Tissue-expressed B7-H1 Critically Controls Intestinal Inflammation

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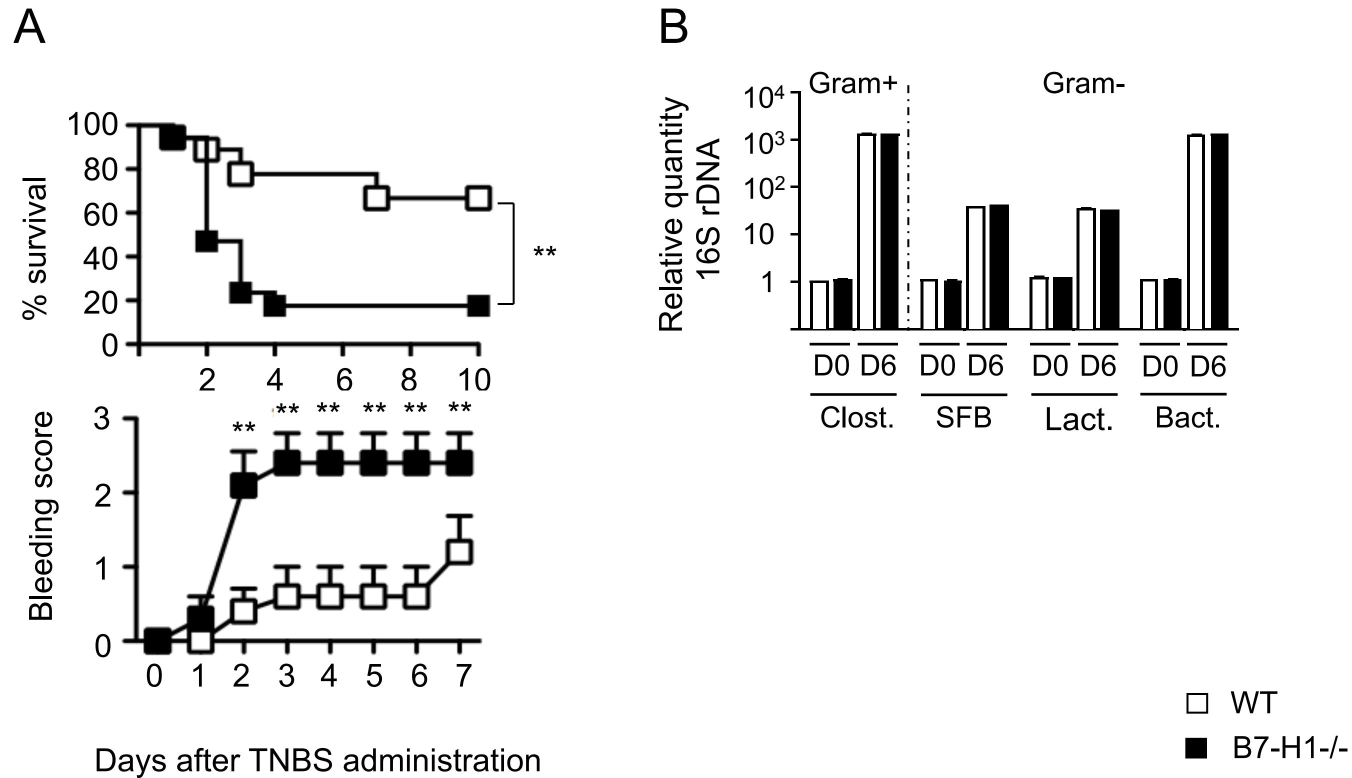


Figure S1. Mortality and morbidity of B7-H1-/- mice upon TNBS treatment and microbiota groups after DSS treatement (A) TNBS treatment in WT and B7-H1-/- mice. Wild-type (n=18) and B7-H1-/- mice (n=17) were intrarectally instilled with TNBS at day 0. Survival and bleeding score were monitored after TNBS administration. Data were pooled from two independent experiments. Survival data are represented as Kaplan-Meier curves (** $P < 0.01$; *** $P < 0.001$; log-rank test). Data represent means ± S.E.M. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Student's *t* test). (B) qPCR analysis of 16S rDNA for microbiota groups during DSS-treatment normalized with values obtained from untreated mice. Data are pooled from two independent experiments with (n=3-4). Data represent means ± S.E.M. Clost. = Clostridiales; SFB = Segmented Filamentous Bacteria; Lact. = Lactobacillaceae; Bact. = Bacteroides.

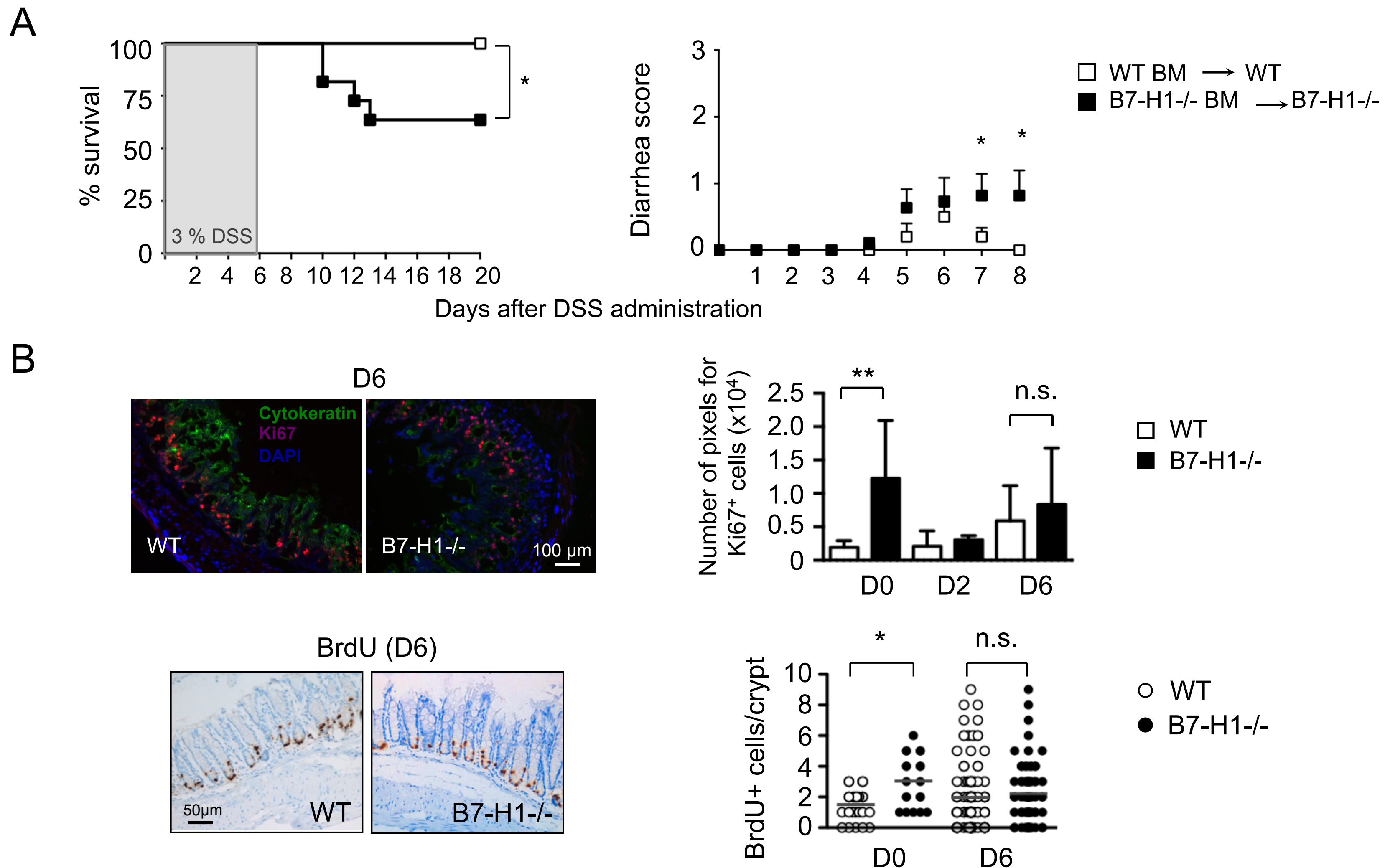


Figure S2. Chimera control mice and epithelial cell proliferation during intestinal inflammation. (A) Survival and morbidity in chimera control mice. Wild-type (WT) mice reconstituted with WT bone marrow (BM) cells and B7-H1-/- mice reconstituted with B7-H1-/- bone marrow cells were fed with 3% DSS in drinking water for 6 days. Survival and diarrhea score were monitored after the beginning of DSS. Data are representative of two experiments with at least 5 mice per group. Survival data are represented as Kaplan-Meier curves (*P < 0.01; log-rank test). (B) Cell proliferation analysis. Upper panel: IF staining on colon tissues from DSS-fed (D6) WT and B7-H1-/- mice for Cytokeratin+ (green) cells, Ki-67+ cells (magenta) and nuclei; scale bar, 100µm. Quantification of Ki-67+ cells was calculated with Volocity® software. 10 fields/tissues sections were quantified per mouse. Lower panel: IHC staining for BdrU on colon section from WT and B7-H1-/- mice during DSS-treatment (D6) and quantification of BrdU+ cells (scale bars, 50µm) at D0 and D6. At least 20 intact crypt/time point were counted. Small horizontal bars indicate the mean. Data represent means ± S.E.M. (* P < 0.05; n.s.=not significant; Student's t test).

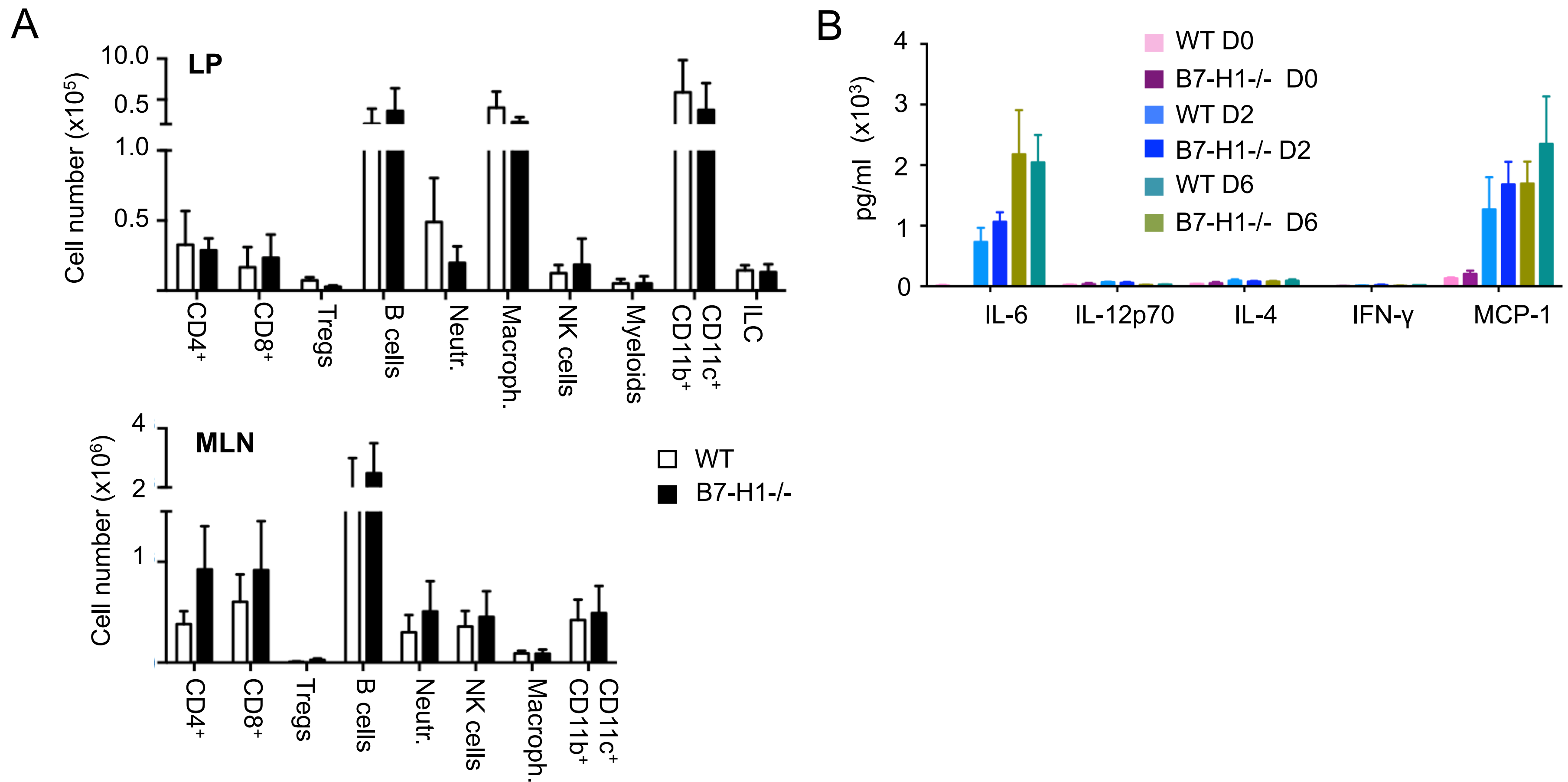
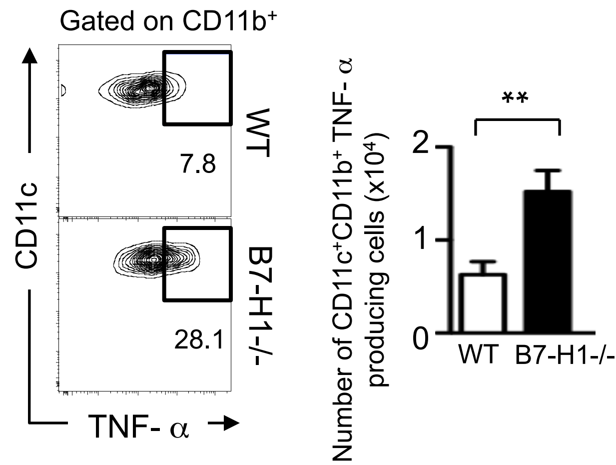


Figure S3. Cell infiltration in lamina propria (LP) and mesenteric lymphonode (MLN) and inflammatory cytokines in colon supernatant. (A) Single cell suspension of cells isolated from lamina propria and mesenteric lymph node were analyzed at day 6 of DSS-mediated inflammation for cell surface markers to detect immune cell populations. Data represent means ± S.E.M. ILC= innate lymphoid cells. (B) Production of IL-6, IL-12p70, IL4, IFN-γ and MCP-1 in supernatant of colons from WT and B7-H1-/- mice at D0, D2 and D6 of DSS-treatment. Data are representative of three independent experiments (n=4).



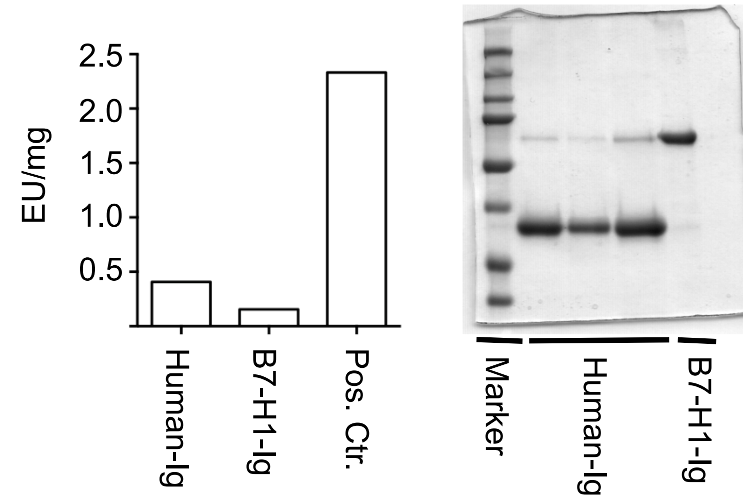


Figure S4. TNF production from CD11c+CD11b+ LP cells and quality check of fusion proteins. (A) FACS plots and number of TNF-α-producing CD11c+CD11b+ cells isolated from DSS-fed (D6) mice. Numbers above bracketed lines indicate percentage of TNF-α + cells. Data are pooled from at least two independent experiments (n=4). Data represent means ± S.E.M. (** $P < 0.01$; Student's *t* test). (B) Left panel: Bacterial endotoxins (EU) quantification per mg of protein in Human-Ig and B7-H1-Ig as compared to a positive control. Measurements were performed using an ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript). Right panel: Coomassie stained gel of Human-Ig and B7-H1-Ig.

Extended Experimental Procedures

Histological analysis, immunohistochemistry and immunofluorescence

Colon tissues were isolated, washed in PBS and either fixed with Zinc Fixative or embedded in OCT compound. Paraffin-embedded sections (5μm) were stained for eosin-hematoxylin (HE) by the Histotechnology and Comparative Pathology Facility. HE stained tissues were blindly analyzed on an AxioCam MRC Zeiss microscope. Each section was evaluated for infiltration, edema and ulceration. A semi-quantitative criterion-based method was used ranging from 0 to 3 where 0=within normal limits or absent; 1=mild changes; 2= moderate changes; 3:=severe changes. Mucosa associated lymphoid tissues (MALTs) area was quantified by matching the size of each MALT to a graduated scale of circles, each with a numerical value. The final values were expressed in μm^2. For immunohistochemistry of human colon tissue, frozen-section slides were used and stained with anti-human B7-H1 biotinylated antibody (R&D). Immunoperoxidase method was used for detection. For immunofluorescence staining we used an anti-FITC-Pan Cytokeratin and an anti-Ki67 antibodies followed by a secondary antibody. DAPI (Invitrogen) was used to detect nucleus. At least 10 fields/mouse were blindly analyzed with an Olympus BX61.

Blockade of TNF-α

100 μg of monoclonal anti-mouse TNF-α blocking antibody (BioXCell, BE0058) or an isotype-matched control rat IgG1 (BioXCell, BE0088) were injected i.p into mice every other day for up to 20 days of DSS treatment starting at day 0.

Analysis of red blood cells

Red blood cell (RBC) concentration and hematocrit were determined by standard hematological analysis in the Histotechnology and Comparative Pathology Facility of Albert Einstein College of Medicine.

Cytokine measurement

All cytokines, except for TGF-β and IL-22, were measured in the colon supernatants using Cytometric Beads Array (BD Biosciences) according to the manufacturer's instructions. Data were acquired on FACscalibur (BD Biosciences) and analyzed with FlowJo software (version 8.8.4). TGF-β was detected using Single Plex Flex Set CBA (BD, Bioscience) according to manufacturer's instructions. IL-22 was detected by ELISA assay using AAM65 (AbDSerotec) as capture antibody and AAM65B (AbDSerotec) as detection antibody according to manufacturer's instruction.

Quantification of B7-H1$^+$ on human samples and of Ki67$^+$ cells on mouse samples

B7-H1 expression on human samples was quantified using Volocity® software based on a 2 steps protocol. Briefly, two images in .tiff format were imported into Volocity® as follows: one isotype control image as negative control and one image positively stained for the antibody of interest as positive control. The object of interest was found using RGB with the following intensity thresholds (first channel: lower 10, upper 137; second channel: lower 27, upper 133; third channel: lower 25, upper 101) and then either excluded or retained based upon color and size (< 2 μm^2). A uniform filter was used to remove noise from the system and to identify positive cells. A similar protocol has been used to identify Ki67$^+$ cells.

Flow cytometry reagents

Single-cell suspensions isolated from colons were washed in FACS buffer (ice-cold 0.5% BSA in PBS) and stained with specific antibodies from eBioscience: FITC-CD62L (MEL-14), APC-CD45R (RA3-6B2), PE-CD11b (M1/70), APC-F4/80 (BM8), alexa fluor 647-F4/80, FITC-B220, FITC-B7-2 (GL1), Alexa-fluor 700-CD3 (17A2), eFluor405- CD45 (2D1), PE-B7-H1 (MIH5), biotin-CD11c (N418), biotin-NK1.1 (PK136), CD49d and PE-Cy7 CD90.2 (53-2.1); three Abs from BD: FITC-Ly-6G (1A8), Annexin V and CD40 (3/23); one Ab from Abd Serotec: F4/80-alexa

fluor 647; one antibody was from Sigma-Aldrich: FITC-conjugated anti-Pan Cytokeratin monoclonal (C-11) and one from Abcam: anti-Ki67 (ab15580). Biotin-conjugated Abs were followed by APC-, PE-, FITC- or PE-Cy7- conjugated streptavidin (eBioscience) staining. For intracellular staining we used PE-conjugated anti-mouse/rat Foxp3 (FJK-16s, Ebioscience), anti-mouse TNF-α (MP6-XT22, Ebioscience) and an anti-mouse IL-22 (Poly5164, BioLegend). To prevent non-specific binding, all samples were pre-incubated with Fc-Block (eBioscience) and isotype-matched antibodies were used. Data were acquired using BD™LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Version 8.8.4).

Scoring of colonic bleeding

Colons isolated from mice at different time points during DSS-treatment were scored as follows: 0= lack of any gross blood visible throughout the entire colon; 1= gross blood present in <1/3 of the colon; 2= <2/3; 3= >2/3 of the colon.

Bacterial culture

Samples of feces, colon, mesenteric lymph nodes (MLN), liver and spleen were collected in 0.01% Triton X-100/PBS and homogenized. Different dilutions of the obtained homogenate were plated on blood agar-plates and incubated for 24 h. Bacterial count was determined by colony-forming assay.

Isolation of epithelial and LP cells

Colons were washed in PBS and chopped into 0.5 cm pieces. Tissues were incubated in 2 mM EDTA with PBS for 30 min at 37°C while shaking at 200 rpm. Samples were filtered in a 70-μm strainer, centrifuged for 15’ at 1700 rpm in 30% Percoll to isolate epithelial cells which were then used for FACS analysis or co-culture assay. The remaining pieces of colons were further digested in 2 mg/ml of collagenase-IV, 5% FBS, 1 mg/ml DNaseI in PBS for 30 min at 37°C shaking at 200 rpm. Samples were passed through 40-μm strainers. LP cells were then separated in a 30% Nycoprep gradient and used for experiments.

qPCR

To analyze commensal bacteria, stool from colon was collected and DNA was extracted with Qiagen DNA isolation kit and quantitative PCR for 16S rDNA was performed. Absolute copy numbers of bacterial 16S rDNA were determined from standard curves established by qPCR of serial dilutions of reference samples harboring the 16S rDNA gene from each of the bacteria strains analyzed. All reactions were performed in 10μl using Power SYBR Green Master Mix (Applied Biosystems) with 1 μM concentration of each primer using the following steps: 90˚C for 3 minutes, 25 cycles of 95˚C for 40 seconds, 60˚C for 40 seconds, and 60˚ for 4 minutes on a ABI-PRISM 7900 (Applied Biosystem). Primer sequences for bacteria groups are the followed: Total Bacteria Fwd: 5' -ACTCCTACGGGAGGCAGCAGT- 3'; Rev: 5' -ATTACCGCGGCTGCTGGC- 3'. Clostridiales Fwd: 5' -ACTCCTACGGGAGGCAGC- 3' Rev: 5' -GCTTCTTAGTCAGGTACCGTCAT- 3'; SFB (Segmented Filamentous Bacteria) Fwd: 5' -GACGCTGAGGCATGAGAGCAT- 3', Rev: 5' -GACGGCACGGATTGTTATTCA- 3'; Lactobacillaceae Fwd: 5' -AGCAGTAGGGAATCTTCCA- 3', Rev: 5' -CACCGCTACACATGGAG- 3'; Bacteroides Fwd: 5' -GGTTCTGAGAGGAAGGTCCC- 3, Rev: 5' -GCTGCCTCCCGTAGGAGT- 3'.

In vivo epithelial permeability assay

Mice were deprived of water and food for 3 h and then gavaged with FITC-dextran (MW 40,000; Sigma-Aldrich) at 0.6mg/g body weight. 3h later fluorescence was measured in sera using a spectrophotometer (Synergy H4, BioTek). FITC-dextran concentration was determined from standard curves generated by serial dilution of FITC-dextran.

Production of B7-H1-Ig Fusion Protein and Functional Assay

B7-H1-Ig fusion protein was produced in an inducible secreted serum-free Drosophila expression system. The coding region of the extracellular domain of B7-H1 was fused to a human Ig-G_1 Fc tag of plasmid pMT/BiP. The construct

was co-transfected into Drosophila cell line S2 with a hygromycin resistance plasmid. The stable transfected cell line was induced with $CuSO_4$ to secrete B7-H1 in Drosophila serum-free medium (Invitrogen). B7-H1-Ig was purified on an ImmunoPure Plus protein G column. The control human IgG1 Fc protein was produced in the same way. The purity of fusion proteins was confirmed by Coomassie blue staining (Figure S4B), immunoblotting with antibodies against human IgG Fc (Jackson Imm. Res.) and by checking the bacterial endotoxin levels using an ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript) (Figure S4B). B7-H1-Ig or control Ig (3μg/ml) in PBS were incubated in a 96-well plate overnight at 4°C. After washing the wells twice with PBS, 2×10^4 LP cells isolated from DSS-treated wild-type or PD1-/-B7-1/-/- mice were added and incubated for 24h at 37°C in 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin in DMEM-F12 medium. Cells were collected and used for flow cytometry analyses.

Co-culture assay

Isolated IEC cells from naïve wild-type or B7-H1-/- mice were plated on a thin layer of matrigel (BD) in a 96-well plate at 1×10^5/well in complete RPMI. LP cells, isolated as previously described from different mice at different time points during DSS-treatment, were added on the top at 1×10^5/well in complete RMPI plus GolgiStop reagents (BD). 24h after LP cells were collected and processed for FACS analysis.

Colon organ culture

Colons were washed in PBS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, cut in 0.5-cm pieces and cultured in 24-well-flat-bottom plates in serum-free RPMI-1640 medium supplemented with 100U/ml penicillin and 100 μg/ml streptomycin, L-glutamine and nonessential amino acids. 24h later supernatant was harvested, centrifuged at 13,000 g for 10 minutes at 4°C and stored at -20°C until analyzed.

Supplemental References

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