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Supplemental Information

PTPN2 Regulates Inflammasome Activation and Controls Onset of Intestinal Inflammation and Colon Cancer

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Supplementary Methods

Mice, colitis, and colitis-associated tumour models. Mice with a floxed PTPN2 exon 3 on a C57/Bl6 background were obtained from the European knockout mouse consortium. C57/Bl6 mice expressing Cre recombinase under the lysozyme promoter were initially obtained from Jackson laboratories and maintained as a breeding colony at our facility. Acute colitis was induced by administration of 2.5% DSS (MP Biomedicals, Carlsbad, CA) in the drinking water for seven days. Chronic colitis was induced by administration of 3-4 cycles of 1.5% DSS in the drinking water for seven days, followed by a recovery phase of 10 days with normal drinking water. For the induction of colorectal carcinoma, Azoxymethane (AOM; 10mg/kg body weight, Sigma-Aldrich, St. Louis, MO) was injected at day 1 and day 8 of each DSS cycle during chronic colitis induction.

H&E, immunohistochemistry and immunofluorescent staining. For H&E, immunohistochemistry and immunofluorescent staining, sections from the most distal 1 cm of the mouse colon were deparaffinized and antigen retrieved using citrate buffer, pH 6.0 (DAKO, Glostrup, Denmark). For immunohistochemistry, endogenous peroxidases were inhibited by incubation with 0.9% hydrogen peroxide for 15 min at room temperature (RT). Blocking was performed using 3% BSA or 3% normal goat serum in PBS as appropriate. Sections were incubated with rabbit anti-p68 antibody (Abcam, Cambridge, UK); rabbit anti-Ki67 antibody (Biolegend, San Diego, CA); mouse anti-PTPN2 antibody (Merck Millipore, Billerica, MA) or rat anti-F4/80 antibody (BMA biomedicals; Augst, Switzerland). For immunohistochemistry, HRP-labeled secondary antibody (Santa Cruz Biotechnology, Dallas, TX) was applied for 1 h at RT and antibody binding visualized by a Liquid DAB+ Substrate Chromogen System (DAKO); samples were counterstained with hematoxylin, incubated in ascending concentrated ethanol and xylol solutions and finally mounted. For immunofluorescence, AlexaFluor647 labeled anti-mouse and AlexaFluor594 labeled anti-rabbit antibody were used as secondary antibodies, and slides mounted in fluorescence mounting medium (DAKO). Microscopic assessment was performed using an AxioCam HRc (Zeiss, Jena, Germany) on a Zeiss Axio Imager.Z2 microscope (Zeiss) with AxioVision Release 4.8.2 software (Zeiss).

siRNA transfections. For knockdown of PTPN2, JNK and Syk, three different silencer predesigned siRNA oligonucleotides targeting the JNK or Syk gene were obtained from Life Technologies. Per transfection, 100 pmol of each of the three gene-specific siRNA oligonucleotides were used. A nonspecific control siRNA SMARTpool (Life Technologies) at a concentration of 100 pmol per transfection was used as negative control. For transfection of BMDM and HT29 (DSMZ, Braunschweig, Germany), siRNA was mixed with Lipofectamine® RNAiMAX (Life Technologies) for 15 min before adding to the cell culture media. After 6 h, medium was changed and cells cultured for 24 h in RPMI supplemented with 10% FCS

Western blot analysis and immunoprecipitation. For Western blotting, equal amounts of protein from each lysate were loaded on polyacrylamide gels and after separation by gel-electrophoresis blotted onto nitrocellulose membranes. Membranes were incubated over night with primary antibody, washed three times with washing buffer (Tris buffered saline, 3 % milk) before incubation with HRP-coupled anti-mouse, anti-rabbit or anti-goat secondary antibodies (Santa Cruz Biotechnologies) for 2 h. Immunoreactive proteins were detected with a Fusion Solo S imager (Vilber Lourmat, Eberhardzell, Germany) using an enhanced chemiluminescence detection kit (Witek AG, Lucerne, Switzerland).

For immunoprecipitation, samples were pre-cleared with Sepharose G beads (GE Healthcare, Little Chalfont, UK) and incubated on a rocker over night at 4 °C with 10 μg/ml rabbit anti-ASC (Enzo life sciences, Farmingdale, NY), 10 μg/ml mouse anti-PTPN2 (Merck Millipore, Darmstadt, Germany), 7 μg/ml anti-ASC (Enzo life sciences) or 10 μg/ml mouse anti-NLRP3 (Enzo lifesciences) antibodies, prior to precipitation with Sepharose G beads (GE Healthcare). After washing, the pellet was re-suspended in 1x loading buffer, boiled for 10 min at 95 °C, and the supernatants loaded on polyacrylamide gels. For detection of precipitated proteins, same procedure was applied as used for development of Western blots.

Cell isolation. For sorting of immune cells from spleen, single cell suspensions were made in PBS by grinding the spleens over a 70 μm nylon mash. Samples were incubated for 3 min in ACK buffer (150 mM Ammonium chloride; 10mM Potassium hydrogen carbonate; 1 mM EDTA) to lyse red blood cells,

resuspended in 5ml complete RPMI (Sigma), washed two times in FACS buffer (PBS supplemented with 1% BSA and 0.2 mM EDTA), and incubated with anti-B220 antibody, anti-F4/80 antibody, and anti-CD3 antibody for 20 min. All antibodies were obtained from eBiosicence. Samples were washed in FACS buffer and B220+, F4/80+, and CD3+ cells sorted on a BD Aria III cell sorter. For isolation of peritoneal macrophages, 5ml RPMI (life technologies) was injected i.p. and recollected medium plated into 24 well plates for 2h. Non-adherent cells were washed away with PBS and attached cells collected using ice cold PBS with 2mM EDTA.

ELISA. Cell culture supernatants and sera were collected and stored at -80°C until use. ELISA kits detecting human and murine IL-1β and IL-1α were obtained from R&D systems (Minneapolis, MN), anti-human and anti-mouse IL-18 ELISA kit was obtained from Promokine (Heidelberg, DE). Assays were performed according to the manufacturer's instructions using a sample volume of 70 µl. Absorbance at 450 nm with a correction wavelength of 610 nm was detected on a SpectraMax M2 Fluorescence Microplate reader using SoftMax Pro v5 Software (Molecular Devices, Sunnyvale, CA). Measurements were performed in duplicates.

Flow cytometry. For IL-1α surface expression, peritoneal macrophages were isolated as described above. IEC were isolated as described previously [17]. In brief, the colon was washed in PBS, and cut into 0.2 cm^2 pieces, and mucus removed by shaking at 37 °C for 30 min. in HBSS supplemented with 2mM EDTA. The remaining pieces were then shaken rigorously with fresh HBSS, strained over a 70um sieve and flow-through fraction containing IEC collected. IEC and peritoneal macrophages were then washed 3 times in ice cold PBS, incubated for 10 min with anti-goat serum to block Fc Receptors and incubated with anti IL-1α antibody (R6D systems) for 20 min. Cells were washed three times in FACS buffer (PBS supplemented with 1% BSA and 0.2 mM EDTA) and analysed on a BD Canto II. Propidium iodide-containing sample acquiring buffer was used to discriminate life from dead cells.

RNA isolation and RT-PCR. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Venlo, NL), and DNA removed by TURBO DNA-free Kit (Ambion, Austin, TX) according to manufacturer's instructions. RNA concentration was assessed by absorbance at 260 and 280 nm. Complementary DNA (cDNA) synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied

Biosystems, Foster City, CA) following the manufacturer's instructions. Real-time PCR was performed using FAST qPCR MasterMix for Taqman Assays (Applied Biosystems) on a Fast HT7900 Real-Time PCR system using SDS Software (Applied Biosystems). Measurements were performed in triplicates, human or mouse β-actin was used as endogenous control, and results were analyzed by ΔΔCT method. The real-time PCR contained an initial enzyme activation step (5 min, 95 °C) followed by 45 cycles consisting of a denaturing (95 °C, 15 sec) and an annealing/extending (60 °C, 1 min) step. Gene expression assays were obtained from Life Technologies.

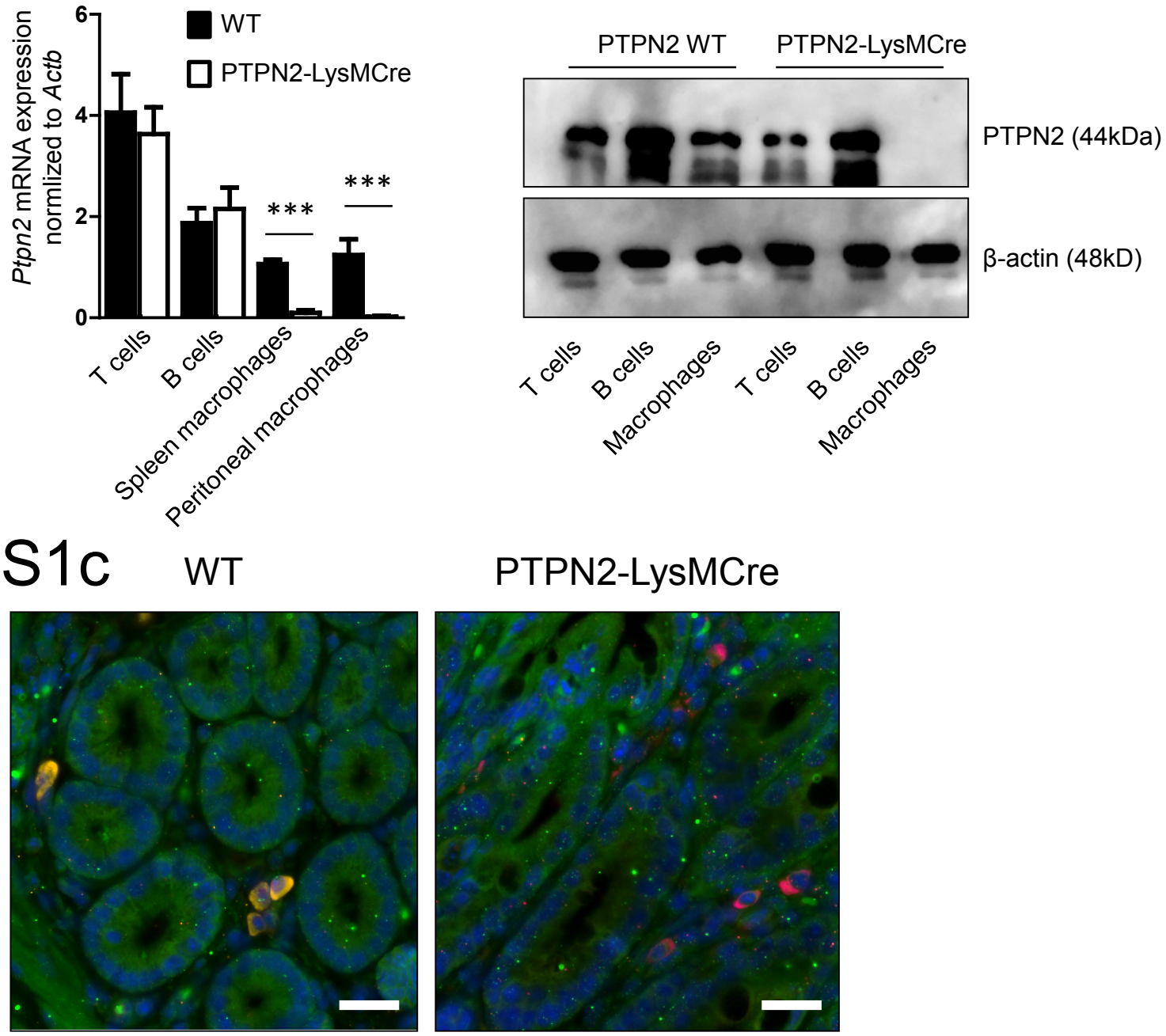
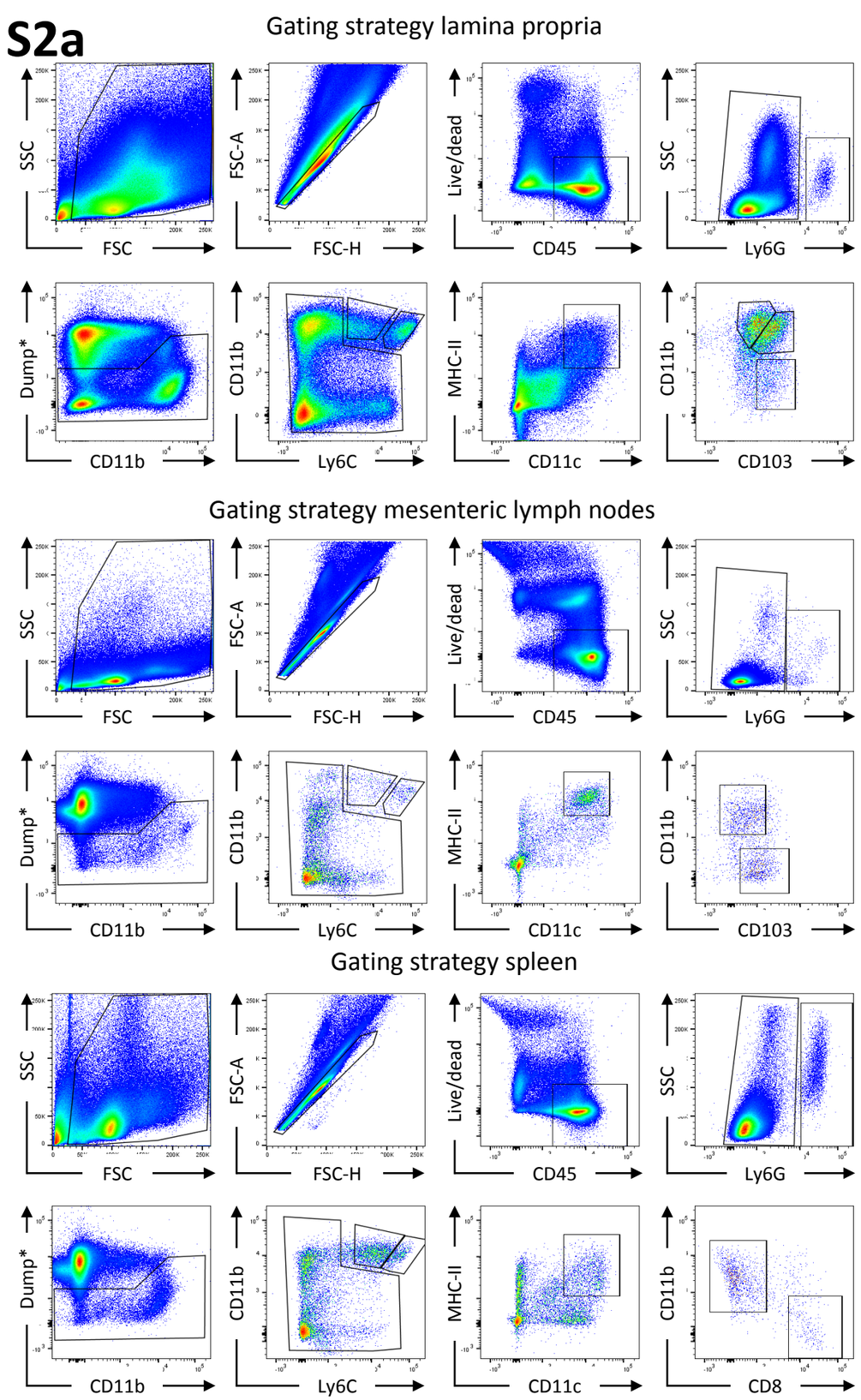


Figure S1. Macrophages from PTPN2-LysMCre mice do not express PTPN2. Related to figures 1 and 3, characterization of the PTPN2-LysMCre mice. a) *Ptpn2* mRNA expression normalized to *Actb* mRNA expression in FACS-sorted CD3+ (T cells), B220+ (B cells), and F4/80+ (macrophages) spleen cells, as well as peritoneal macrophages from WT and PTPN2-LysMCre mice. **b)** Western blot analysis for PTPN2 in FACS sorted T cells, B cells and macrophages from WT and PTPN2-LysMCre mice. **c)** Immunfluorescent staining for PTPN2 (green) and F4/80 (red) of terminal colon pieces from WT and PTPN2-LysMCre mice. Scale bars represents 50 μm.



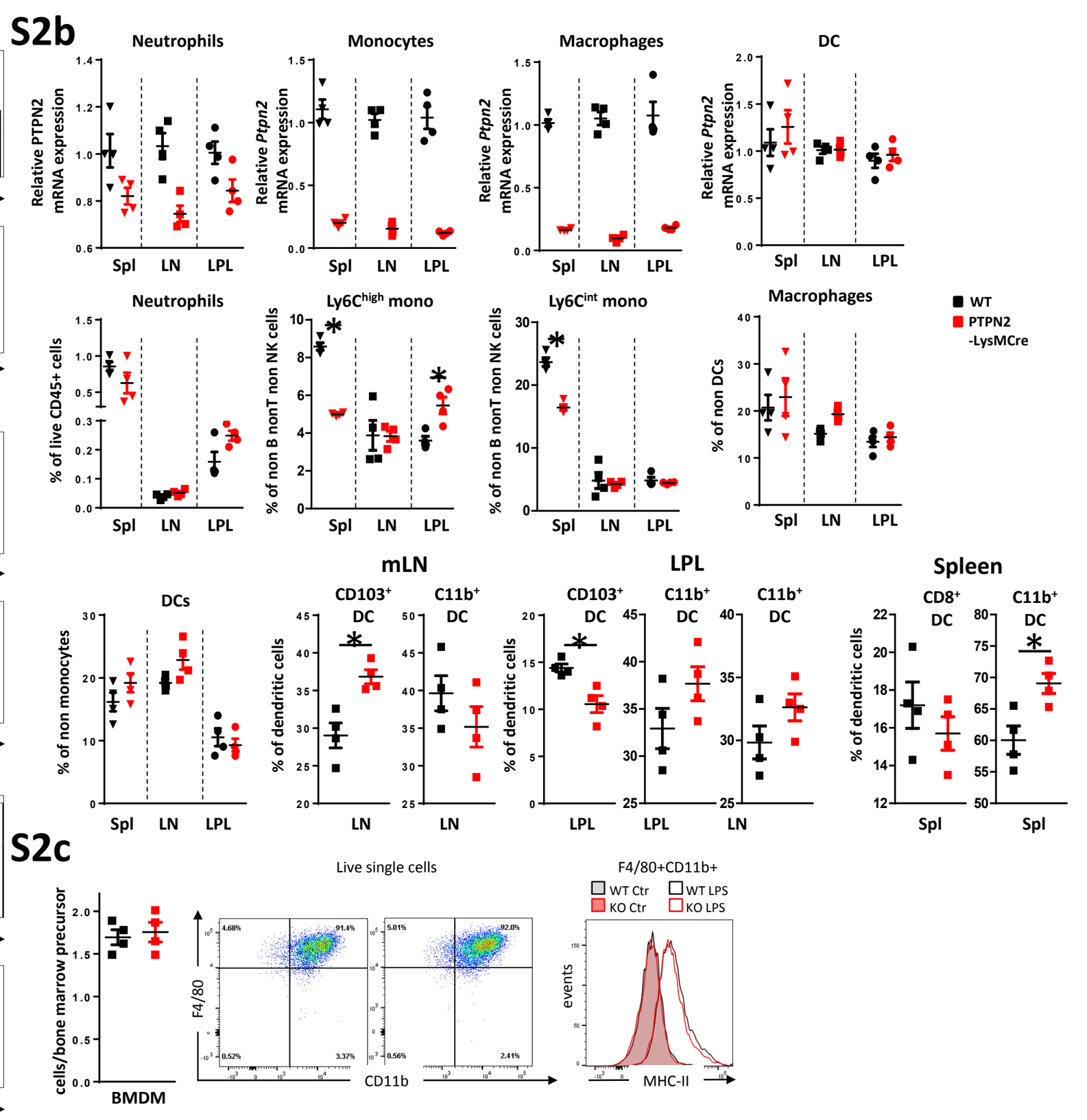
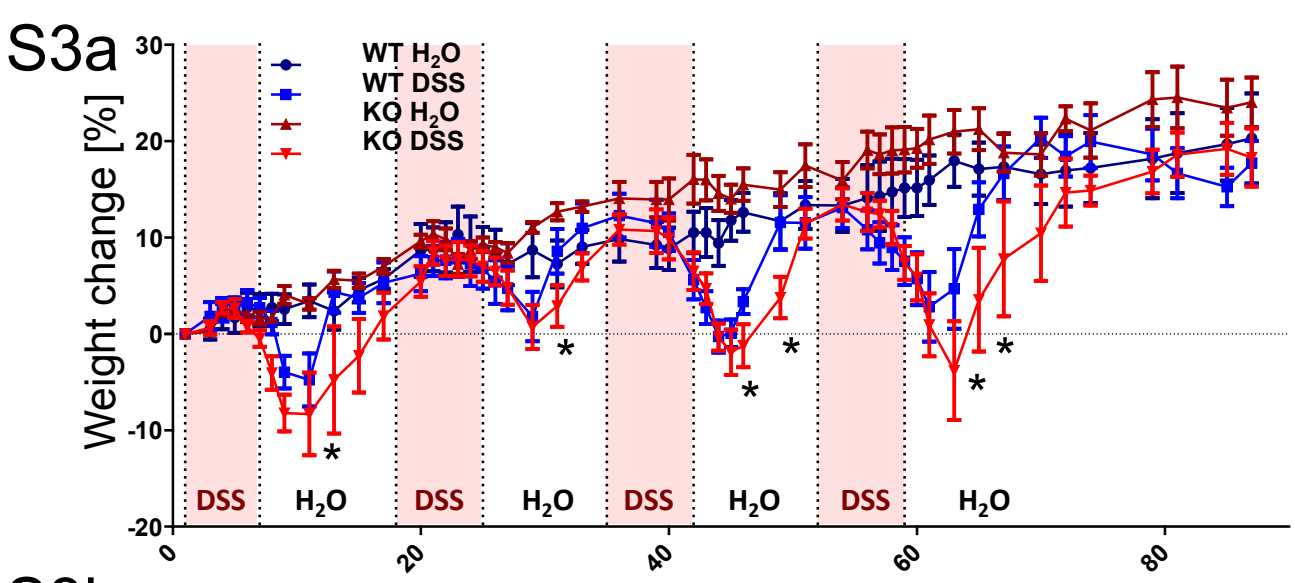
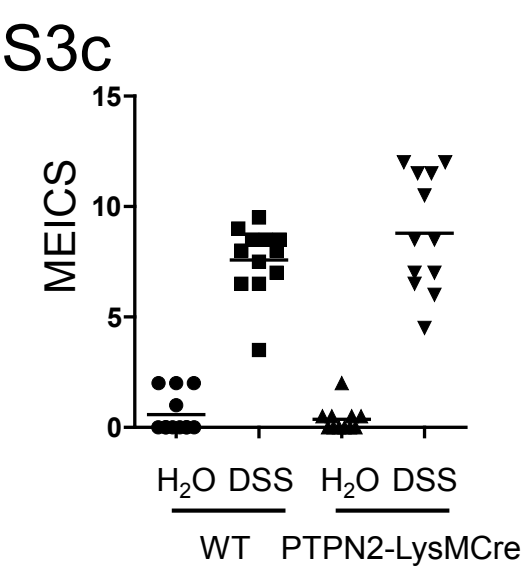
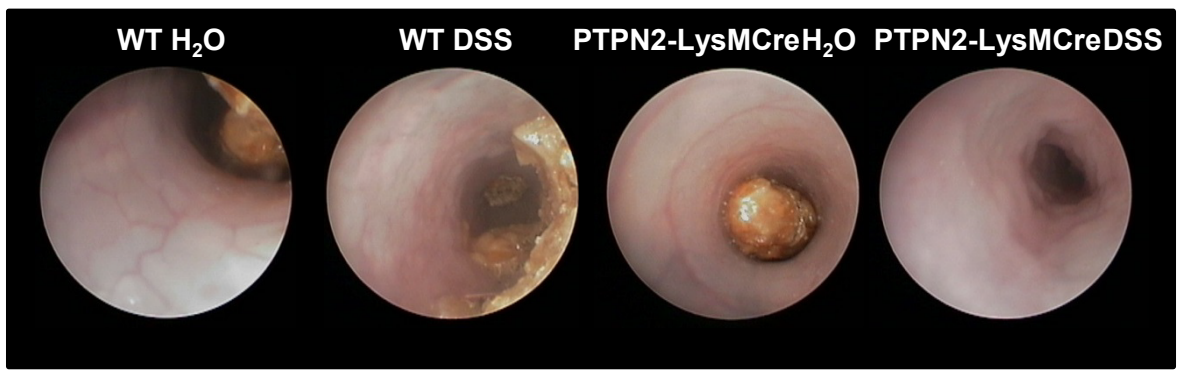
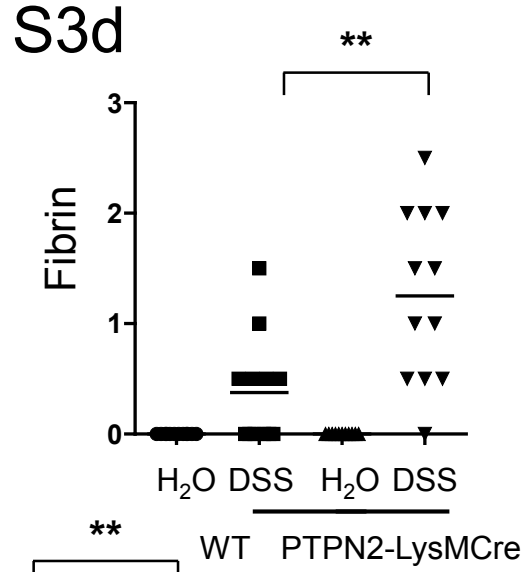


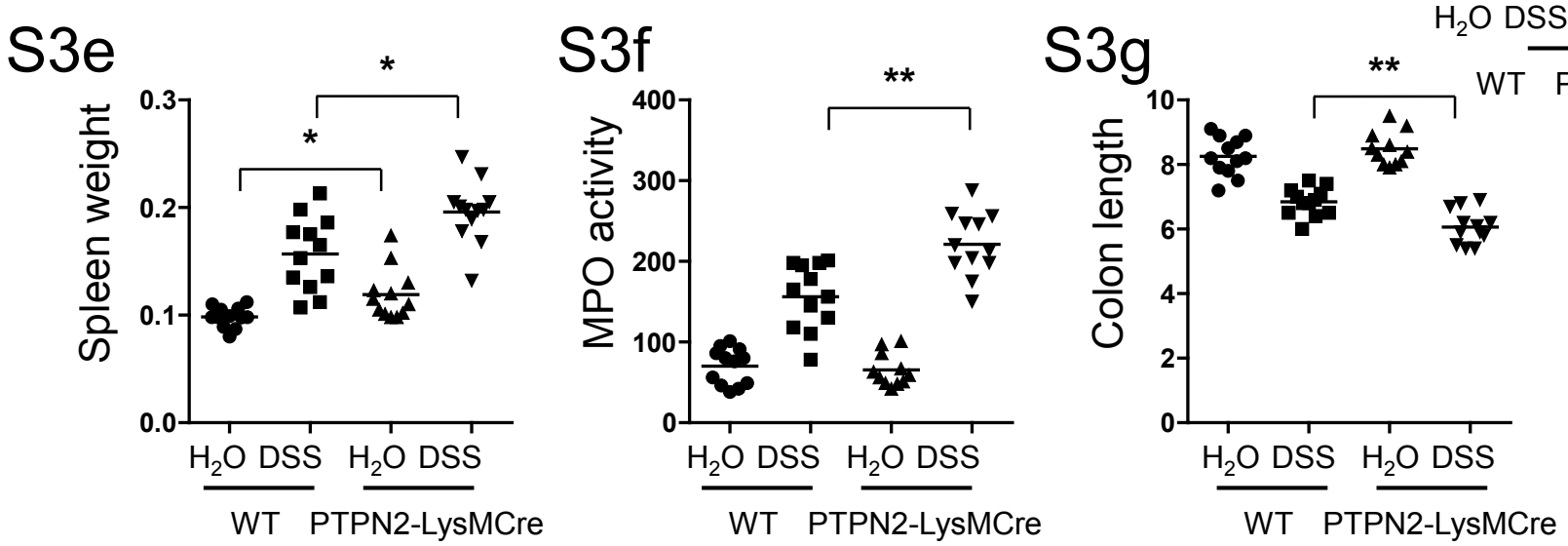
Figure S2. Alterations in the composition of myeloid cell subsets in the intestine of PTPN2-LysMCre mice. Related to figures 1-3, characterization of the PTPN2-LysMCre mice. a) + b) Lymphocytes were isolated from the lamina propria, mesenteric lymph nodes and the spleen of PTPN2-LysMCre and WT littermates and analysed for myeloid cell populations. To address PTPN2-deletion, indicated cells were sorted prior to isolation of mRNA and RT-PCR for PTPN2. Shown is **a)** gating strategy used to discriminate immune cell populations; and **b)** relative cell numbers in the indicated gates, or relative *Pton2* mRNA expression normalized to *Actb* and the respective cell population from WT mice. Please note that CD103 staining is rather low in our hands, which might result from compensation. **c)** Macrophages were differentiated from the bone marrow of WT and PTPN2-LysMCre mice. Depicted are obtained cell numbers (left), and representative FACS analysis of F4/80 and CD11b (middle), as well as MHC-II expression after LPS treatment for 12 h.

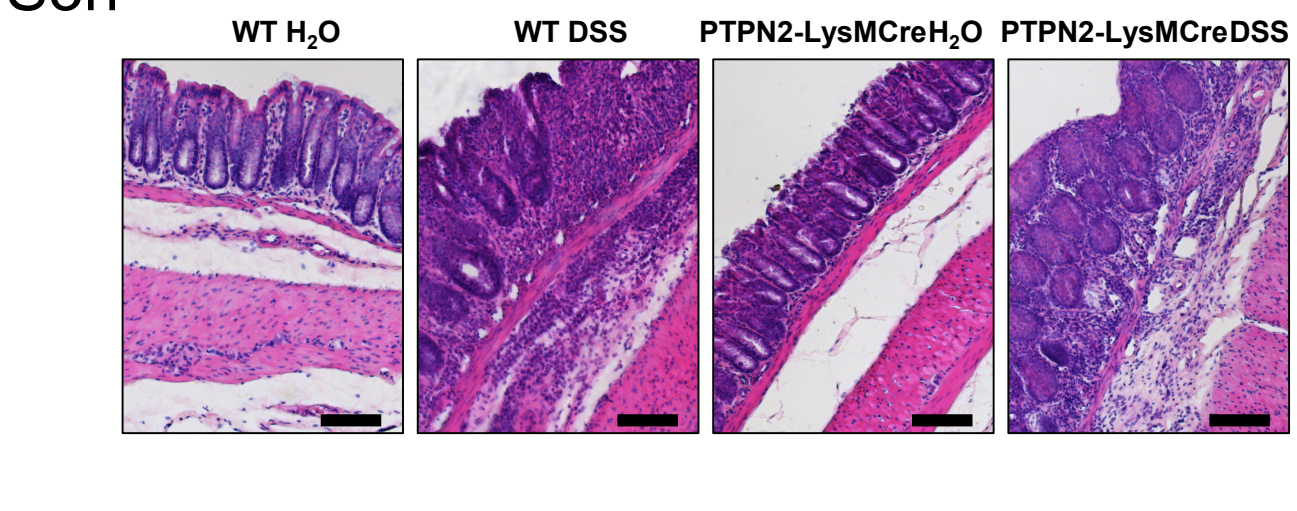












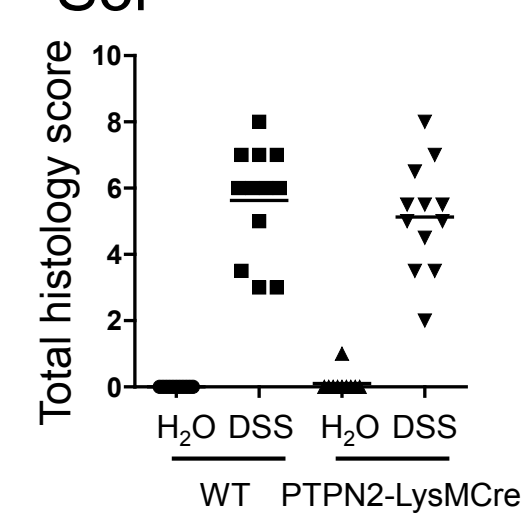
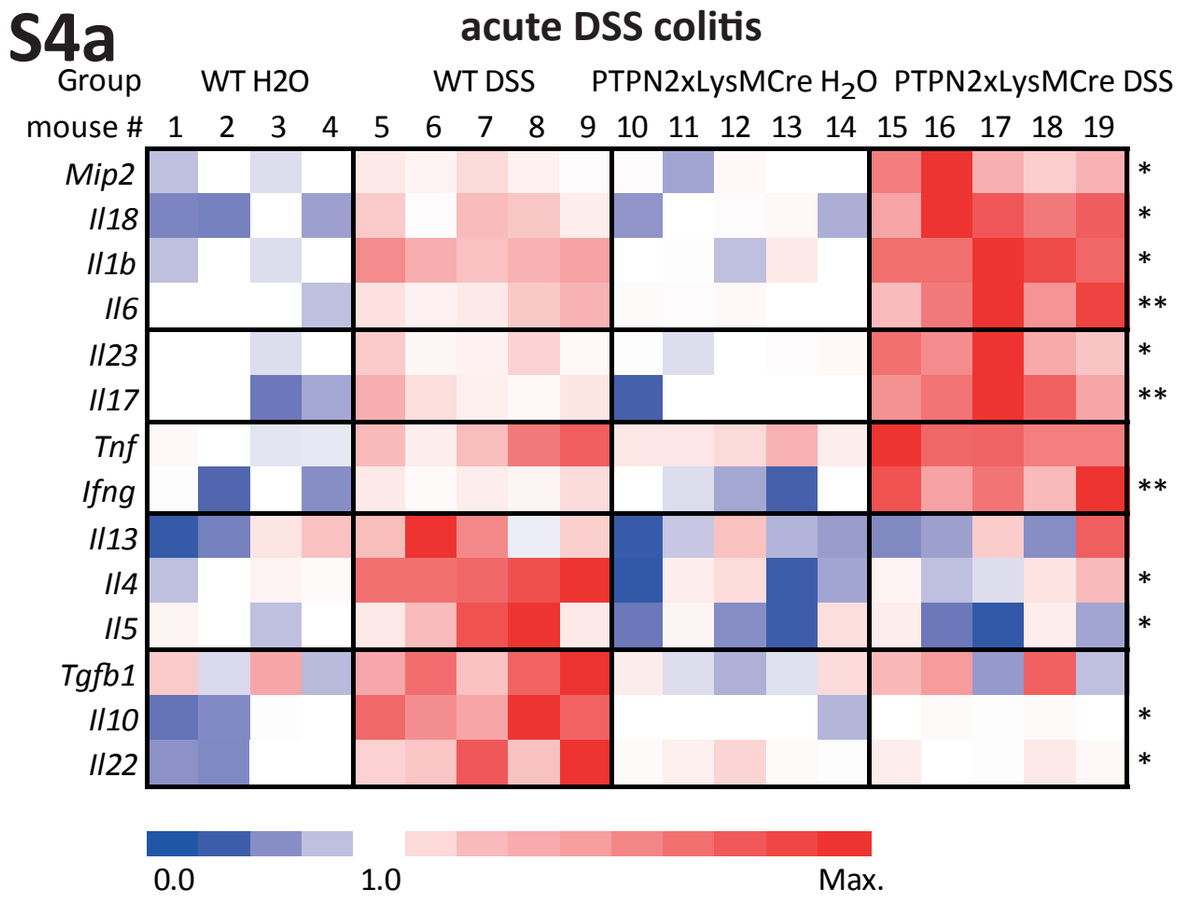
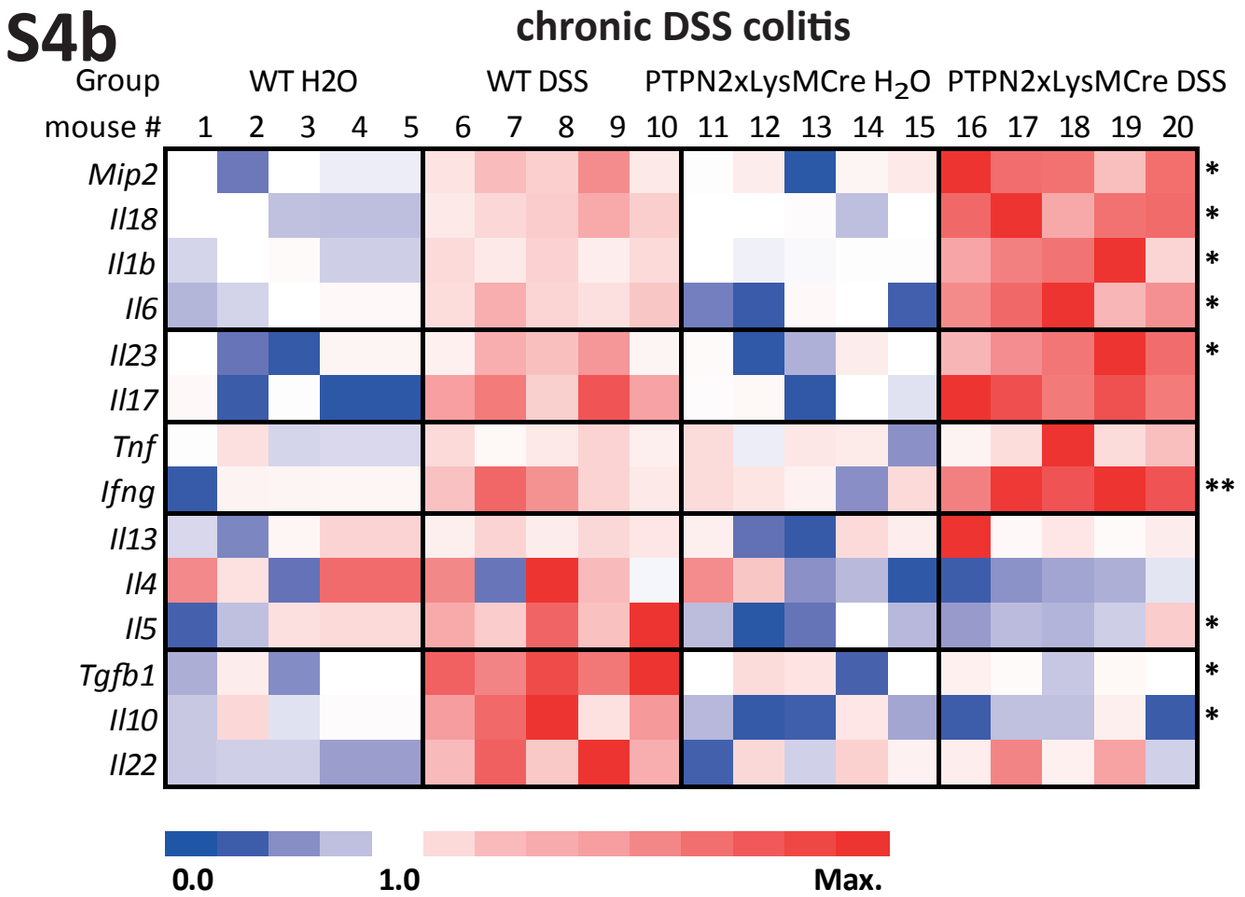
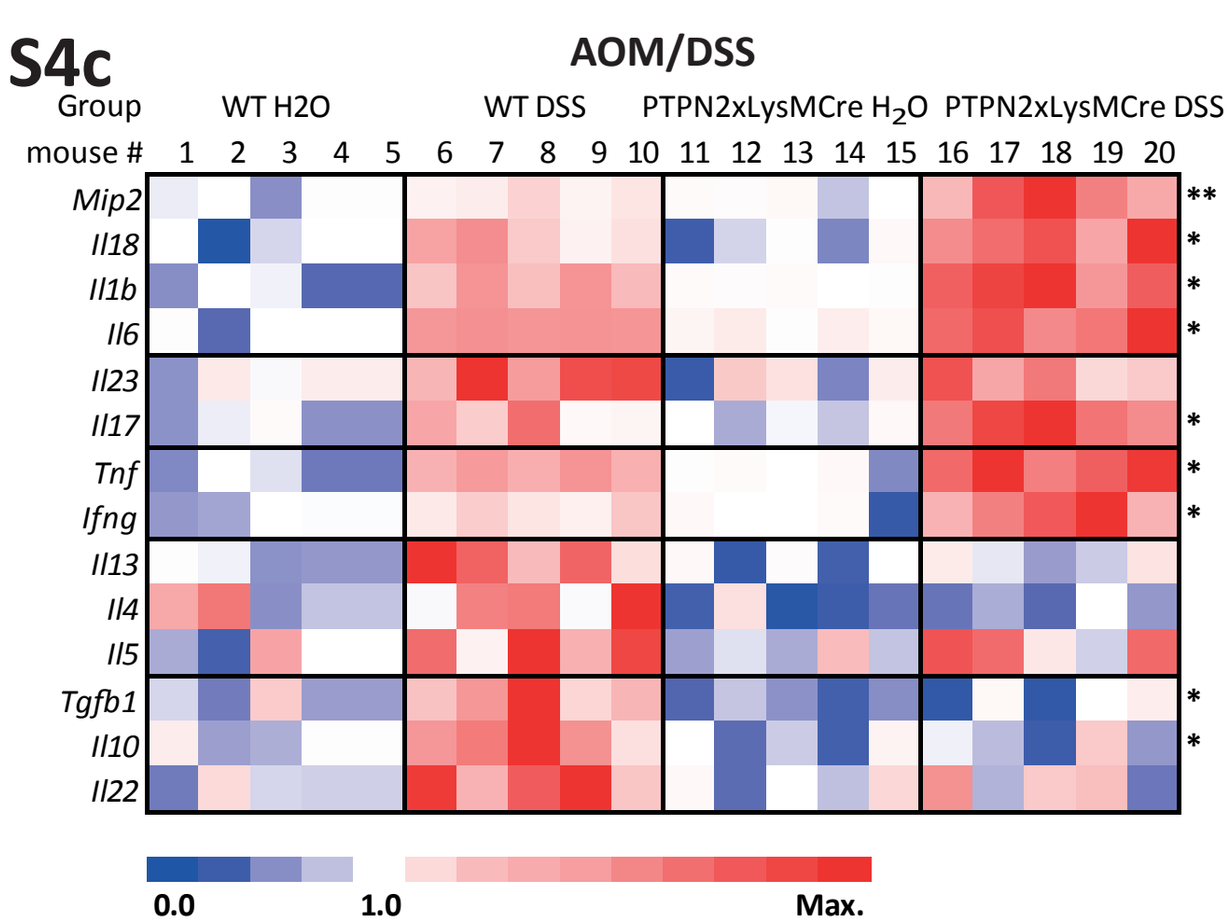


Figure S3. Loss of PTPN2 in macrophages promotes chronic DSS-induced colitis. Related to figure 1. Chronic colitis was induced in WT and PTPN2-LysMCre mice by administration of four cycles of 1.5% DSS in the drinking water for 7 days, followed by 10 days of recovery period, each. The graphs show **a)** weight development, **b)** representative pictures from colonoscopy and **c)** respective statistical analysis; **d)** scoring of fibrin observed in colonoscopy; **e)** spleen weight; **f)** myeloperoxidase (MPO) activity; **g)** colon length; **h)** representative pictures of H&E stained sections and **i)** scoring of epithelial damage and inflammatory infiltration in the terminal colon. Data are pooled from two independent experiments with 3-5 mice per group. Asterisks denote statistical significance (*=$p<0.05$, **=$p<0.01$; Mann-Whitney-U-test with Bonferroni correction). Scale bars represents 100µm.







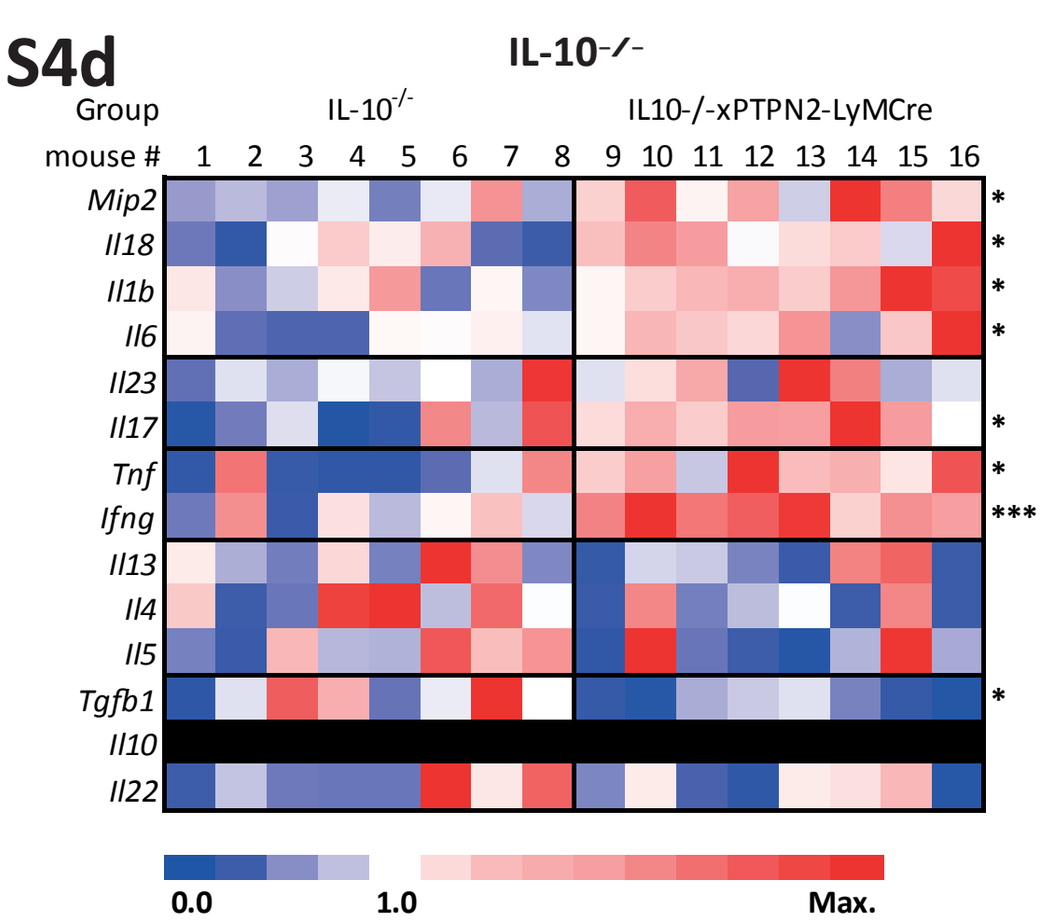
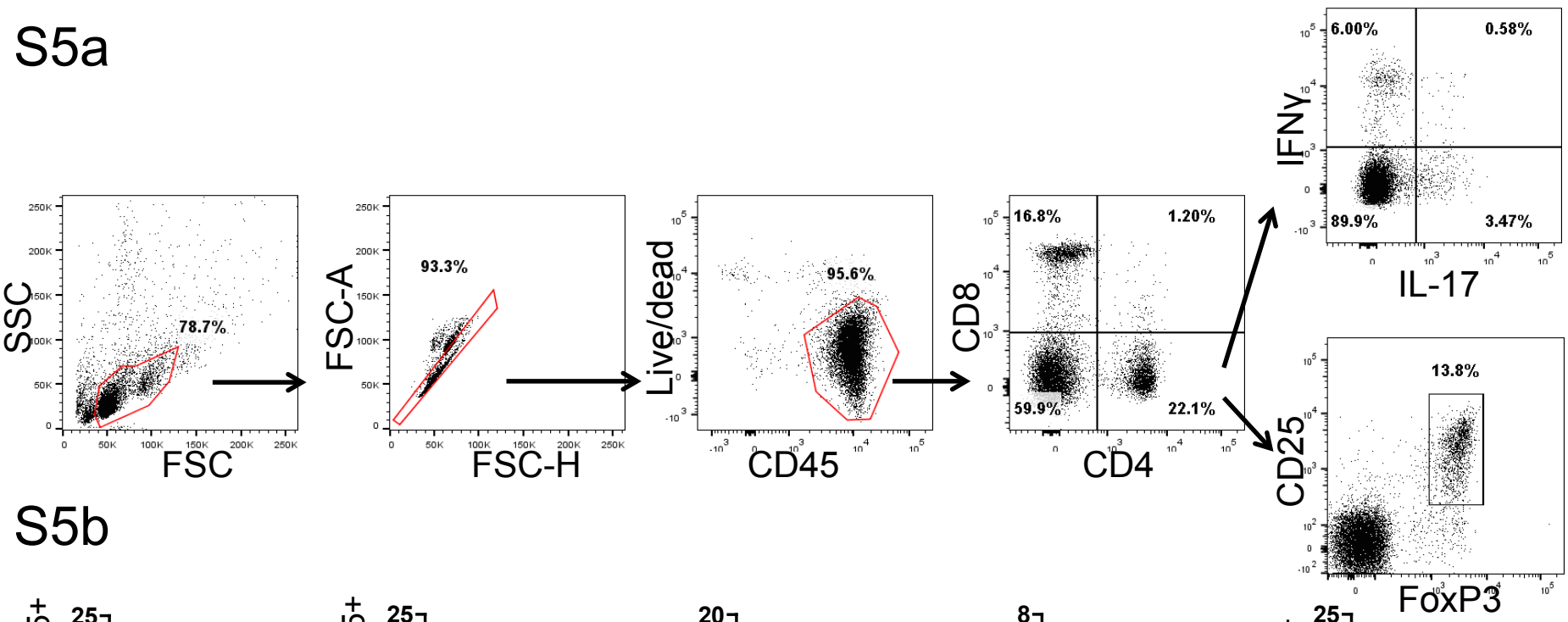
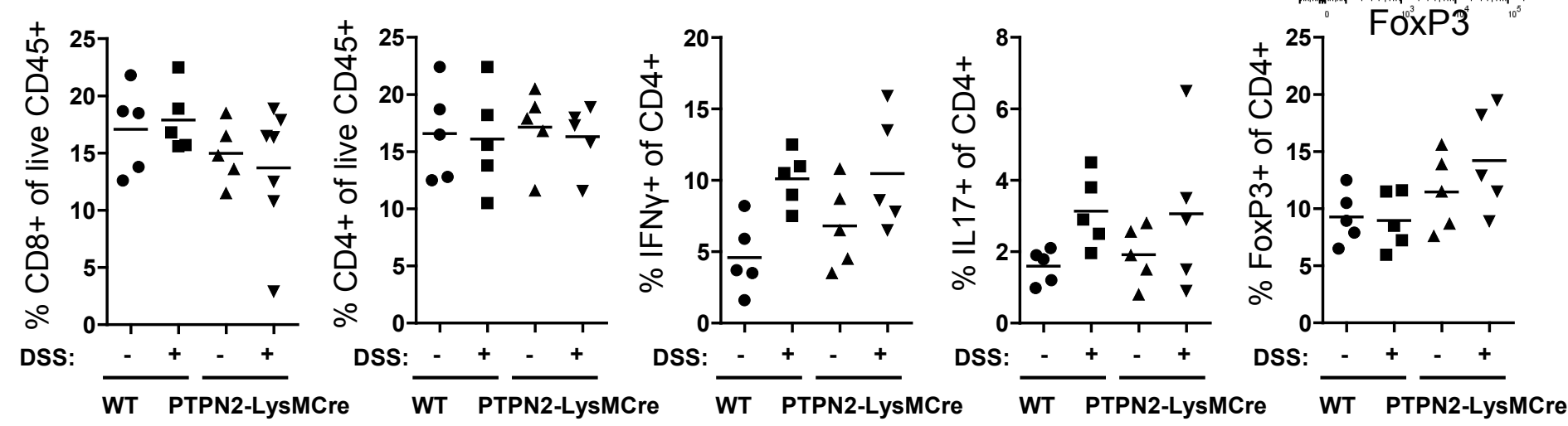


Figure S4. Altered cytokine secretion in PTPN2-LysMCre mice upon colitis induction. Related to figures 1-3. Colon pieces from WT and PTPN2-LysMCre mice with a) acute DSS-induced colitis; b) chronic DSS-induced colitis; c) AOM-DSS induced colon tumours; and d) IL-10-/- as well as PTPN2-LysMCre x IL-10-/- mice were analysed for mRNA expression of the indicated cytokines. Heat maps show relative change of expression normalized to Actb and the median of untreated WT mice. Asterisks denote significant differences between WT and PTPN2-LysMCre mice treated with DSS or AOM/DSS, respectively (*=p<0.05, **=p<0.01, Mann-Whitney-U-test with bonferroni correction).

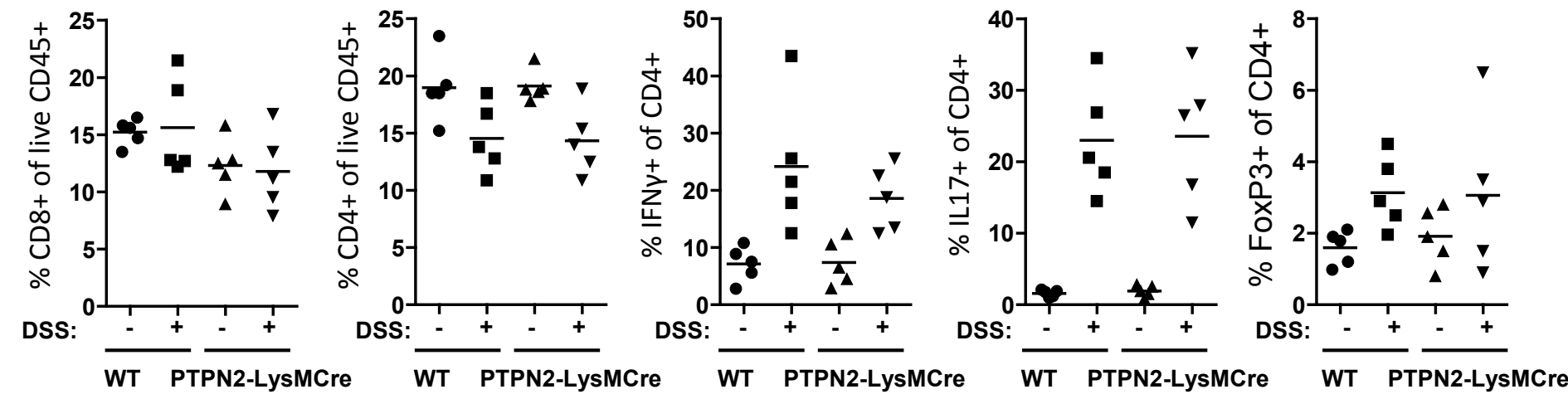
S5a



S5b



S5c



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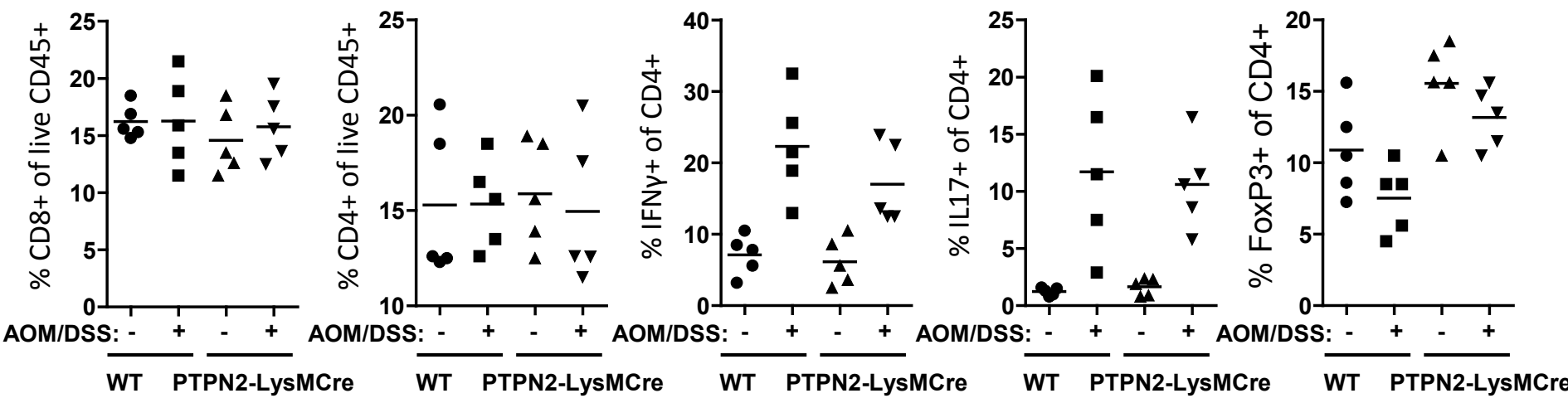


Figure S5. Loss of PTPN2 in macrophages does not alter T helper cells subsets. Related to Figures 1-3. Lymphocytes were isolated from the lamina propria of WT and LysMCre mice with acute **(a+b)** or chronic **(c)** DSS-induced colitis, or **(d)** AOM-DSS induced tumours and analysed for the abundance of CD4+ and CD8+ cells among live, CD45+ cells; and for the abundance of IFN-γ+, IL-17+ and FoxP3+ cells within the CD4+ population. Dot plots in **a)** show the gating strategy used to analyse these subsets.

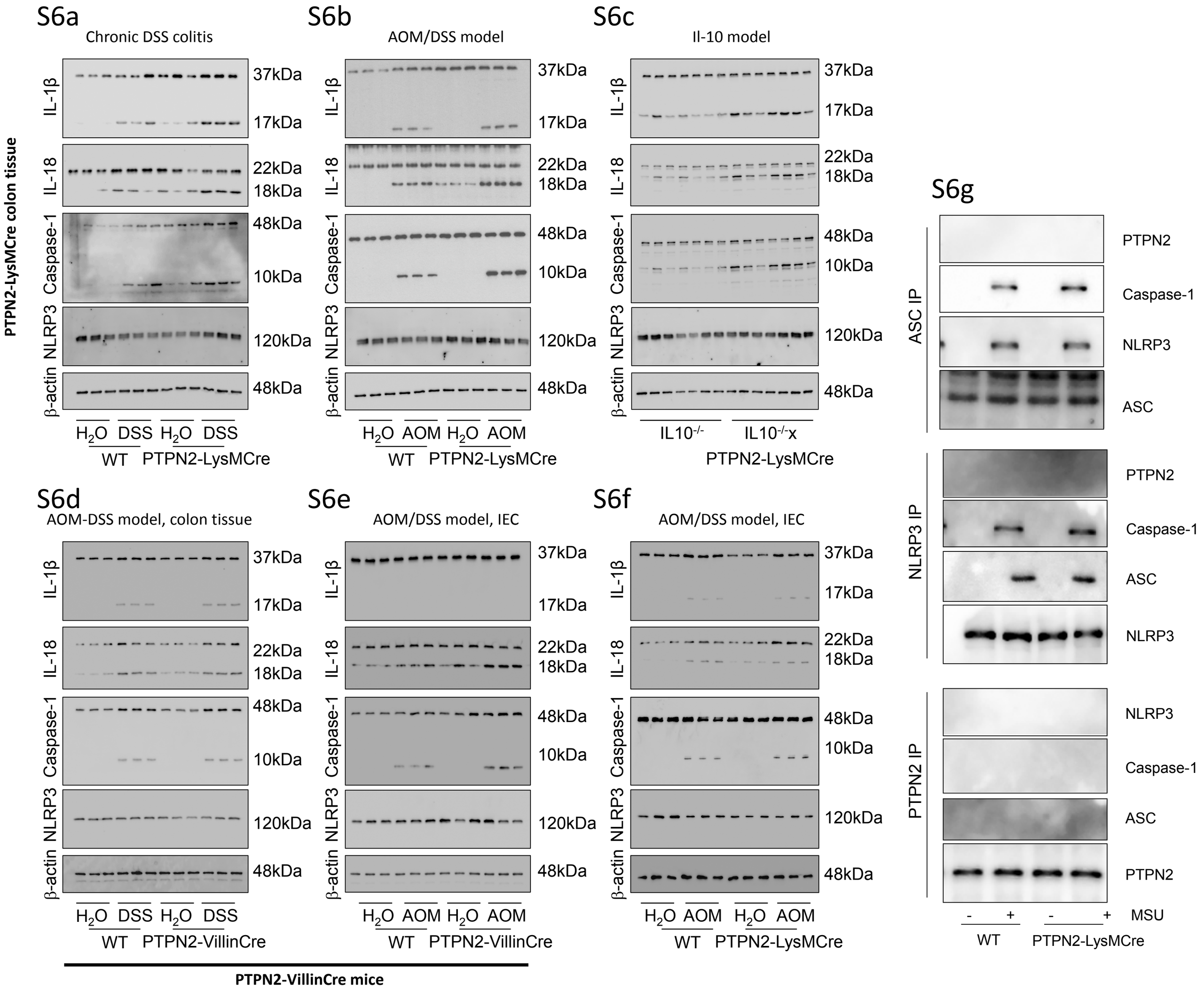
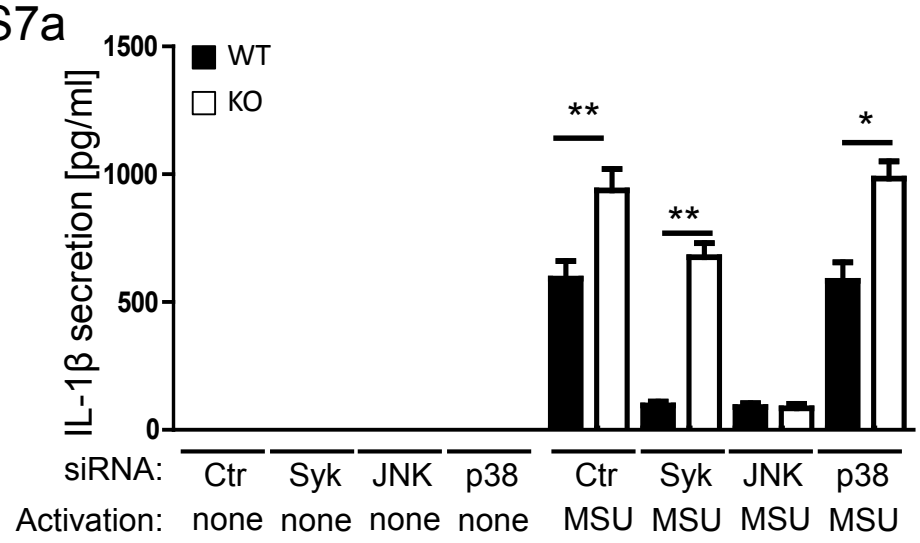


Figure S6. Loss of PTPN2 in macrophages promotes IL-1β, IL-18, and caspase-1 maturation in chronic DSS colitis, in the AOM/DSS induced tumour model, and in IL-10$^{-/-}$ mice. Related to Figure 4. a-d: Colon lysates from WT and PTPN2-LysMCre (**a-c**) or PTPN2-VillinCre (**d**) mice with chronic DSS colitis **(a)**, from mice in the AOM/DSS model **(b+d)**, and from 150 days old IL-10$^{-/-}$ and IL-10$^{-/-}$xPTPN2-LysMCre mice **(c)** were analysed by Western blot for the maturation of IL-1β, IL-18, and caspase-1 as indicated. **e+f:** Intestinal epithelial cells (IEC) were isolated from WT and PTPN2-VillinCre (**e**) or PTPN2-LysMCre **(f)** mice and analysed by Western blot for the maturation of IL-1β, IL-18, and caspase-1 as indicated. **g:** BMDC from WT and PTPN2-LysMCre mice were activated with MSU to induce inflammasome activation. ASC, NLRP3 and PTPN2 were precipitated from cell culture lysates and analysed for co-precipitation of PTPN2, caspase-1, NLRP3 and ASC, as indicated.

S7a



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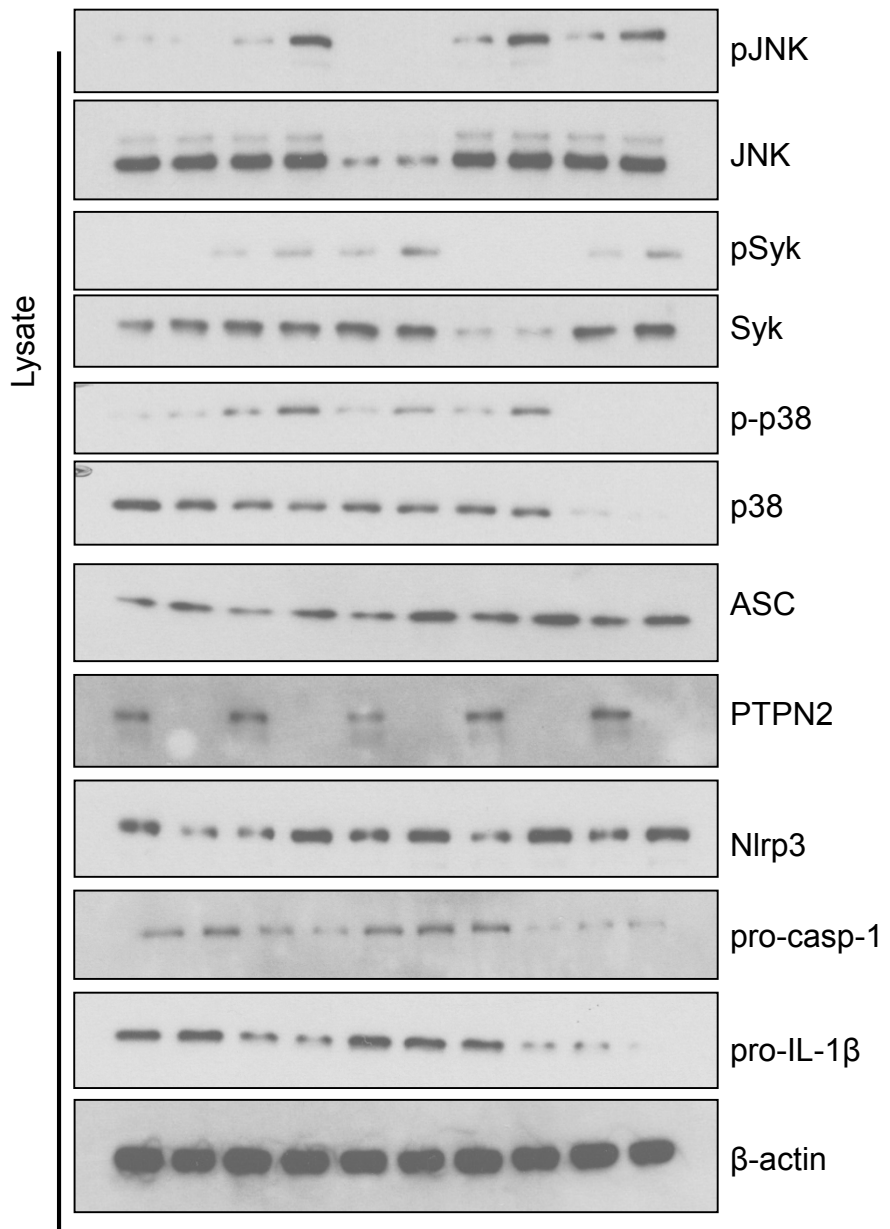

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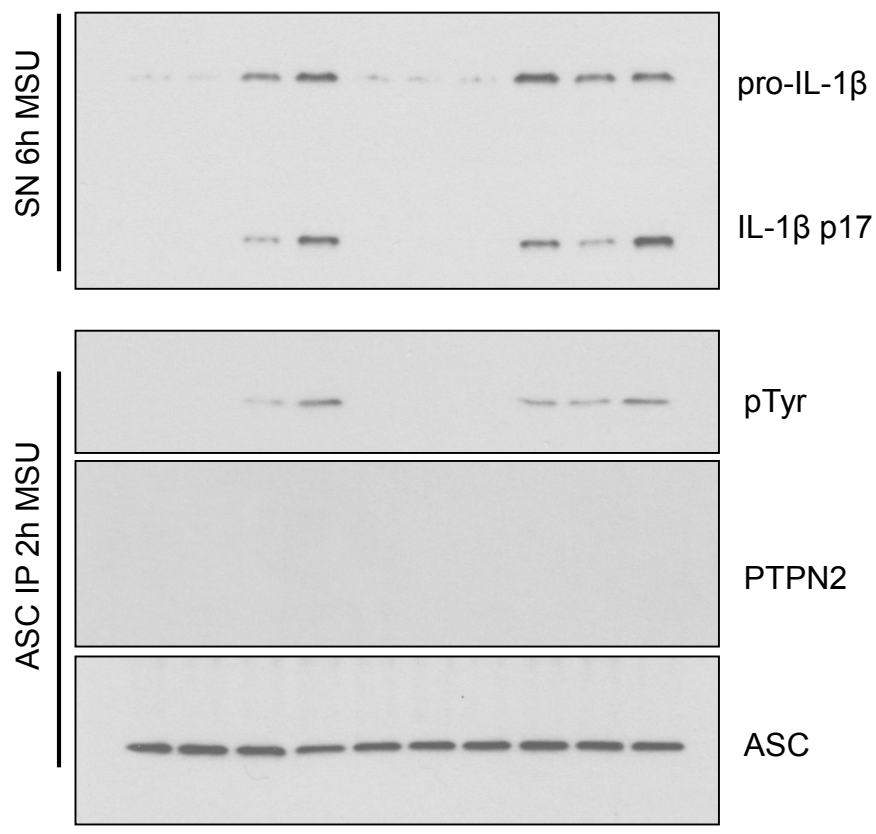

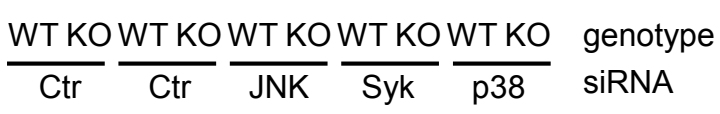

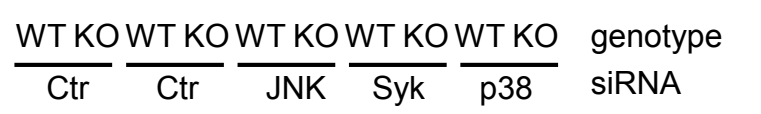

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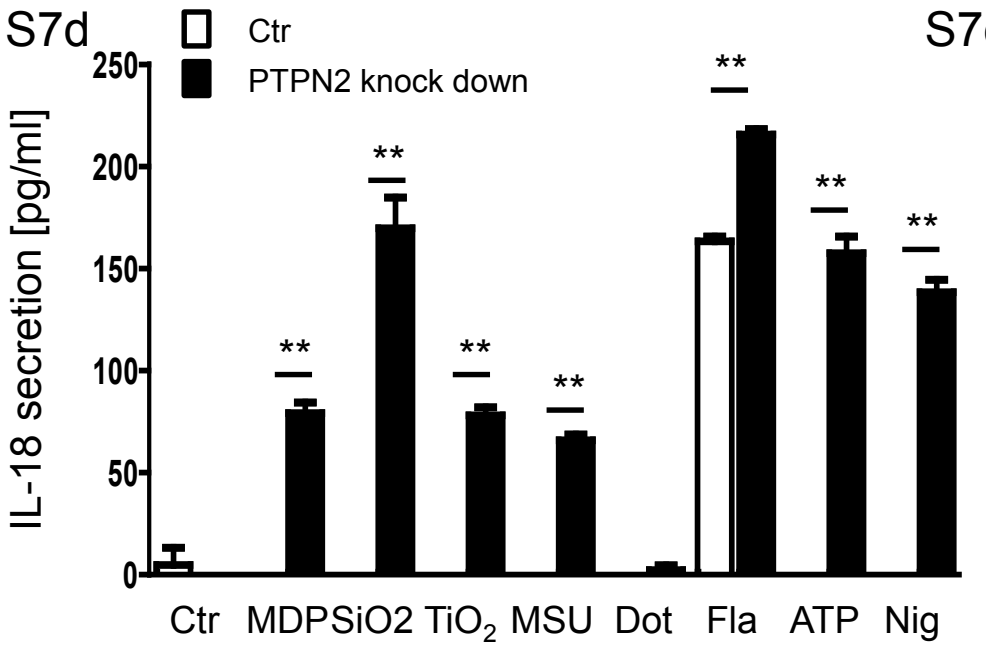

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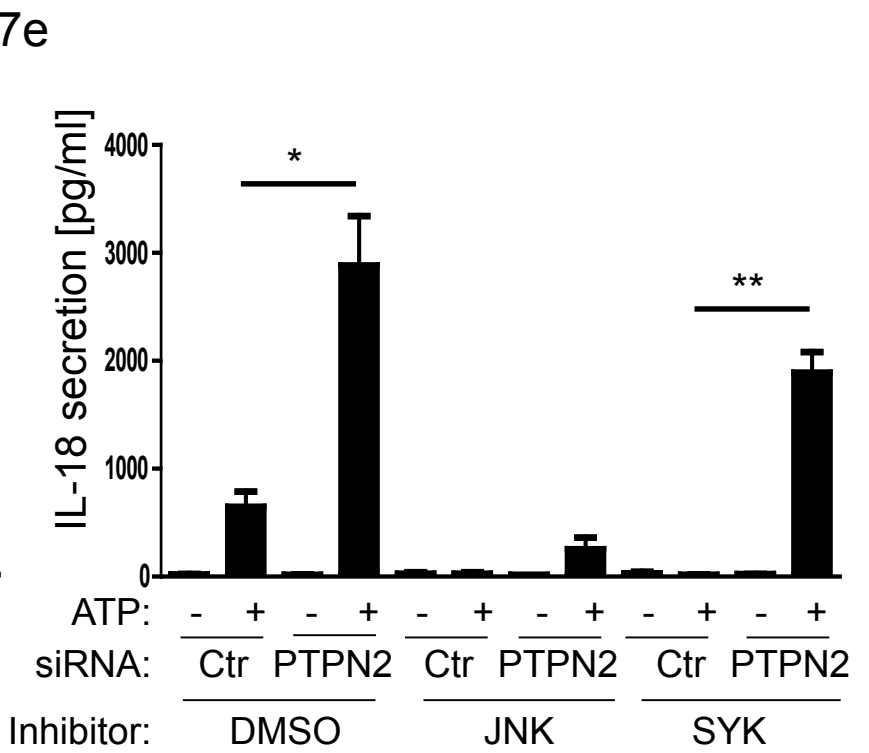
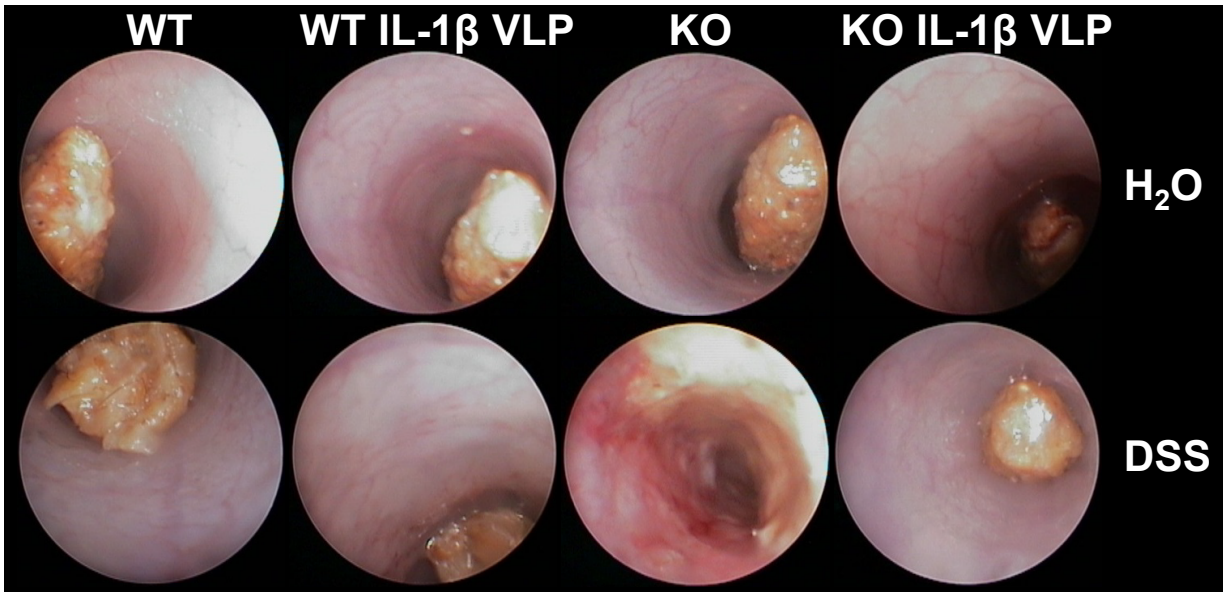
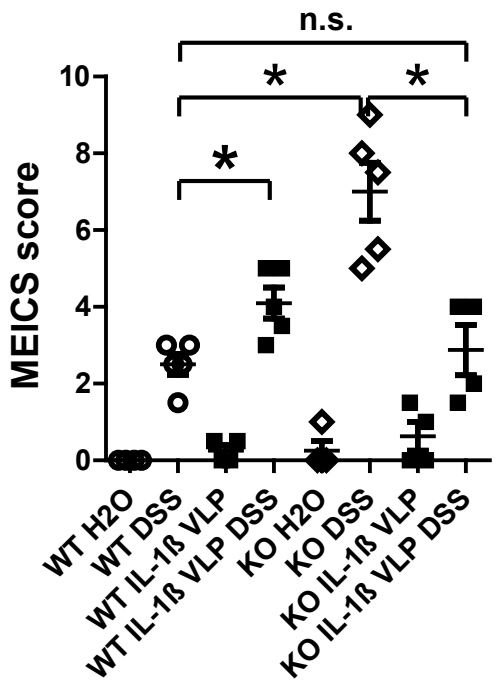


Figure S7. Silencing of JNK, but not Syk abrogates inflammasome activation in PTPN2-deficient cells. Related to figure 5. a-c: BMDC from WT and PTPN2-LysMCre (KO) mice were treated with siRNA for JNK, Syk, or p38 as indicated and activated with MSU. **a+c:** The cell culture supernatant was analysed for IL-1β secretion **(a)** and maturation **(c)** by ELISA and Western blot, respectively. **b)** Cell lysates were analysed for the indicated proteins; and **c)** ASC was precipitated from the cell lysate and analysed for tyrosine phosphorylation and co-precipitation of PTPN2. **d)** PTPN2 was silenced in HT-29 cells using PTPN2-specific siRNA prior to activation with the indicated inflammasome activators. Cell culture supernatant was collected and analysed for IL-18. **e)** PTPN2 was silenced in HT-29 cells using PTPN2-specific siRNA. The cells were treated for 1h with JNK or Syk inhibitors as indicated prior to activation of the NLRP3 inflammasome using ATP. Cell culture supernatants were collected and analysed for IL-18. Asterisks denote significant different results Asterisks denote significant different results (*= $p<0.05$, **=$p<0.01$; Student's t test with bonferroni correction).

S8a





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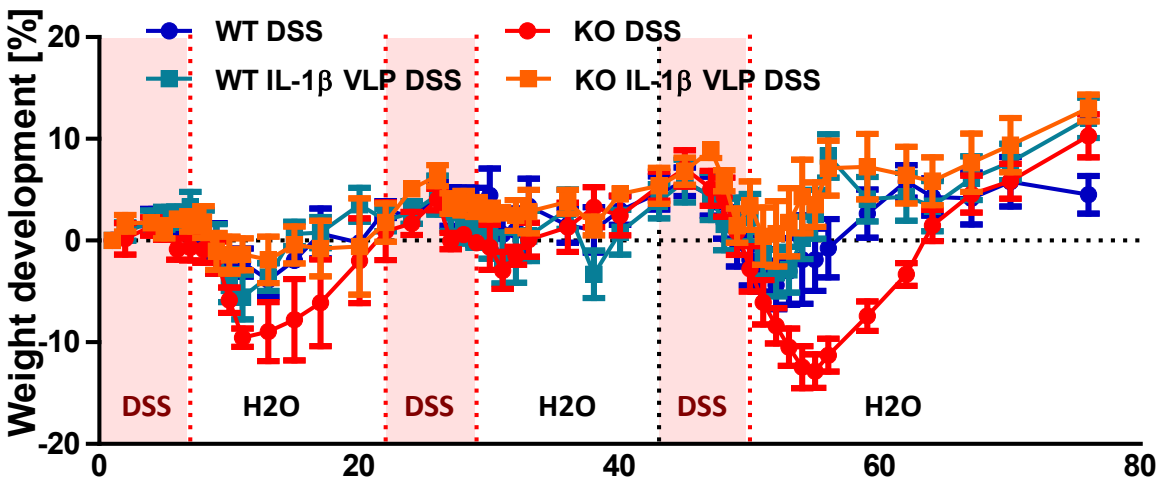
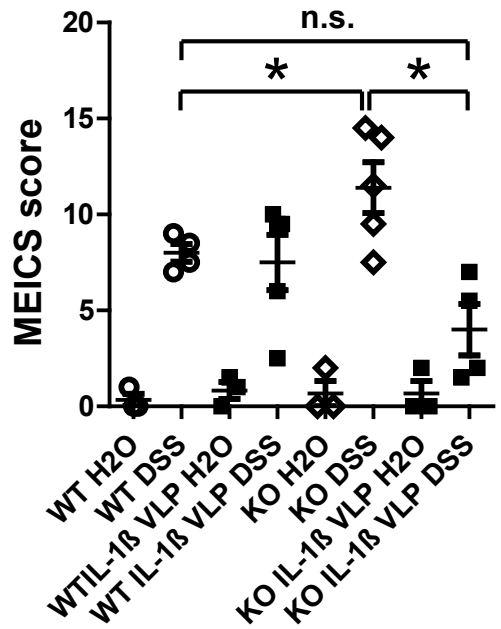




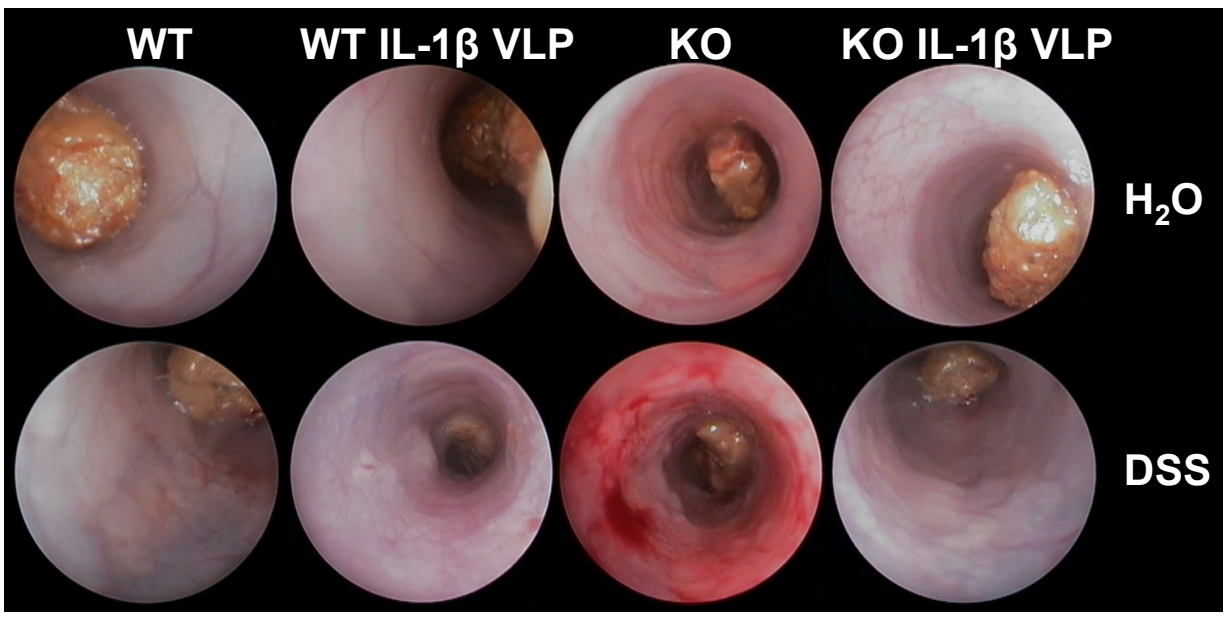
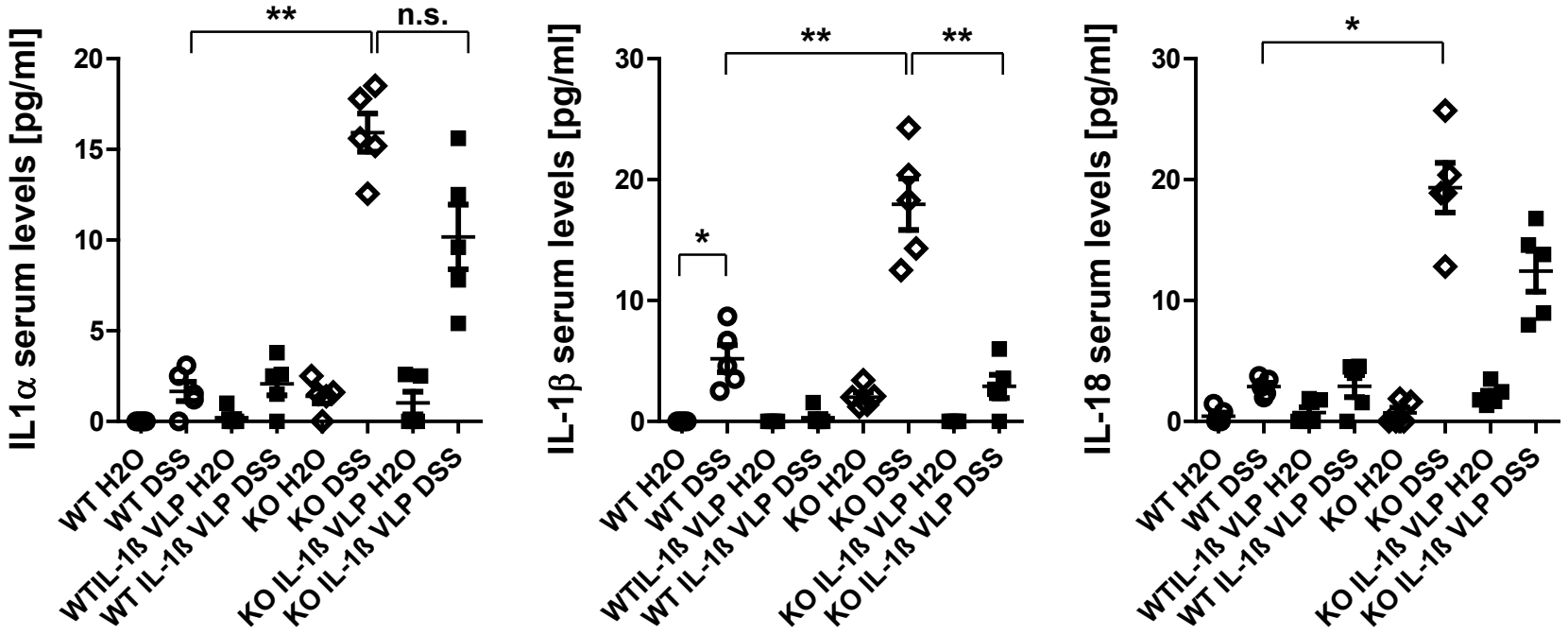
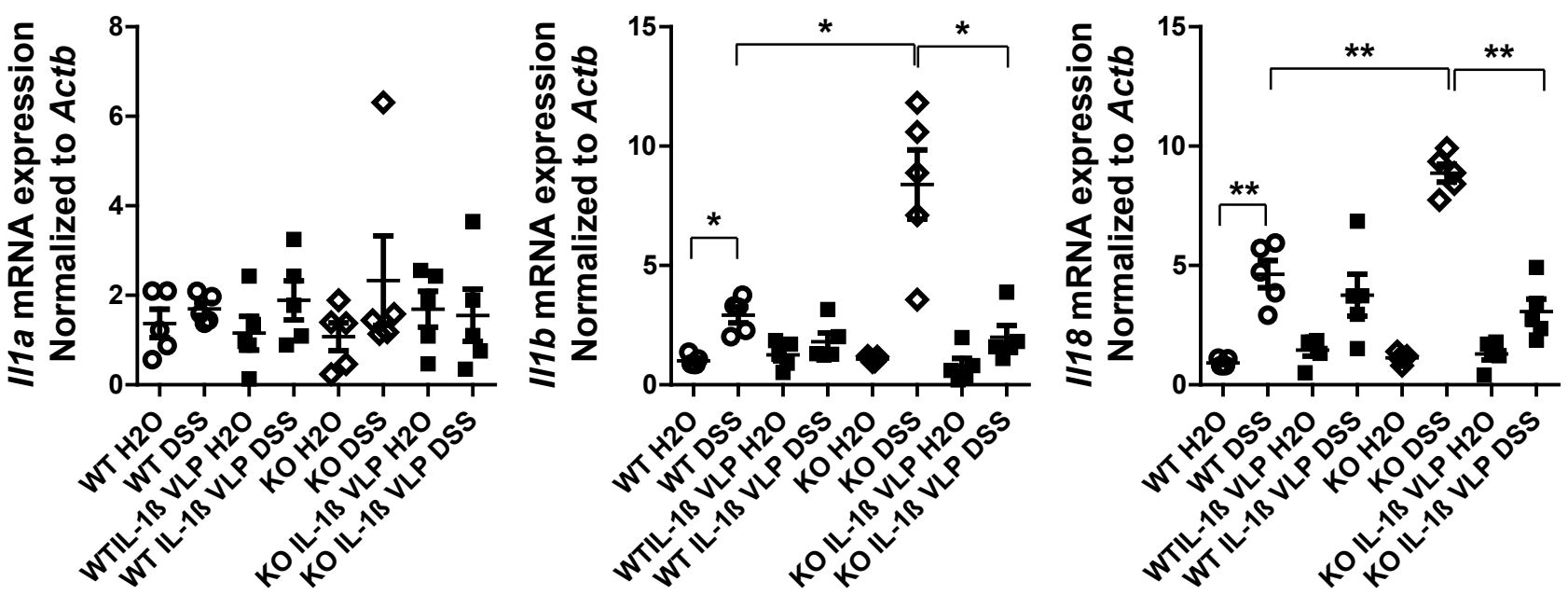


Figure S8. Inhibition of IL-1β protects PTPN2-LysMCre mice from increased colitis severity. Related to figure 6. Mice were immunized against IL-1β using IL-1β-Qβ virus like particles five, three, and one week(s) prior to acute or chronic colitis induction. **a:** representative pictures of colonoscopy and respective scoring in acute DSS-induced colitis; **b:** Weight development, representative pictures of colonoscopy, scoring of endoscopic colitis severity and evaluation of fibrin in chronic DSS-induce colitis. Asterisks indicate statistically significant differences (*=$p<0.05$, Mann-Whitney-U-test with bonferroni correction).

S9a



S9b



S9c

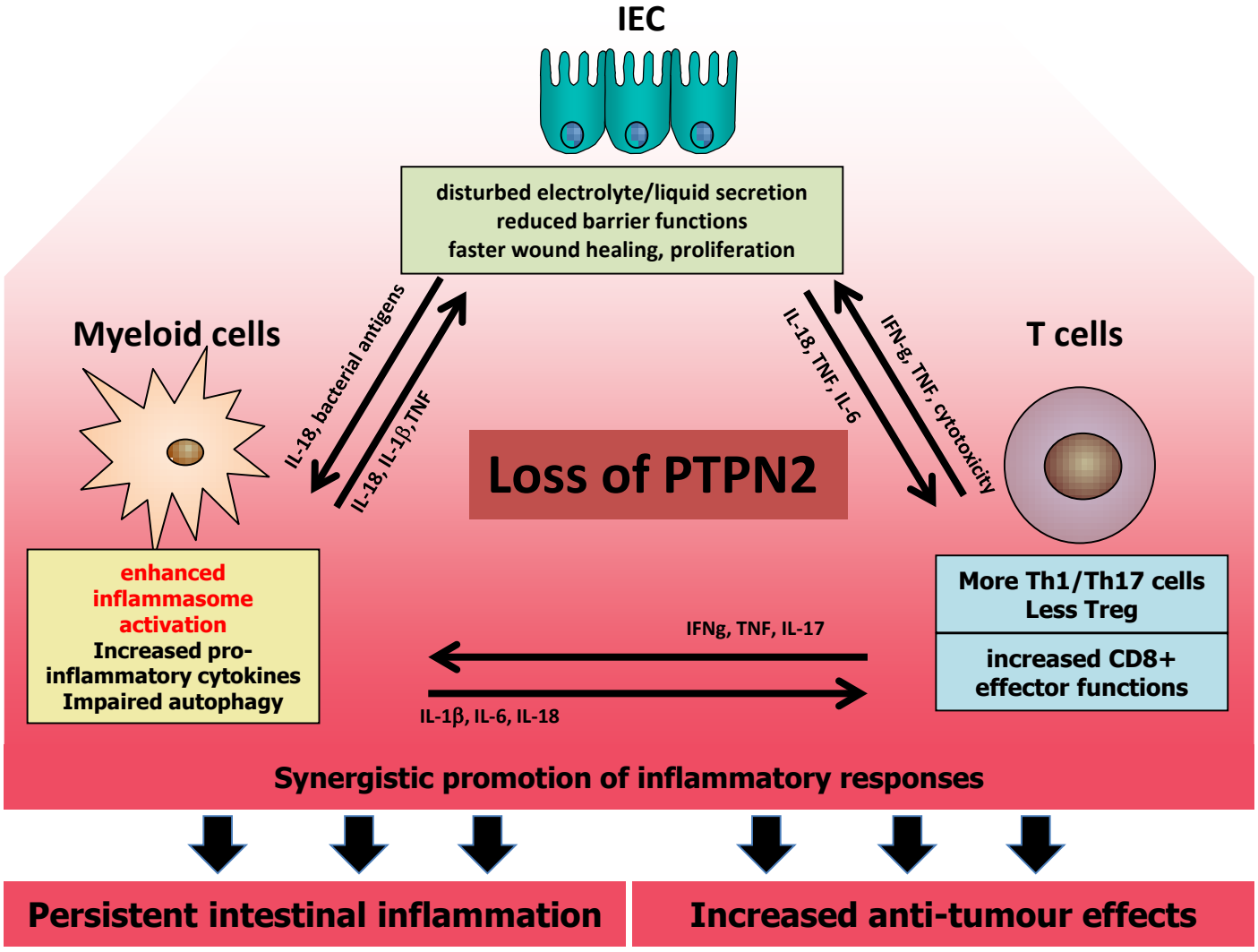


Figure S9. Serum levels and mRNA expression of IL-1α, IL-1β and IL-18 upon inhibition of IL-1β. Related to figure 6. Mice were immunized against IL-1β using IL-1β-Qβ virus like particles five, three, and one week(s) prior to induction of chronic colitis. The graphs show **a:** serum levels for IL-1α, IL-1β and IL-18, and **b:** mRNA expression of *Il1a*, *Il1b*, and *Il18* in the colon. Asterisks indicate statistically significant differences (*=p<0.05, **=p<0.01; Mann-Whitney-U-test with bonferroni correction). **c:** Summary schematic of how loss of PTPN2 in different cell types synergistically contributes to enhanced inflammation but protects from tumour formation.