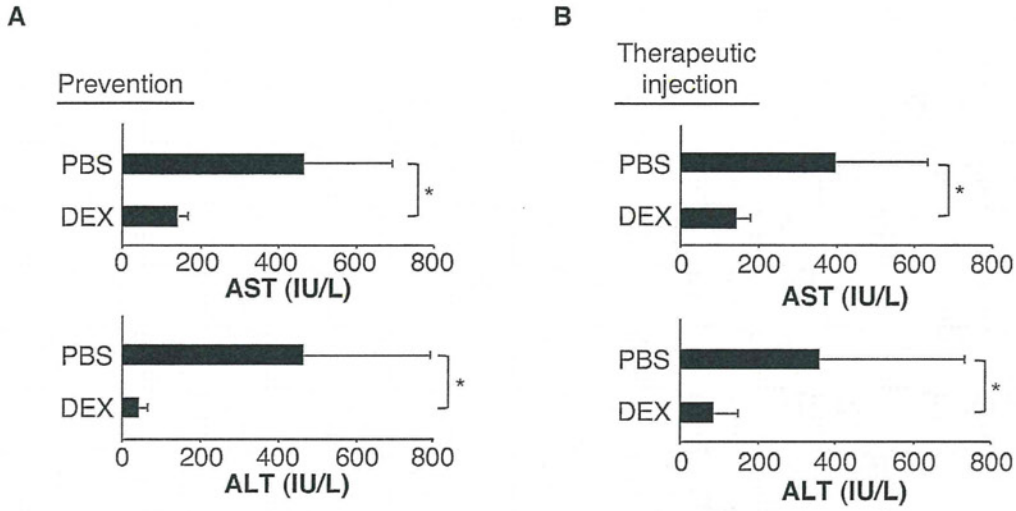
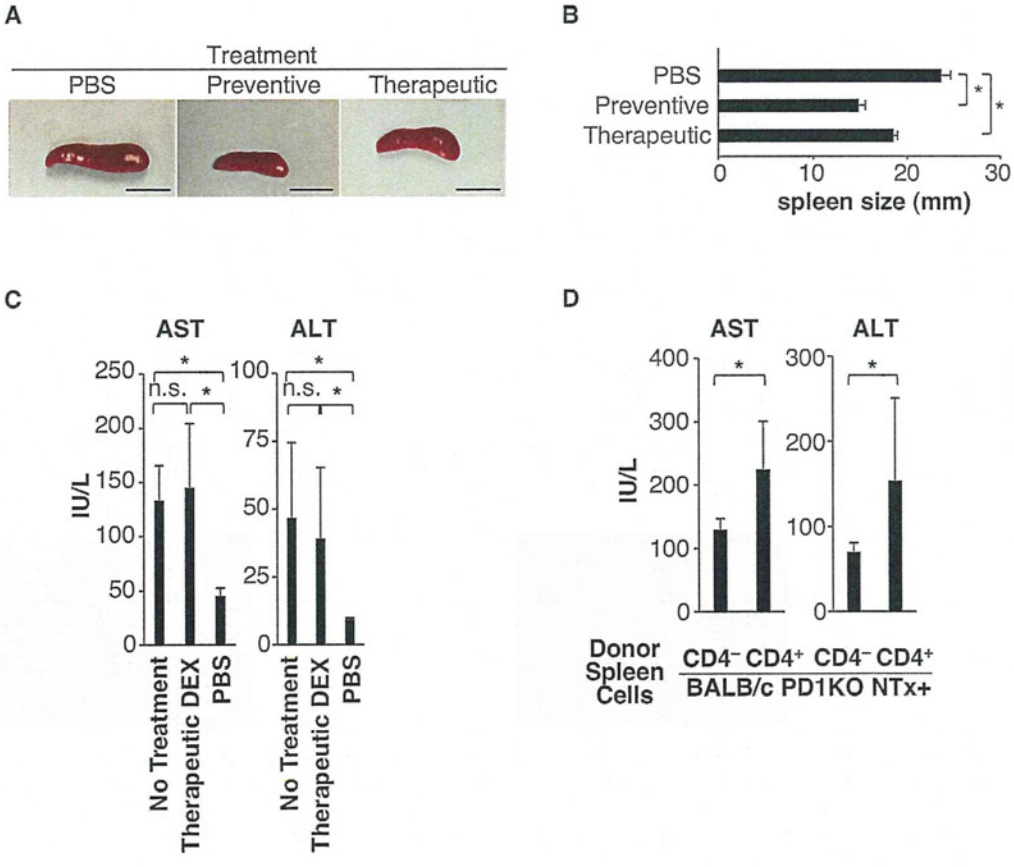
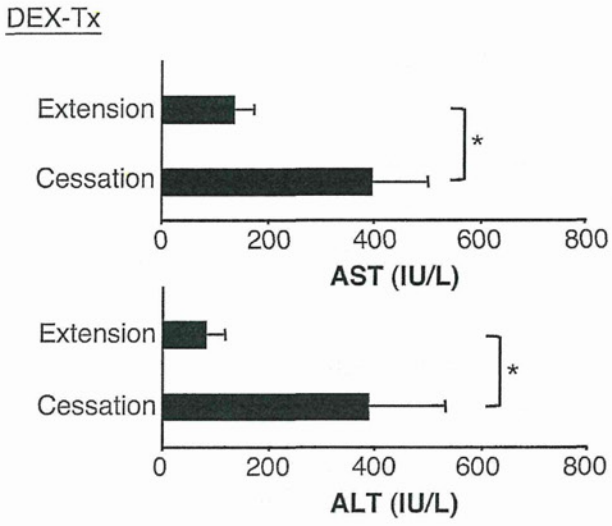


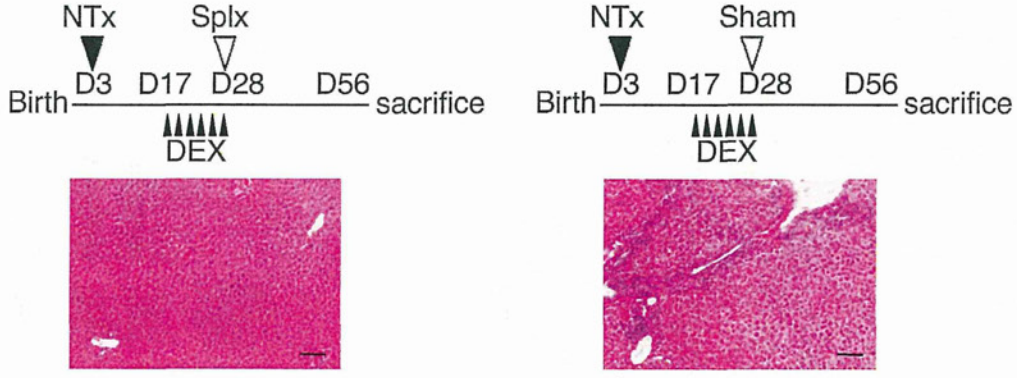
Supplementary Figure 1. Either preventive or therapeutic injection of DEX suppresses fatal AIH in BALB/c-NTx-*PD-1*$^{-/-}$ mice. (*A*) Starting 1 day after thymectomy, BALB/c-NTx-*PD-1*$^{-/-}$ mice were intraperitoneally injected every other day with 1.0 mg/kg DEX diluted in PBS (n = 5) or PBS alone (n = 17). After 13 injections, mice at 4 weeks of age were killed and sera were harvested. Serum levels of the liver transaminases AST and ALT are shown. (*B*) Starting 14 days after thymectomy, NTx-*PD-1*$^{-/-}$ mice were intraperitoneally injected every other day with 1.0 mg/kg DEX diluted in PBS (n = 11) or PBS alone (n = 13). After 6 injections, mice at 4 weeks of age were killed. Serum levels of AST and ALT are shown. *Bars* indicate the mean of each group, and the *error bars* indicate SD. *$P < .05$.



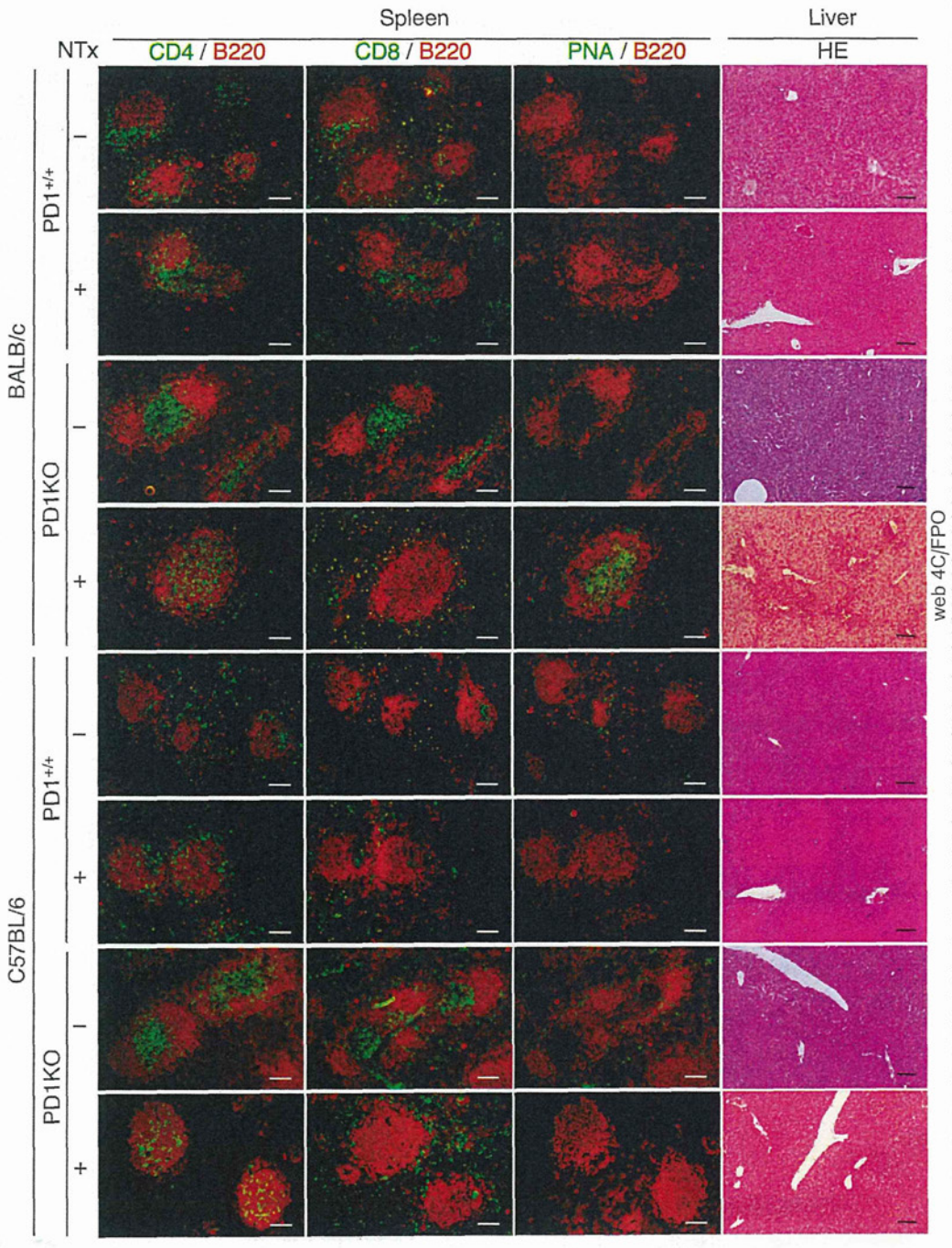
Supplementary Figure 2. Therapeutic administration of DEX reduces the size of the spleen, and residual CD4$^+$ T cells in the spleen of BALB/c-NTx-*PD-1*$^{-/-}$ mice can induce hepatitis. BALB/c-NTx-*PD-1*$^{-/-}$ mice were injected with DEX or PBS alone preventively or therapeutically, as described in Figure 1. (*A*) Macroscopic view of spleen. (*B*) Splenic sizes. (*C* and *D*) Serum levels of the liver transaminases AST and ALT in recipient BALB/c-*RAG2*$^{-/-}$ mice 3 weeks after transfer. Total splenocytes were solated and transferred from BALB/c-NTx-*PD-1*$^{-/-}$ mice with or without therapeutic injections of DEX (*C*). Purified splenic CD4$^+$ T cells or CD4$^+$ T cell–depleted splenocytes (CD4$^-$ cells) were transferred from BALB/c-NTx-*PD-1*$^{-/-}$ mice therapeutically treated with DEX (*D*). *Bars* indicate the mean of each group, and the *error bars* indicate SD. *$P < .05$. n.s., not significant. *Scale bars* = 1 cm.



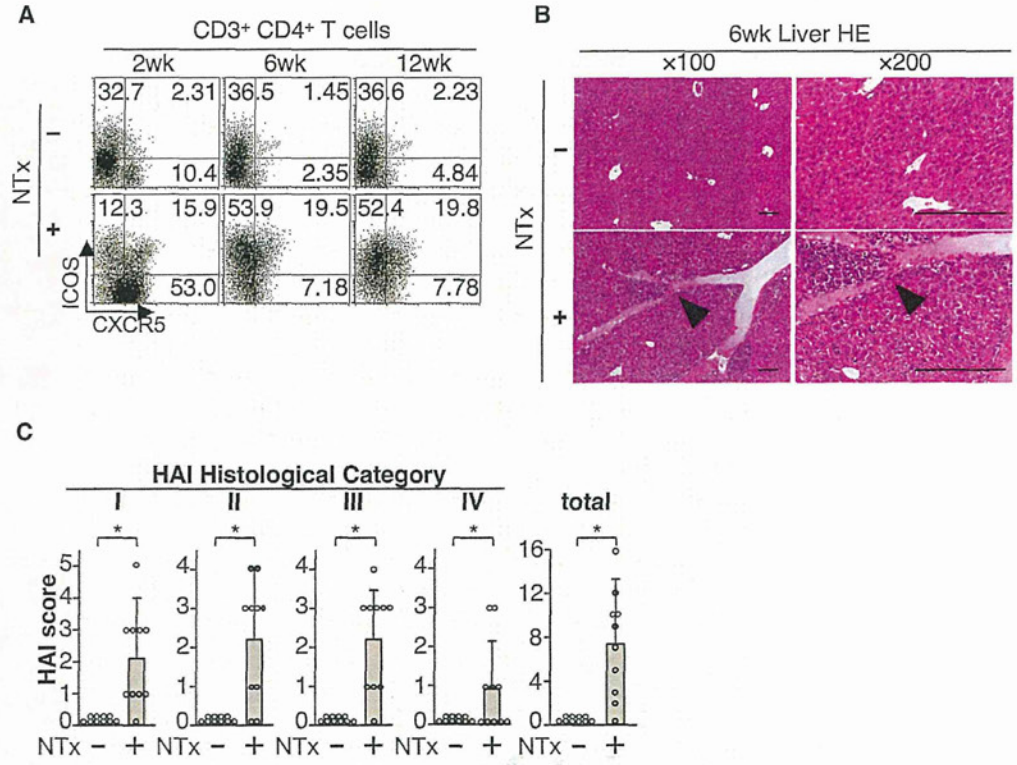
Supplementary Figure 3. Cessation of DEX therapy induces relapse of hepatitis in BALB/c–NTx–*PD-1*$^{-/-}$ mice. Starting 14 days after thymectomy, BALB/c–NTx–*PD-1*$^{-/-}$ mice were intraperitoneally injected every other day with 1.0 mg/kg DEX. Mice with DEX injections extended until 40 days of age (Extension, n = 7) and mice with cessation of DEX injections at 4 weeks of age (Cessation, n = 10) were killed at 40 days of age, and sera were harvested. Serum levels of the liver transaminases AST and ALT are shown. The *bars* indicate the mean of each group, and the *error bars* indicate SD. *$P < .05$.



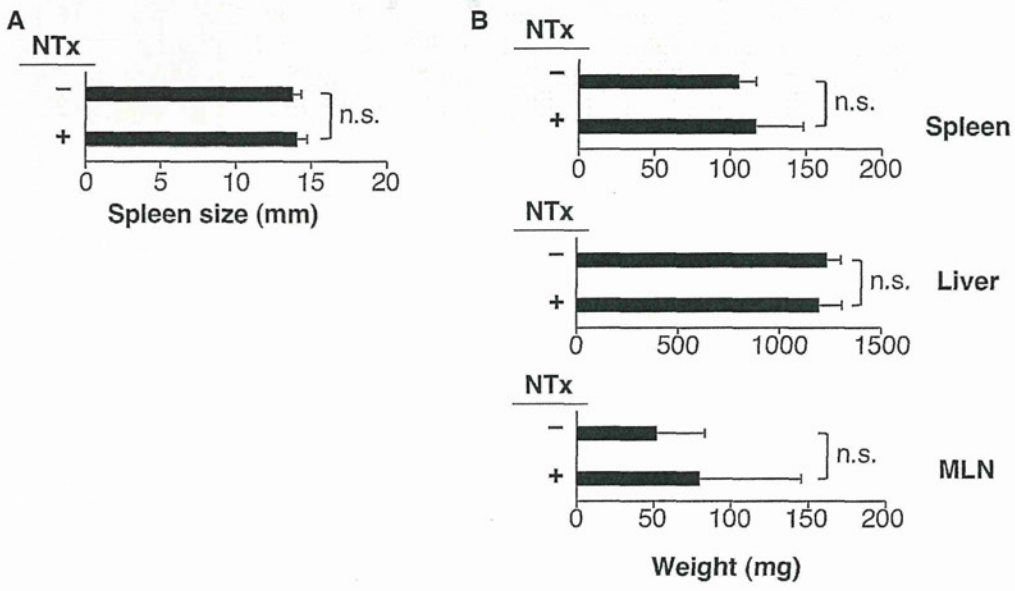
Supplementary Figure 4. Splenectomy overcomes therapeutic insufficiency of corticosteroids and induces prolonged remission of AIH in BALB/c–NTx–*PD-1*$^{-/-}$ mice. Starting 14 days after thymectomy, BALB/c–NTx–*PD-1*$^{-/-}$ mice were intraperitoneally injected every other day with 1.0 mg/kg DEX. After 6 injections, mice stopped receiving DEX injections and underwent splenectomy (Splx, n = 3) or sham operation (n = 3) at 28 days of age. Mice at 56 days of age were killed, and the livers were harvested. Histological analysis of the liver from each group is shown, with staining of the liver with H&E. All *scale bars* = 100 μm.



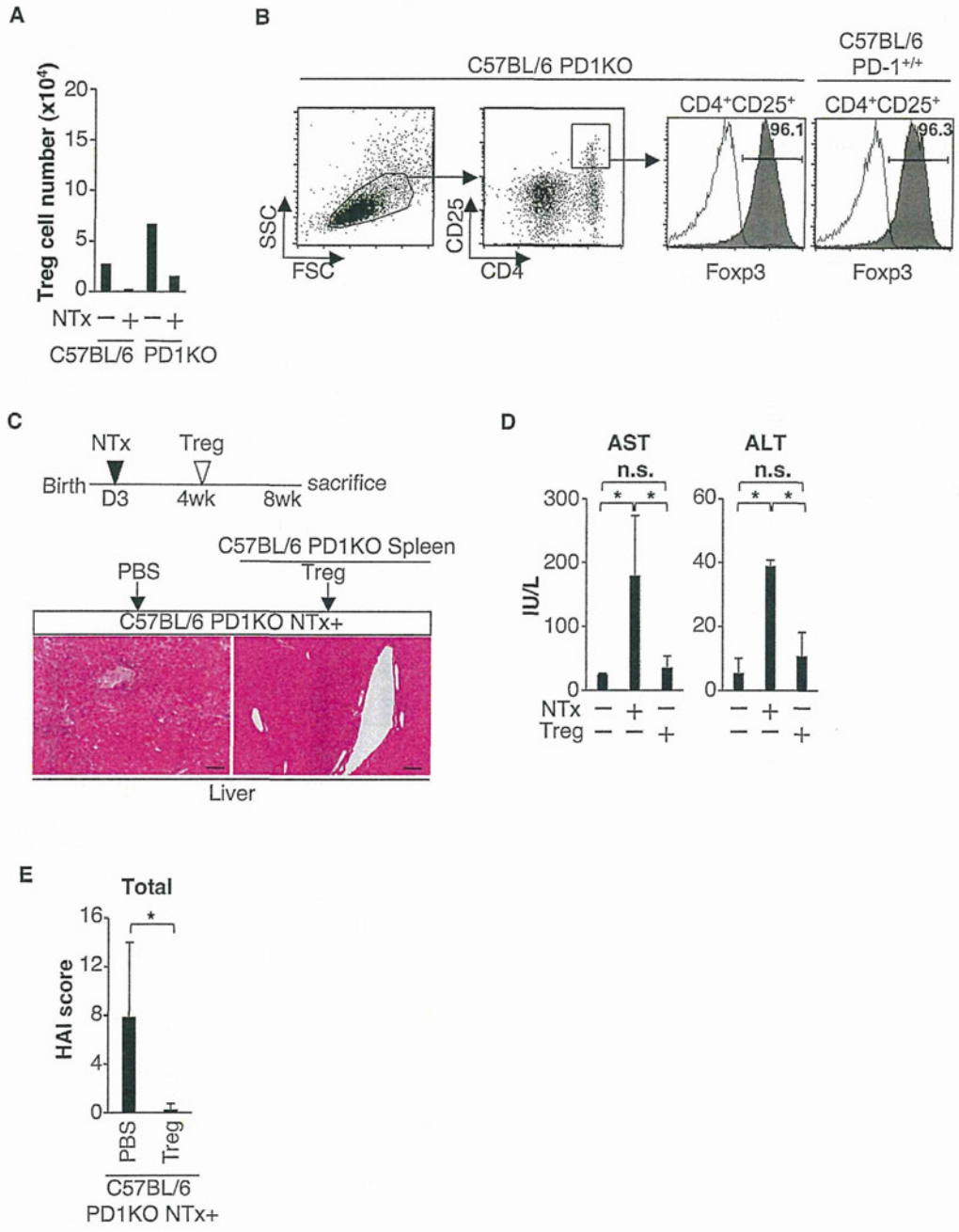
Supplementary Figure 5. Splenic $CD4^+$ T cells are preferentially localized within $B220^+$ B-cell follicles in NTx–*PD-1*$^{-/-}$ mice at 2 weeks of age, whereas PNA^+ germinal centers exist in $B220^+$ B-cell follicles in the spleen of AIH-bearing NTx–*PD-1*$^{-/-}$ mice on the BALB/c background but in not those on the C57BL/6 background. Immunohistological staining of the spleen (*left panels*) and H&E (HE) staining of the liver (*right panels*) is shown. The spleens and livers were from 2-week-old *PD-1*$^{+/+}$ mice or *PD-1*$^{-/-}$ mice with or without NTx on the BALB/c or C57BL/6 background. The 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*). All *scale bars* = 100 μm.



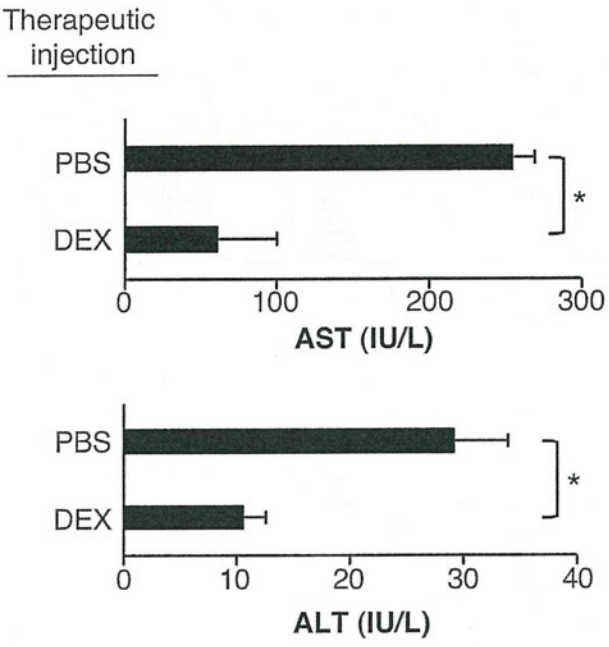
Supplementary Figure 6. Adult C57BL/6–NTx–*PD-1*$^{-/-}$ mice with increased numbers of splenic T_{FH} cells develop chronic hepatitis. (*A*) Flow cytometric analysis of splenic CD4$^+$ T cells in C57BL/6–*PD-1*$^{-/-}$ mice with or without NTx at indicated ages. The cells were stained with FITC/anti-CXCR5, PE/anti-ICOS, and APC-Cy7/anti-CD4. The numbers in plots indicate the percentage of cells in each gate in the CD4$^+$ T-cell population. (*B*) Histological findings from the livers of 6-week-old C57BL/6–*PD-1*$^{-/-}$ mice with or without NTx. In contrast to mice without NTx, those with NTx showed dense packing of inflammatory cells marked in portal branches (*black arrowhead*). *Scale bars* = 100 μm. (*C*) Knodell's HAI score for livers in 8-week-old C57BL/6–*PD-1*$^{-/-}$ mice with (n = 10) or without NTx (n = 10). HAI score is graded in 4 categories: I, periportal and/or bridging necrosis; II, intralobular degeneration and focal hepatocellular necrosis; III, portal inflammation; and IV, fibrosis. All are described in Supplementary Materials and Methods.



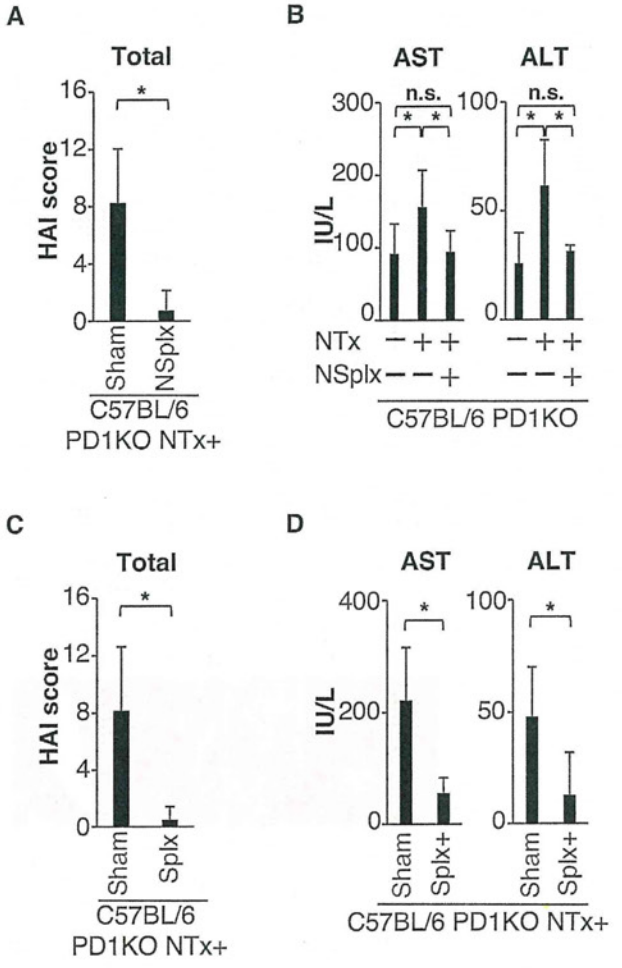
Supplementary Figure 7. Adult C57BL/6–NTx–*PD-1*$^{-/-}$ mice do not show obvious splenomegaly. (*A*) Splenic sizes and (*B*) weights of the spleen, liver, and mesenteric lymph node (MLN) in 12-week-old C57BL/6-*PD-1*$^{-/-}$ mice with or without NTx. *Bars* indicate the mean of each group, and the *error bars* indicate SD. n.s., not significant.



Supplementary Figure 8. Transfer of Tregs has therapeutic efficacy for chronic AIH in C57BL/6–NTx–*PD-1*$^{-/-}$ mice. (*A*) Flow cytometric analysis of Tregs in the liver from the indicated mice at 6 weeks of age. The cells were stained with FITC/anti-CD4, PE/anti-CD3, and APC/anti-CD25. The numbers of $CD3^+CD4^+CD25^+$ Tregs were calculated as follows: (Percentage of Cells in the Cell Types) × (Number of Viable Cells). Data shown are from one of 3 separate experiments. (*B*) Flow cytometric analysis of $CD4^+CD25^+$ T cells in the spleen from the 8-week-old C57BL/6–*PD-1*$^{-/-}$ or *PD-1*$^{+/+}$ mice. The cells were isolated from the spleen and stained with FITC/anti-CD4, APC/anti-CD25, and PE/anti-Foxp3. *Filled histograms* represent staining of $CD4^+CD25^+$ T cells with Foxp3, and *open histograms* represent the isotype control. Data represent one of 5 experiments. The numbers in histograms indicate percentages of $Foxp3^+$ cells in viable $CD4^+CD25^+$ T cells. (*C–E*) For transfer of Tregs, 1×10^6 of Tregs were prepared from 8-week-old C57BL/6–*PD-1*$^{-/-}$ mice. Four-week-old C57BL/6–NTx–*PD-1*$^{-/-}$ mice were intravenously injected with 1×10^6 Tregs (n = 5) or PBS (n = 5). Recipient mice were analyzed at 8 weeks of age. (*C*) Histological findings of the liver. *Scale bars* = 100 μm. (*D*) Serum levels of the liver transaminases AST and ALT. (*E*) Knodell's HAI score. *Bars* indicate the mean of each group, and the *error bars* indicate SD. *$P < .05$. n.s., not significant.



Supplementary Figure 9. Therapeutic injections of DEX suppress chronic AIH in C57BL/6–NTx–*PD-1*$^{-/-}$ mice. Intraperitoneal injections of DEX (n = 5) or PBS (n = 5) were started at 4 weeks of age in C57BL/6–NTx–*PD-1*$^{-/-}$ mice. After 14 injections every other day at 8 weeks of age, mice were killed and examined. Serum levels of the liver transaminases AST and ALT are shown. *Bars* indicate the mean of each group, and the *error bars* indicate SD. *$P < .05$.



Supplementary Figure 10. The spleen is the induction site of chronic AIH in C57BL/6–NTx–*PD-1*$^{-/-}$ mice, and splenectomy suppresses chronic AIH. (*A* and *B*) C57BL/6–NTx–*PD-1*$^{-/-}$ mice underwent a splenectomy (NSplx, n = 5) or a sham operation (n = 5) at 1 day after NTx and were analyzed at 8 weeks of age. (*C* and *D*) Four-week-old C57BL/6–NTx–PD-1$^{-/-}$ mice underwent a splenectomy (Splx, n = 5) or a sham operation (n = 5) and were analyzed at 8 weeks of age. (*A* and *C*) Total HAI scores for livers were as described in Supplementary Materials and Methods. (*B* and *D*) Serum levels of the liver transaminases AST and ALT are shown. *Bars* indicate the mean of each group, and *horizontal short bars* indicate SD. *$P < .05$. n.s., not significant. *Scale bars* = 100 μm.

Supplementary Table 1. Incidence of Autoimmunity in Various Organs of C57BL/6 PD1KO Mice With or Without NTx

Mice	C57BL/6 PD1KO NTx+				C57BL/6 PD1KO NTx−			
Age (Month) (Total number)	~1M (n=14)	1~2M (n=19)	2~3M (n=15)	3M~ (n=12)	~1M (n=12)	1~2M (n=15)	2~3M (n=16)	3M~ (n=16)
Hepatitis	6 (42.9%)	16 (84.2%)	15 (100%)	12 (100%)	0 (0%)	0 (0%)	0 (0%)	2 (12.5%)
Sialoadenitis	0 (0%)	9 (47.4%)	5 (33.3%)	4 (33.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Pancreatitis	0 (0%)	7 (36.8%)	4 (26.7%)	2 (16.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Myocarditis	0 (0%)	3 (15.8%)	2 (13.3%)	2 (16.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Nephritis	0 (0%)	2 (10.5%)	2 (13.3%)	2 (16.7%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Gastritis	0 (0%)	0 (0%)	0 (0%)	1 (8.3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Enteritis	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Pneumonitis	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)

The tissues of various organs in C57BL/6 PD1KO mice with or without NTx at the indicated ages (n = 12~19 in each group) were evaluated histologically. Incidence was determined by inflammation characterized by slight infiltration of mononuclear cells in the organs by lymphocytes.

Supplementary Table 2. Survival Rate With or Without Splenectomy

Mice	C57BL/6 PD1$^{-/-}$NTx+ Splx 6 week	
Splenectomy	+	−
Total number	n = 8	n = 16
Survival rate at 20 weeks of age	8 (100%)	16 (100%)