Supplemental information

Intestinal barrier regulates immune responses in the liver via interleukin-10-producing macrophages

Nobuhito Taniki[1]、 Nobuhiro Nakamoto[1*], Po-Sung Chu[1], Yohei Mikami[1], Takeru Amiya[1, 2], Toshiaki Teratani[1], Takahiro Suzuki,[1] Tomoya Tsukimi[3], Shinji Fukuda[3,4], Akihiro Yamaguchi[1], Shunsuke Shiba[1], Rei Miyake[1], Tadashi Katayama[1], Hirotoshi Ebinuma[1], and Takanori Kanai[1*]

Supplemental Materials and Methods

Experimental Protocols of α-Galcer-Induced Hepatitis

α-Galcer was purchased from Kirin (Tokyo, Japan). α-Galcer (0.1 mg/kg) were intravenously administered 24 hours before liver resection.

Experimental Protocols of TNBS-Induced colitis

Mice were applied 150 ml of the TNBS presensitization solution (Mixture of acetone/olive oil (4:1) with 5% TNBS solution, final concentration of TNBS was 1% (w/v)) to the shaved abdominal skin on day 1, control mice are treated with presensitization solution without TNBS and leaved until day 8. Anesthetize the mouse by intraperitoneal injection of 80 ml of ketamine/xylazine solution per 10 g body weight. Mice were administered 100 m l of TNBS solution (Mixture of absolute ethanol with 5% TNBS solution, final concentration of TNBS was 1% (w/v)) into the colon lumen using 3.5 F catheter and kept with the head down in a vertical position for 60 s on day 8; 48hr before Con A administration.

Measurement of serological and histological liver injury

Serum alanine aminotransferase (ALT) levels were measured using Fuji DRI-CHEM (Fuji Film, Tokyo, Japan) according to the instructions provided by the manufacturer. Livers were fixed in 10 % formalin and embedded in paraffin. Sections were stained with H&E and examined.

Experimental Protocols of Terminal deoxynucleotidyl transferase–mediated deoxyuridine nick-end labeling (TUNEL) assay

Terminal deoxynucleotidyl transferase–mediated deoxyuridine nick-end labeling staining (Chemicon International, Temecula, CA) was performed on specimens to assess apoptosis. Apoptosis was quantified by

counting positively stained cells in 5 random fields at 40_magnification. Apoptosis was measured for each specimen as a percentage of total cells per field. Antibody binding was detected by incubation with biotinylated anti-mouse immunoglobulin G antibody and visualized with a Vectastain Elite ABC Kit (Vector Laboratories, Inc) by reaction with Vectastatin DAB Substrate (Vector).

Preparation of liver mononuclear cells (MCs)

Briefly, livers were perfused through the portal vein with phosphate-buffered saline and then minced and passed through 100 μm nylon mesh. The filtrate was centrifuged at 50 x g for 1 min, and the supernatant was washed once. Cells were suspended in a Histopaque solution (Sigma-Aldrich, St. Louis, MO, USA) and overlaid on an HBSS solution. After centrifugation at 2,500 rpm for 20 min, the cells were collected from the upper face of the Histopaque.

Preparation of Intestinal lamina propria mononuclear cells (LPMCs)

The colon was removed and placed in Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution (HBSS; Nacalai Tesque, Japan). After removal of the mesentery, the intestine was opened longitudinally, thoroughly washed in HBSS and cut into small pieces. The dissected mucosa was incubated with HBSS containing 1 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO) and 5 mM EDTA (Gibco, Carlsbad, CA, USA) for 30 min at 37 °C to remove the epithelial layer. The pieces of intestine were washed and placed in digestion solution containing 1.5 % FBS, 1.0 mg/mL collagenase A (Roche Diagnostics GmbH, Germany) and 0.1 mg/mL DNase (Sigma-Aldrich, St. Louis, MO) for 1 hr at 37 °C Intestine supernatants were washed, resuspended in 40 % Percoll and overlaid on a 75 % Percoll fraction. Percoll gradient separation was performed by centrifugation at 840 × g for 20 min at room temperature. Mononuclear cells were collected at the interphase, washed, and resuspended in FACS buffer or RPMI-1640 medium (Sigma-Aldrich) containing 10 % FBS and 1 % penicillin/streptomycin

(Nacalai Tesque, Japan).

Antimouse mAbs

FITC-, PE-, APC-, Percp-, APC-Cy7-, PE-Cy7- labeled antibodies to CD3e (clone 145-2C11), CD4 (RM4-5), CD8α (53-6.7), CD11b (M1/70), CD11c (HL3), Ly6C (AL-21), Ly6G (1A8), IA^b (AF6-120.1), CD62L (MEL-14), CD25 (PC61), 7-AAD were purchased from BD Pharmingen. F4/80 (BM8), CCR9 (eBioCW-1.2), CSF1R (AFS98), Siglec H (551), Foxp3 (FJK-16S), NKp46 (29A1.4) were purchased from eBioscience. CLEC4F polyclonal Goat IgG antibody was purchased from R&D systems and FITC- labeled Donkey anti-Goat IgG polyclonal secondary antibody was purchased from invitrogen. Background fluorescence was assessed by the irrelevant anti-rat isotype (BD Pharmingen). The stained cells were analyzed and sorted using FACS Canto II and FACS Aria (Becton Dickinson, NJ, USA), and the data were analyzed using Flowjo software (Tree star Inc., OR, USA). For intracellular cytokine staining, cells were stimulated for 4 h with lipopolysaccharide (LPS, from Escherichia coli B5, Sigma-Aldrich) in the presence of brefeldin A (10 μg/ml) before surface staining, followed by permeabilization and intracellular staining with anti TNF (MP6-XT22) and IL-10 (JES5-16E3) mAb (BD Pharmingen). To detect IFN-γ production from $CD3^+$ T cells, hepatic MCs were stimulated with PMA (50 ng/ml) and Ionomycin (500 ng/ml) for 4 h in the presence of brefeldin A (10 μg/ml), followed by surface staining, permeabilization and intracellular staining with anti- IFN-γ (XMG1.2) mAb (BD Pharmingen).

Quantitative RT-PCR (qPCR)

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized from 100 ng of total RNA using TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). The level of target gene expression was normalized to the β-actin gene (Applied Biosystems). The

following probes were purchased from Applied Biosystems: *Actb* (01205647_g1), *Tnf* (00443258_m1), *Il-6* (00446190_m1), *Il-10* (01288386_m1), *Tgfb1* (03024053_m1), *Irak3* (00518541_m1), *Nnmt* (00447994_m1)

In vitro proliferation assay

$CD11b^+F4/80^+$ cells from the livers of DW-Con A and DSS-Con A mice as antigen presenting cells, and $CD4^+$ $CD62L^+$ splenocytes obtained from OT-II mice as enriched naïve $CD4^+$ T cells were isolated using FACS Aria (Becton Dickinson Co. Franklin Lakes, NJ, USA). Enriched naïve $CD4^+$ T cells labeled with 1 mM Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) (Molecular Probes, Eugene, OR, USA) for 10 min at 37 °C followed by the addition of 1.0 ml of FCS for 2 min and washed three times in PBS. CFSE-labeled $CD4^+$ naïve cells (1×10^5 cells/well) were co-cultured with isolated macrophages (2×10^4 cells/well) in 96-well round-bottom plates for 72 h in the presence of OVA peptides (1 μM). After incubation, cells were collected, incubated with anti-CD4-PE-Cy7 and anti-CD3-APC-Cy7 and analyzed by flowcytometry; 7-AAD was added to exclude dead cells. Proliferation analysis is based on division times of $CFSE^+$ $CD4^+$ T cells.

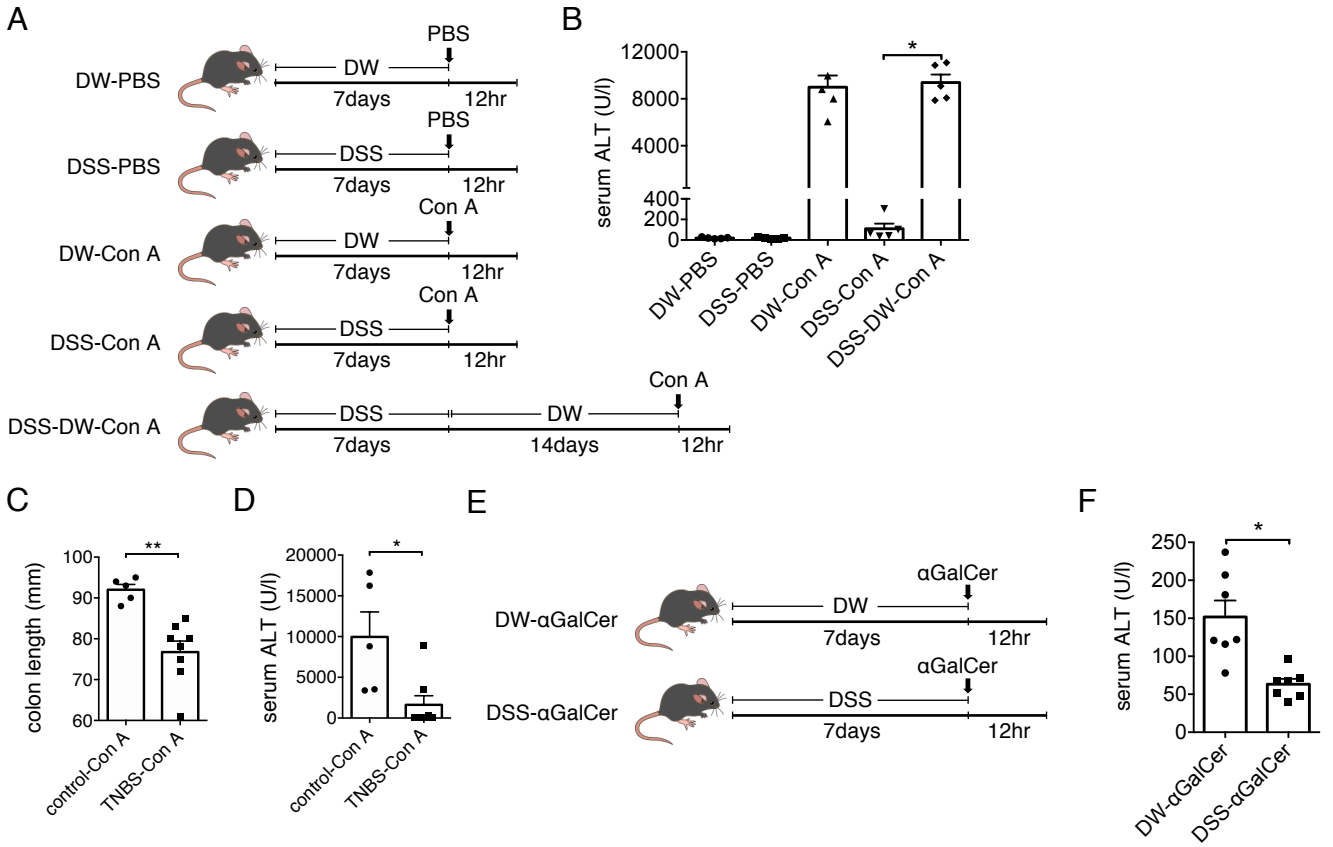
In vitro lymphocytes stimulation with plasma

The cells at a concentration of 3×10^5 /well were resuspended in plasma at a total volume of 100 μl in flat 96-well culture plates and incubated at 37 °C with 5 % CO_2 6 hours, followed by LPS stimulation and intracellular IL-10 staining.

CE-TOFMS-based metabolome analysis

Briefly, plasma metabolites were extracted by vigorous shaking with methanol containing 20 μM each of methionine sulfone and D-camphol-10-sulfonic acid as internal standards. All CE-TOFMS experiments were performed using an Agilent CE capillary electrophoresis system (Agilent Technologies), an Agilent G3250AA

LC/MSD TOF system (Agilent Technologies), an Agilent 1100 series binary HPLC pump, a G1603A Agilent CE-MS adapter, and a G1607A Agilent CE-ESI-MS sprayer kit. Hierarchical cluster analysis of metabolome data was performed based on Person's correlation using the Multiexperiment Viewer (MeV) version 4.9.0. Metabolite concentrations were plotted using R (version 3.1.2) along with the ggplot2 (version 2.2.1) package.



Supplementary Figure 1. Mice recovered from DSS-induced colitis develop severe Con A-related hepatitis and tandem model of TNBS-Con A and DSS-α-Galcer also exhibited significantly milder liver injury. (A) DSS-DW-Con A-treated mice received DW for 3 weeks following DSS treatment, followed by Con A administration. (B) Serum ALT levels in mice from each experimental group (n = 5/group). Data represent the mean ± SEM. *P < 0.05 according to one-way analysis of variance with Tukey's multiple-comparison correction. (C) Colon length of control-Con A or TNBS-Con A mice. (D) Serum ALT levels in mice from each experimental group (n = 5-8/group). Data represent the mean ± SEM. *P < 0.05, **P < 0.01 according to one-way analysis of variance with Tukey's multiple-comparison correction. (E) Mice were either untreated or orally administrated 2.0% DSS, followed by α-Galcer (0.1 mg/kg) administration. (F) Serum ALT levels in mice from of each experimental group (n = 7/group). Data represent the mean ± SEM. *P < 0.05 according to a two-tailed Student's t test.

Supplementary Figure 1
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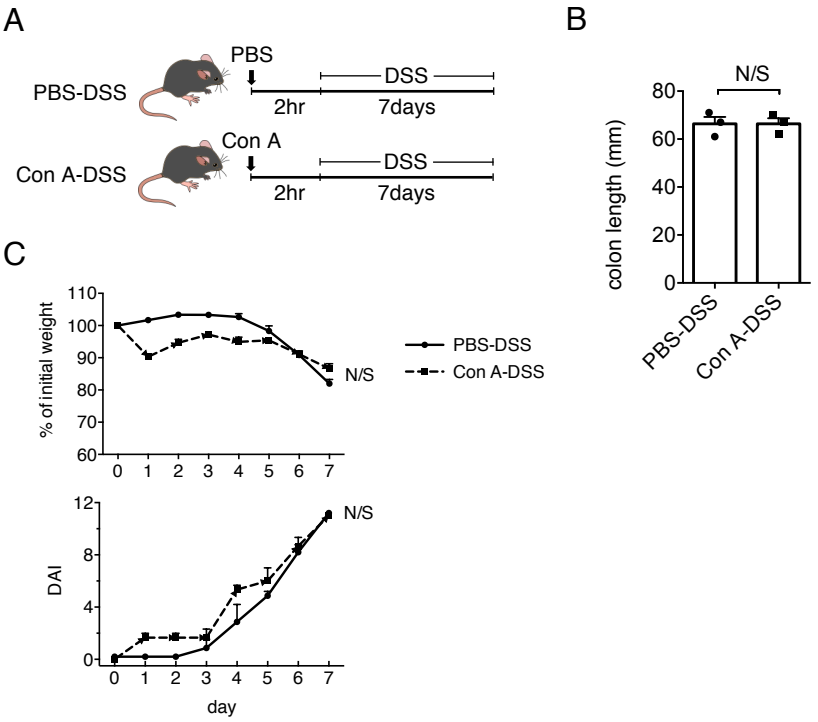

Supplementary Figure 2. Con A pretreatment does not ablate DSS-induced colitis. (A) Mice were administered Con A (20 mg/kg), followed by oral administration of DSS. (B) Colon length in mice from each experimental group (n = 3/group). Data represent the mean ± SEM. Differences were calculated according to a two-tailed Student's t test. (C) Mean body weight and DAI on days 0 to 7 in mice from each experimental group (n = 3/group).

Supplementary Figure 2
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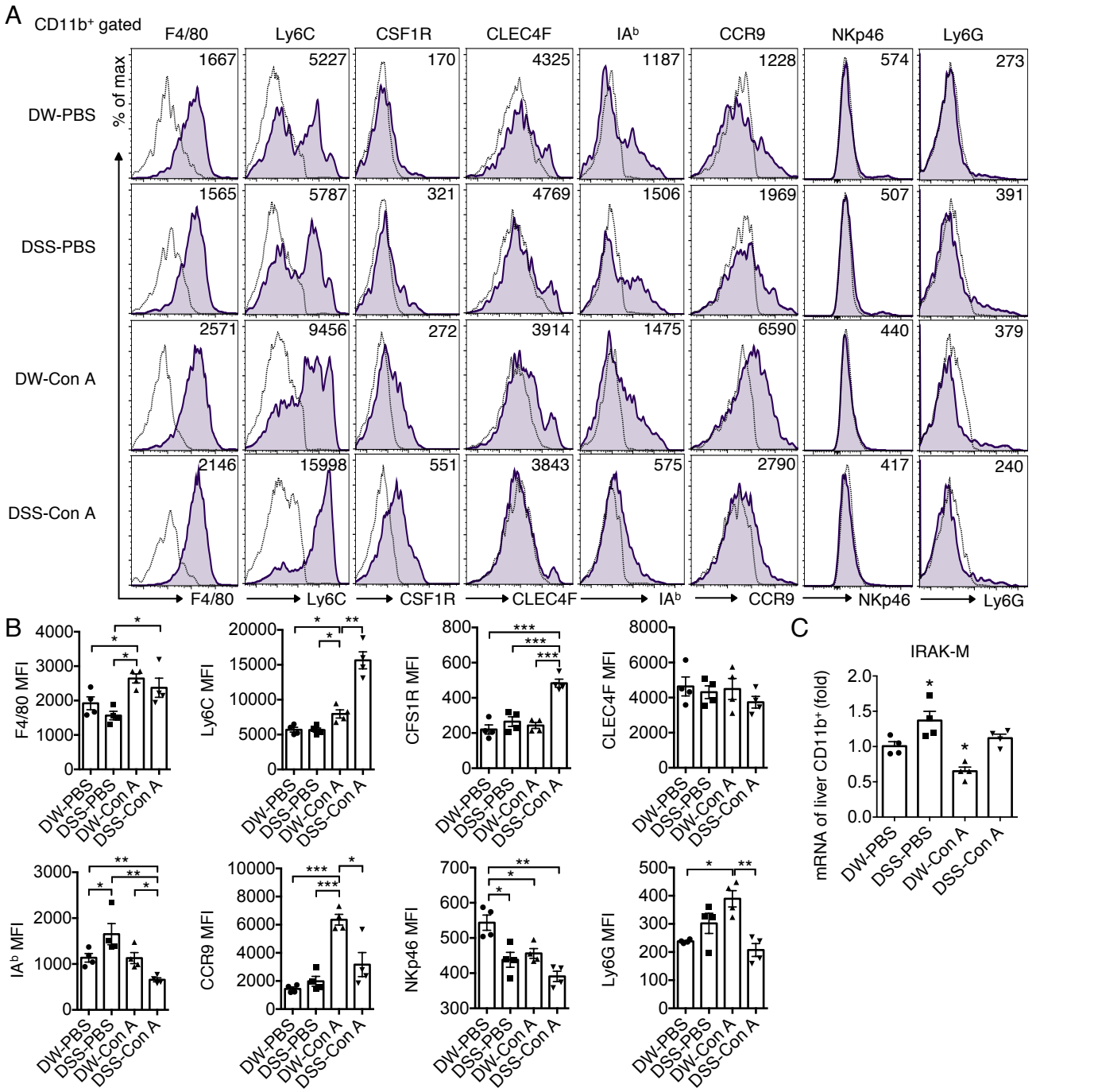

Supplementary Figure 3. Phenotypic characteristics of DSS-Con A macrophages. (A) Representative F4/80, Ly6C, CSF1R, IA^b, CLEC4F, CCR9, NKp46 and Ly6G staining of gated CD11b$^+$ cells in the livers of mice in each experimental group. Each number indicates the mean fluorescent intensity (MFI) of each marker in CD11b$^+$ cells. (B) Mean fluorescence intensity of F4/80, Ly6C, CSF1R, CLEC4F, IA^b, CCR9, NKp46 and Ly6G in gated CD11b$^+$ cells isolated from the livers of mice in each experimental group (n = 4/group). Data represent the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 according to one-way analysis of variance with Tukey's multiple-comparison correction. (C) IRAK3 mRNA expression in CD11b$^+$ cells in the livers of mice from each experimental group (n = 4/group). Data represent the mean ± SEM. *P < 0.05 according to one-way analysis of variance with Tukey's multiple-comparison correction compared with DW-PBS group.

Supplementary Figure 3
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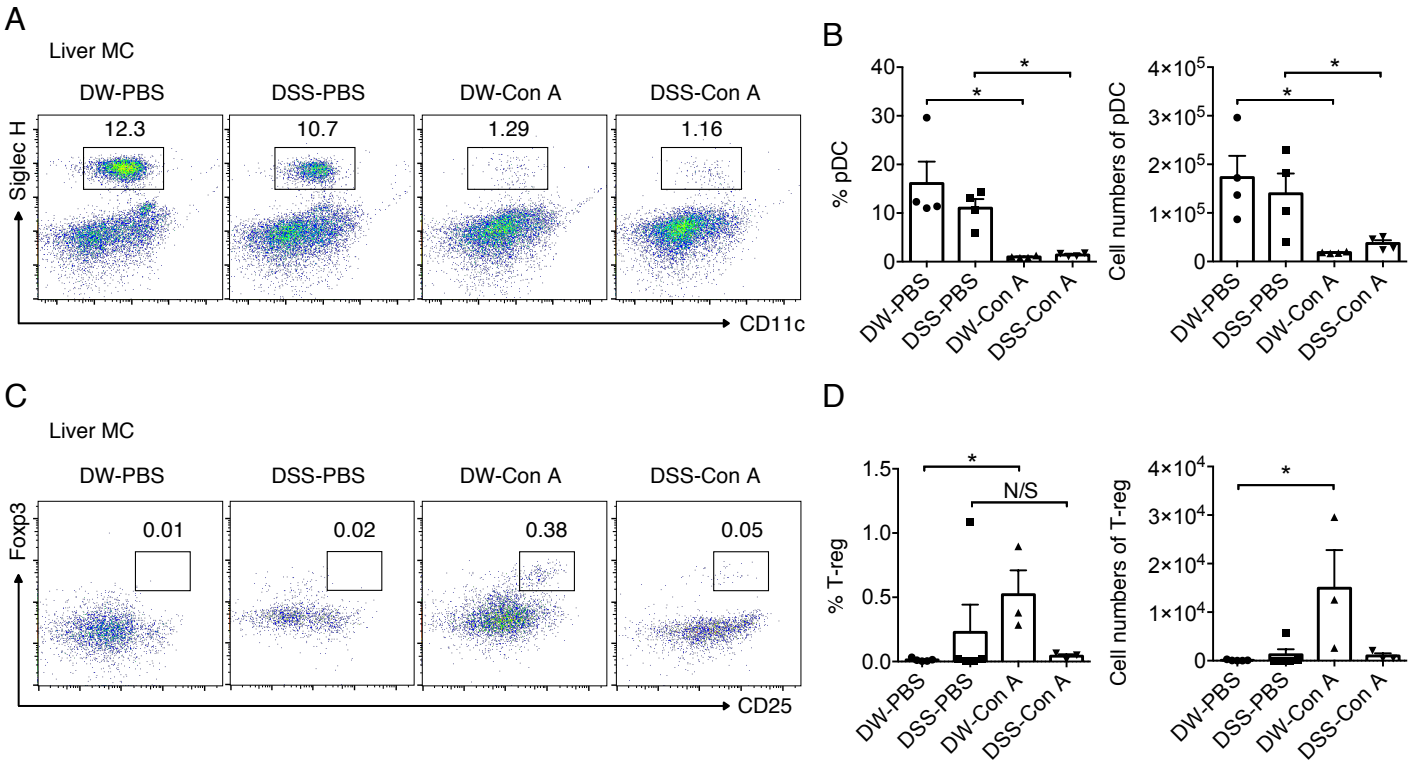

Supplementary Figure 4. The number of pDCs and regulatory T cells are decreased in the livers of DSS-Con A-treated mice. (A) Representative CD11c$^+$ and Siglec H$^+$ staining of whole-liver MCs isolated from mice in each experimental group. Each box and number indicates the population and the percentage of pDCs in whole-liver MCs. (B) Percentage and number of pDCs in whole-liver MCs (n = 4/group). (C) Representative surface CD25 and intracellular forkhead box P3 staining of whole-liver MCs isolated from each experimental group. Each box and number indicates the population and the percentage of regulatory T cells in whole-liver MCs. (D) Percentage and number of regulatory T cells in whole-liver MCs (n = 3–5/group). Data represent the mean ± SEM. *P < 0.05 according to one-way analysis of variance with Tukey's multiple-comparison correction.

Supplementary Figure 4
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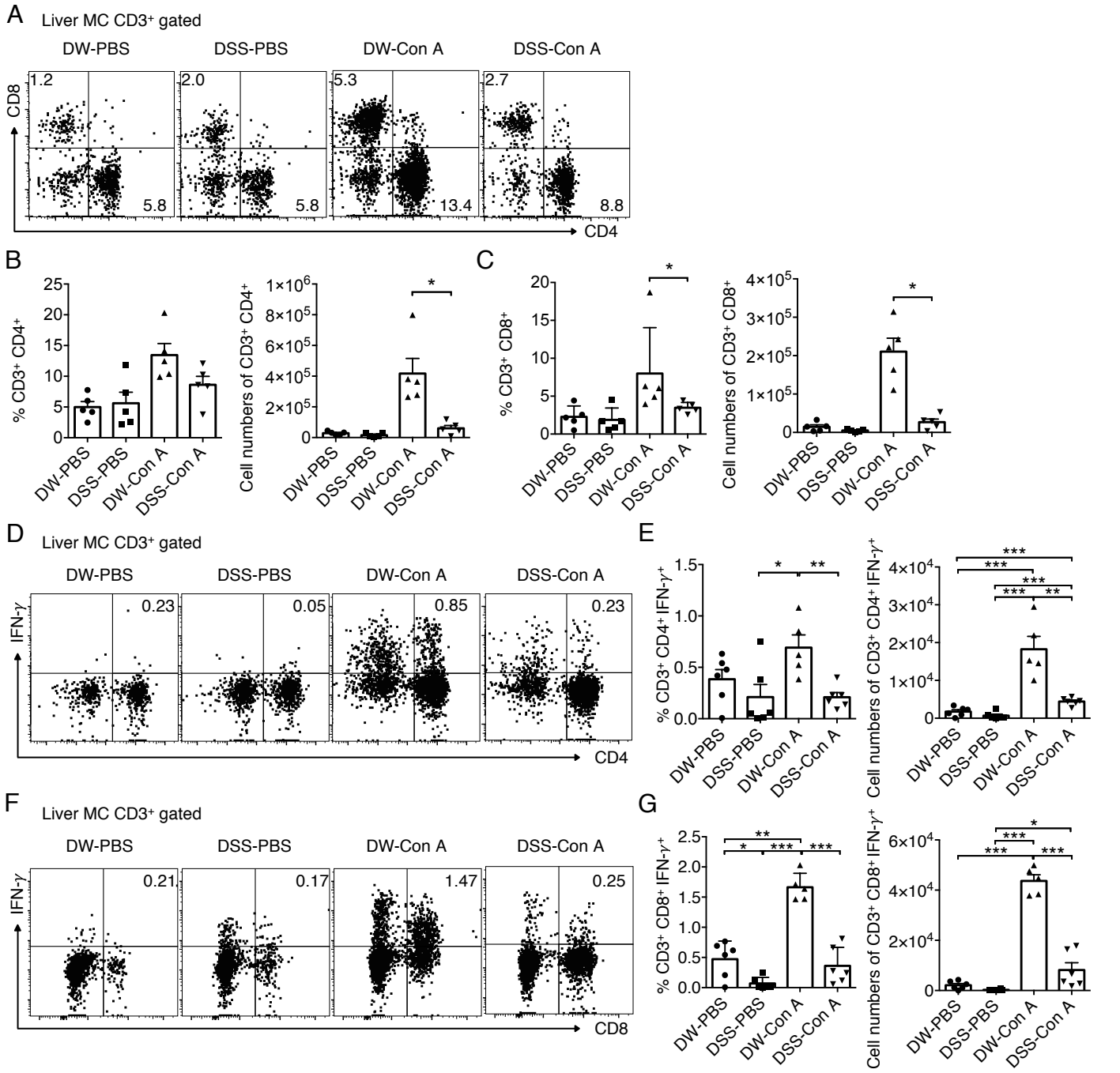

Supplementary Figure 5
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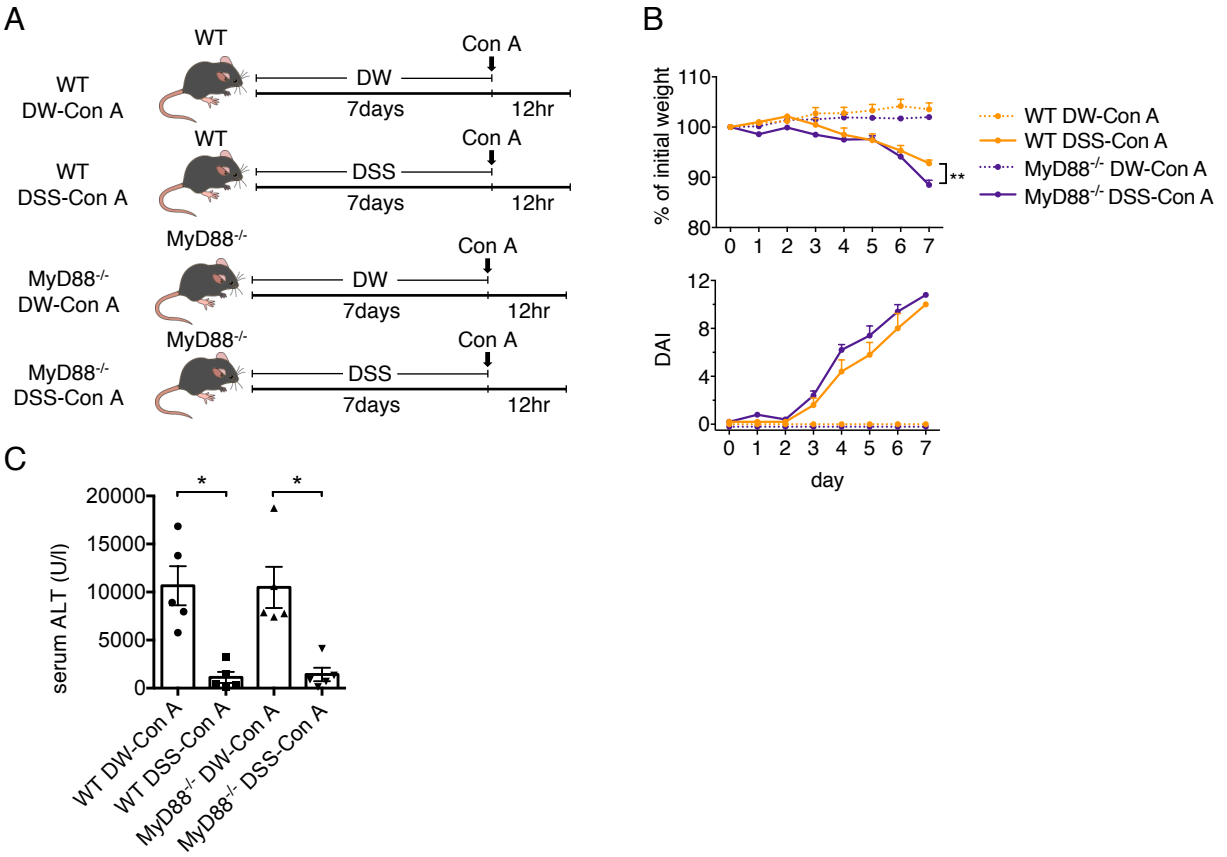
Supplementary Figure 5. Hepatic Th1 accumulation is attenuated following DSS-Con A treatment. (A) Representative CD4 and CD8 staining of gated $CD3^+$ cells from mice in each experimental group. Each number indicates the percentage of positive cells in whole-liver MCs. (B) Percentage and number of $CD3^+CD4^+$ cells in whole-liver MCs from mice in each experimental group (n = 5/group). (C) Percentage and number of $CD3^+CD8^+$ cells in whole-liver MCs from mice in each experimental group (n = 5/group). (D) Representative surface CD4 and intracellular IFN-γ staining of gated $CD3^+$ cells from mice in each experimental group after 4 h of PMA/Ionomycin stimulation. Each number indicates the percentage resident in whole liver MCs. (E) Percentage and number of $CD3^+CD4^+$ IFN-γ^+ cells in the livers of mice from each experimental group (n = 5-6/group). (F) Representative surface CD8 and intracellular IFN-γ staining of gated $CD3^+$ cells from mice in each experimental group after 4 h of PMA/Ionomycin stimulation. Each number indicates the percentage resident in whole liver MCs. (G) Percentage and number of $CD3^+CD8^+$ IFN-γ^+ cells in the livers of mice from each experimental group (n = 5-6/group). Data represent the mean ± standard error of the mean. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ according to one-way analysis of variance with Tukey's multiple-comparison correction.



Supplementary Figure 6. MyD88-deficient mice exhibit similar induction of immune tolerance in the liver following DSS-Con A treatment. (A) Myd88$^{-/-}$ mice were either untreated or orally administrated 2.0% DSS, followed by Con A (20 mg/kg) administration. (B) Mean body weight and DAI on days 0 to 7 in mice from each experimental group (n = 5–7/group). (C) Serum ALT level in mice from each experimental group (n = 5/group). Data represent the mean ± SEM. *P < 0.05 according to one-way analysis of variance with Tukey's multiple-comparison correction.

Supplementary Figure 6
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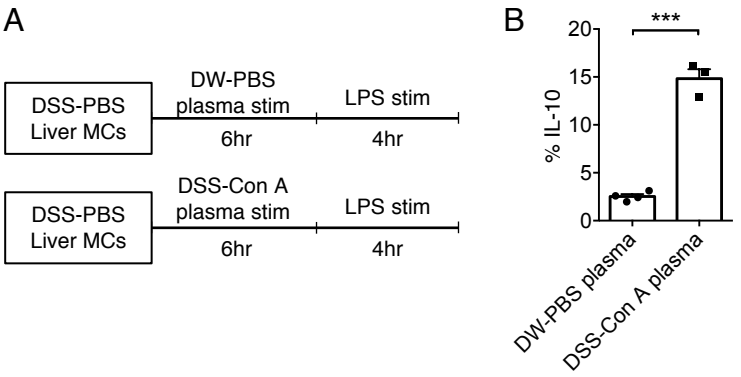

Supplementary Figure 7. Plasma derived from DSS-Con A mice induced higher IL-10 production from DSS-PBS liver CD11b$^+$ cells. (A) Liver MCs from DSS-PBS-treated mice were cultured with plasma derived from DW-PBS- or DSS-Con A-treated mice for 6 h, followed by 4-h LPS stimulation. (B) Percentage of IL-10$^+$ cells in liver CD11b$^+$ cells (n = 3–4/group). Data represent the mean ± SEM. ***P < 0.001 according to a two-tailed Student's t test.

Supplementary Figure 7
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