

SUPPLEMENTARY DOCUMENT**Bile Acids Modulate Colonic MAdCAM-1 Expression in a Murine Model of Combined Cholestasis and Colitis**

Rachel Y. Gao^{1, 2}, Colin T. Shearn³, David J. Orlicky⁴, Kayla D. Battista¹, Erica E. Alexeev¹, Ian M. Cartwright^{1,5}, Jordi M. Lanis¹, Rachael E. Kostelecky¹, Cynthia Ju⁶, Sean P. Colgan^{1,5}, and Blair P. Fennimore¹

SUPPLEMENTARY METHODS

Bile acid analysis. Chenodeoxycholic acid, lithocholic acid, ursodeoxycholic acid, and deoxycholic acid were obtained from Sigma Aldrich (St. Louis, Missouri). Alpha-muricholic acid and beta-muricholic acid were obtained from Cayman Chemical (Ann Arbor, Michigan). Chenodeoxycholic acid-d4 and glycochenodeoxycholic acid-d4 were obtained from Cambridge Isotope labs (Tewksberry, Massachusetts) to use as internal standards.

External standards were prepared by combining all bile acids over a range of 0.05uM-50µM in methanol. For the sample preparation, 140µl of methanol, 15-30µl of water and 10µl of internal standard were added to each sample. The sample was then vortexed for 5 seconds and incubated in a -20°C freezer for 20 minutes. The sample was then centrifuged at 6000RPM for 15 minutes at 4°C. The supernatant (185-200µl) was then transferred to a reduced surface activity (RSA) autosampler vial (Microsolv Technology Corporation, Leland, NC) for immediate analysis or frozen at -70°C until analysis.

High performance liquid chromatography/quadrupole time of flight mass spectrometry (HPLC/QTOF) mass spectrometry was performed on a 1290 series HPLC from Agilent (Santa Clara, CA). Bile acids were separated using an Agilent SB-C18 2.1X100mm 1.8um column with a 2.1X 5mm 1.8um guard column. Buffer A consisted of 90:10 water:acetonitrile with 1mM ammonium acetate adjusted to pH=4 with acetic acid, and buffer B consisted of 50:50 acetonitrile:isopropanol.

Mass spectrometric analysis was performed on an Agilent 6520 quadrupole time of flight mass spectrometer in negative ionization mode. Retention time and m/z for each bile acid was determined by injecting authentic standards individually. Calibration curves

for each bile acid was constructed using Agilent Masshunter Quantitative Analysis software.

Histologic colitis severity. Four independent parameters were assessed, including inflammation (0-3), extent of injury (0-3), crypt damage (0-4), and regeneration (0-4). The score for each parameter was then multiplied by a factor reflecting the percentage of tissue involvement and combined for a maximum possible score of 40. Liver fibrosis was assessed by picosirius red stain (PSR) under polarized light.

IFC Quantification. PSR fibrosis and immunofluorescence were quantified using SlideBook v. 6.0 (Intelligent Imaging Innovations, Denver, Colorado). For IFC quantification, multiple random images were obtained from the distal third of the colon for each mouse in a blinded fashion using an LSM 710 confocal microscope (Zeiss, Thornwood, NY). MAdCAM-1 staining was quantified using SlideBook v. 6.0 (Intelligent Imaging Innovations) and expressed as a percentage per 20x high power field and normalized to a CD31 stain. $\alpha 4\beta 7$ staining was quantified using SlideBook v. 6.0 (Intelligent Imaging Innovations) and expressed in area (μM^2) per 20x high power field.

Cytokine Analysis. RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) was used to isolate RNA from TSECs, liver, and colon tissue. TRIzol reagent (Invitrogen, Carlsbad, CA) was used to isolate RNA from bEnd.3 cells. cDNA was reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). PCR analysis of beta actin, ICAM-1, MAdCAM-1, and VCAM-1 used SYBR Green (Applied Biosystems, Carlsbad, CA) with a 384-well 7900 HT Fast Real-Time PCR machine (Applied Biosystems). Primer sequences are described in Supplemental Table 1. PCR analysis of IL-17A, IL-17F, and IL-

23(Mm00439618_m1, Mm00521423_m1, Mm00518984_m1; ThermoFisher) used the TaqMan™ Fast Advanced Master Mix (ThermoFisher).

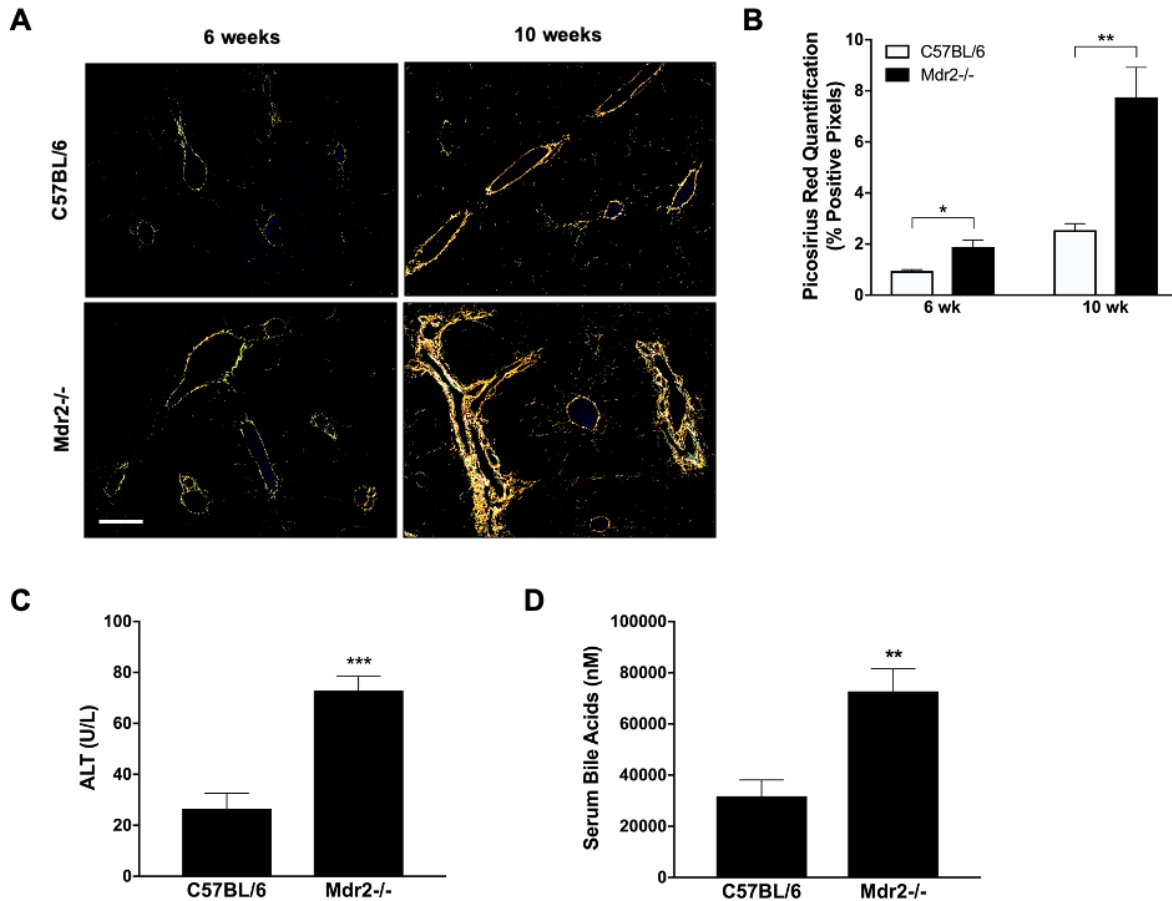
Cell Lines and treatment. TSECs were cultured in 1:1 DMEM-Ham's F12 with 2.5 mM L-glutamine 10% heat inactivated FBS plus endothelial growth supplement (Cat. #1052, ScienCell, Carlsbad, CA). bEnd.3 cells were cultured in DMEM with 2.5 mM L-glutamine and 10% FBS. Both cell types were cultured in 95% air with 5% CO₂ at 37°C. Human recombinant TNF- α (eBioscience, San Diego, CA), IL-1 β (ThermoFisher), and IFN- γ (Biotum, Hayward, CA) were diluted in culture media from a stock of 0.1 mg/ml at the time of treatment. Bile acids (Ursodeoxycholic acid, UDCA; Cholic acid, CA; Chenodeoxycholic acid, CDCA, Deoxycholic acid, DCA; and Lithocholic acid, LCA; Sigma-Aldrich) in DMSO were diluted 1:1,000 in culture media to a working concentration of 100 μ M. For NF- κ B luciferase reporter assays, lipofectamine® LTX Reagent with PLUS™ Reagent (ThermoFisher) was used to transfect the pNF- κ B cis-reporter plasmid (Stratagene, La Jolla, CA, Cat. #219078-51) into TSECs, following manufacturer directions. NF- κ B luciferase levels were measured using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI), according to manufacture directions. For cell viability assays, a cell counting kit-8 (Sigma-Aldrich) colorimetric assay was used to determine cell viability, according to manufacturer directions.

Supplemental Table 1: QPCR primer sequences.

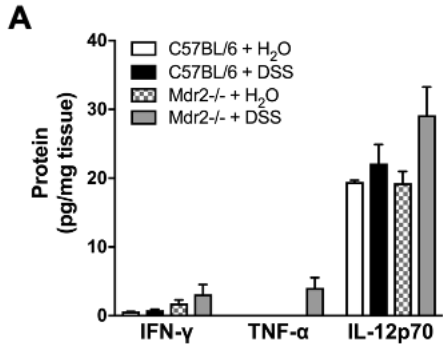
Primers	Sequences
ICAM-1	F - GGCATTGTTCTCTAATGTCTCCG
	R - TGTCGAGCTTTGGGATGGTAG
MAdCAM-1	F - CAGCTTGGGCAGTGTACAGAC
	R - TCTGCAGGCCAGATGTTGTGG
VCAM-1	F - GCCCACTAAACGCGAAGGT
	R - ACTGGGTAAATGTCTGGGAGCC

SUPPLEMENTARY FIGURES

Supplementary 1

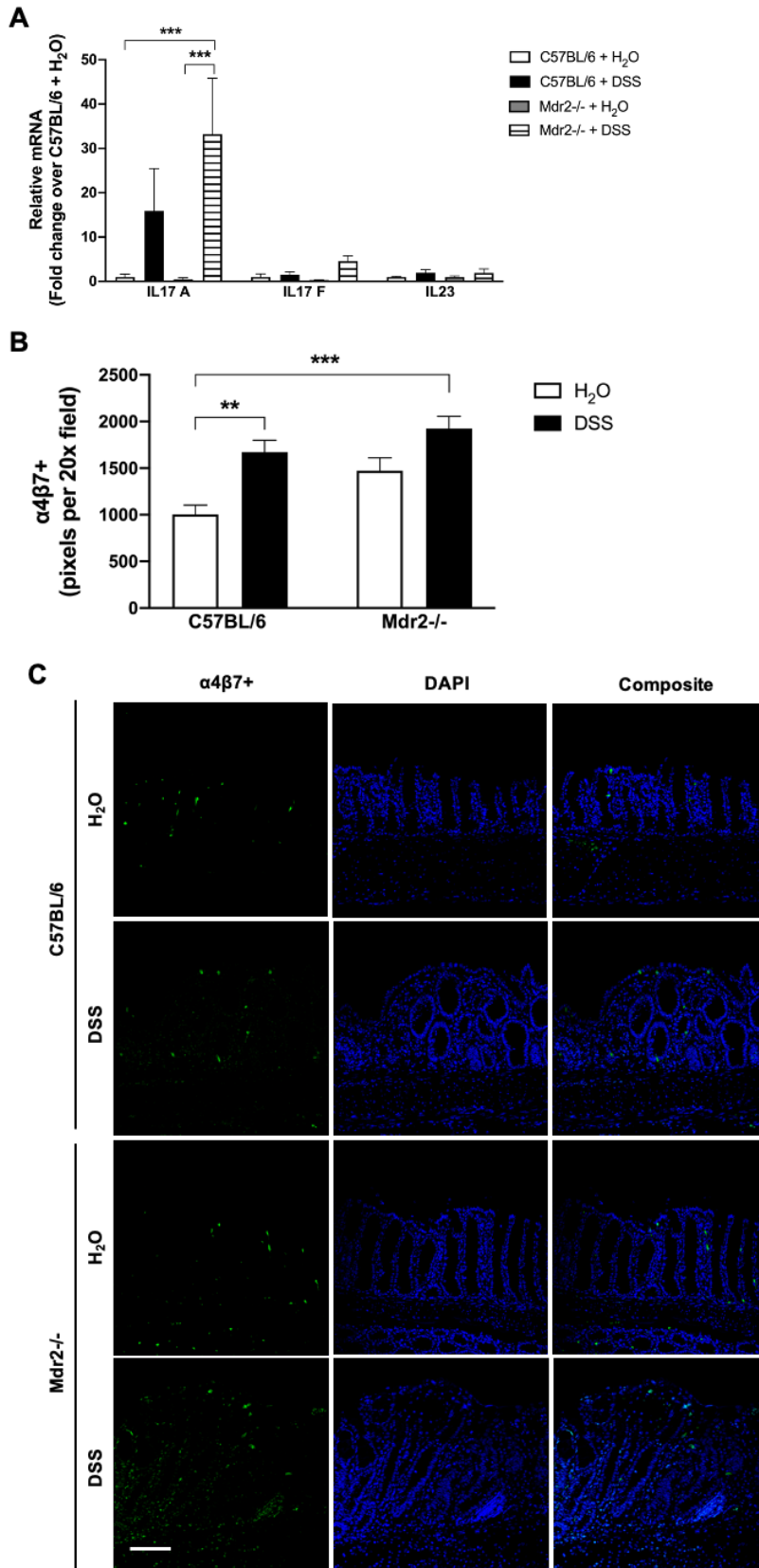


Supplementary Fig. S1. Liver disease assessment in Mdr2^{-/-} mice. (A, B) Liver fibrosis quantified by picosirius red stain (PSR; 200X) seen under polarized light. Representative images are shown in panel A. Quantitation of picosirius red staining is shown in panel B. **(C)** Serum alanine transaminase (ALT). **(D)** Serum bile acids measured using mass spectrometry. $n \geq 4$ mice per group. Data are expressed as means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test.

Supplementary 2

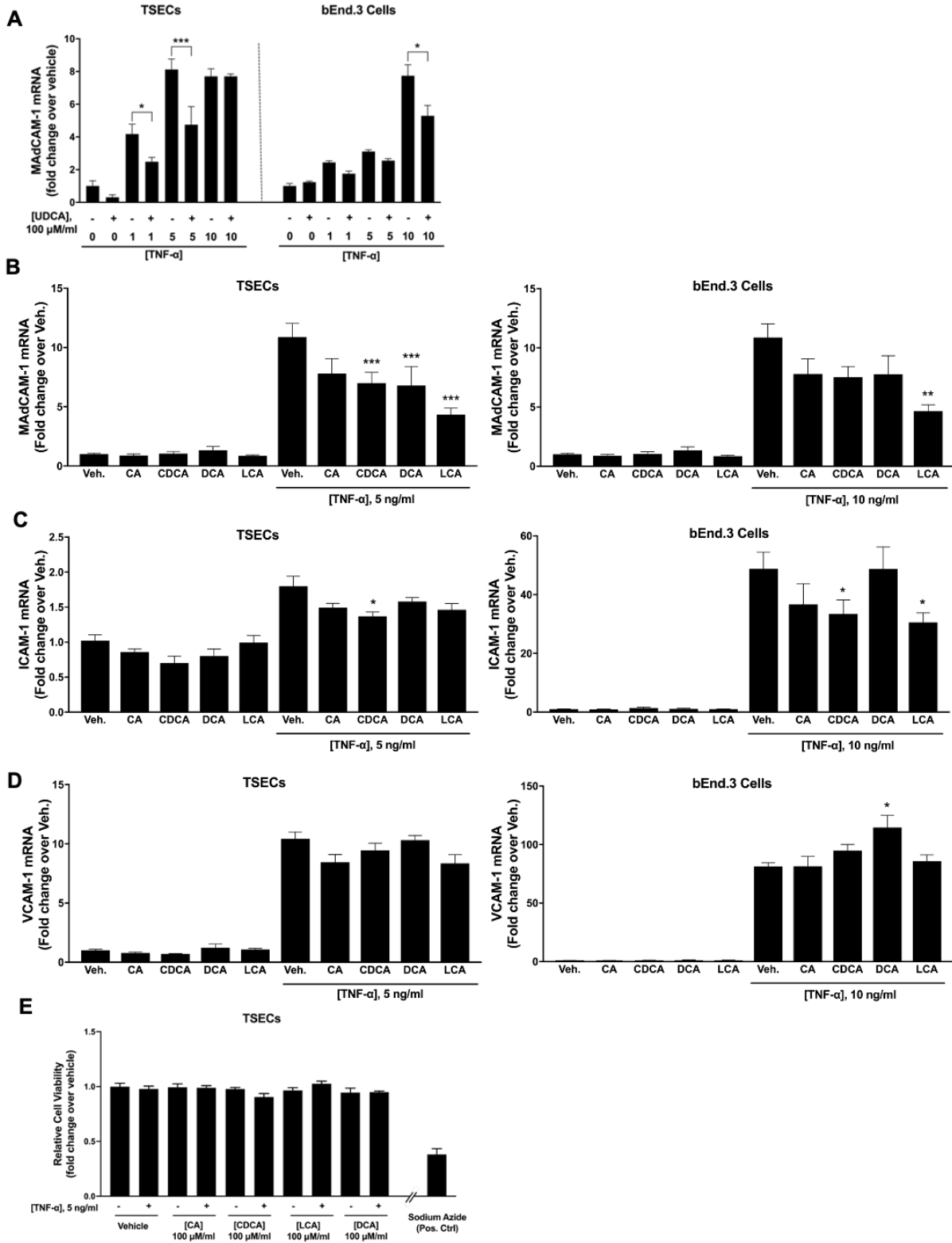
Supplementary Fig. S2. Colonic cytokine levels in DSS-treated Mdr2^{-/-} mice. Colon tissue was homogenized, and cytokine protein levels were measured by Meso Scale Discovery analysis. $n \geq 5$ mice per group. Data are expressed as means \pm SEM.

Supplementary 3



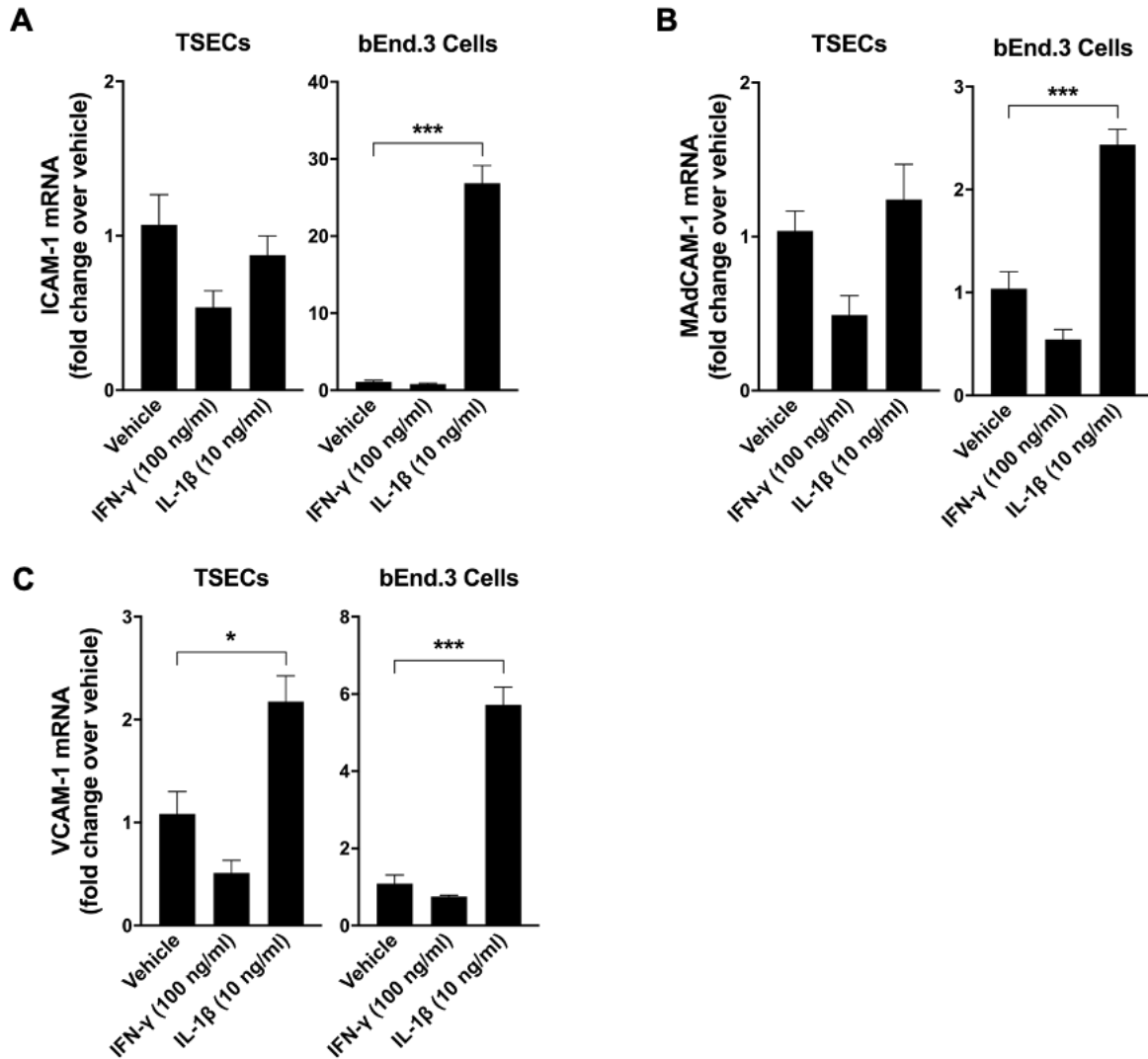
Supplementary Fig. S3. Colonic adaptive immune response in DSS-treated WT and Mdr2^{-/-} mice. WT and Mdr2^{-/-} mice were treated as described in Fig. 2. **(A)** Colon tissue was homogenized, and colonic cytokines were measured by RT-qPCR. **(B, C)** Colon tissue was stained with an $\alpha 4\beta 7$ antibody, the density of $\alpha 4\beta 7^+$ cells was quantified. $n \geq 10$ mice per group. Data are expressed as means \pm SEM. ** $P < 0.01$, *** $P < 0.001$, two-way analysis of variance.

Supplementary 4



Supplementary Fig. S4. Effects of bile acids on TNF- α -induced adhesion molecule expression *in vitro* (A) TSECS or bEnd.3 cells were co-treated with UDCA and titrating doses of TNF- α . Cells were collected and subjected to RT-qPCR analysis of MAdCAM-1 expression. (B-D) TSECS or bEnd.3 cells were co-treated with TNF- α and various bile acids (chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) or lithocholic acid (LCA) for four hours. Cells were collected and subjected to RT-qPCR analysis of MAdCAM-1, (B), ICAM-1 (C), and VCAM-1 (D) expression. (E) TSECS cells were treated with various bile acids with or without TNF- α . Cell viability was assessed. Positive control is treatment of cells with 1% sodium azide (0.76 M). Data are expressed as means \pm SEM. **P<0.001, ***P<0.0001, two-way ANOVA and Student's *t*-test.

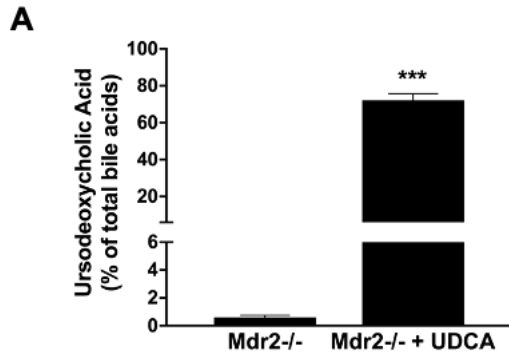
Supplementary 5



Supplementary Fig. S5. Effects of IFN- γ and IL-1 β on adhesion molecule expression. (A-C) TSECs or bEnd.3 cells were treated with IFN- γ or IL-1 β for four hours.

Cells were collected and subjected to RT-qPCR analyses of adhesion molecules.

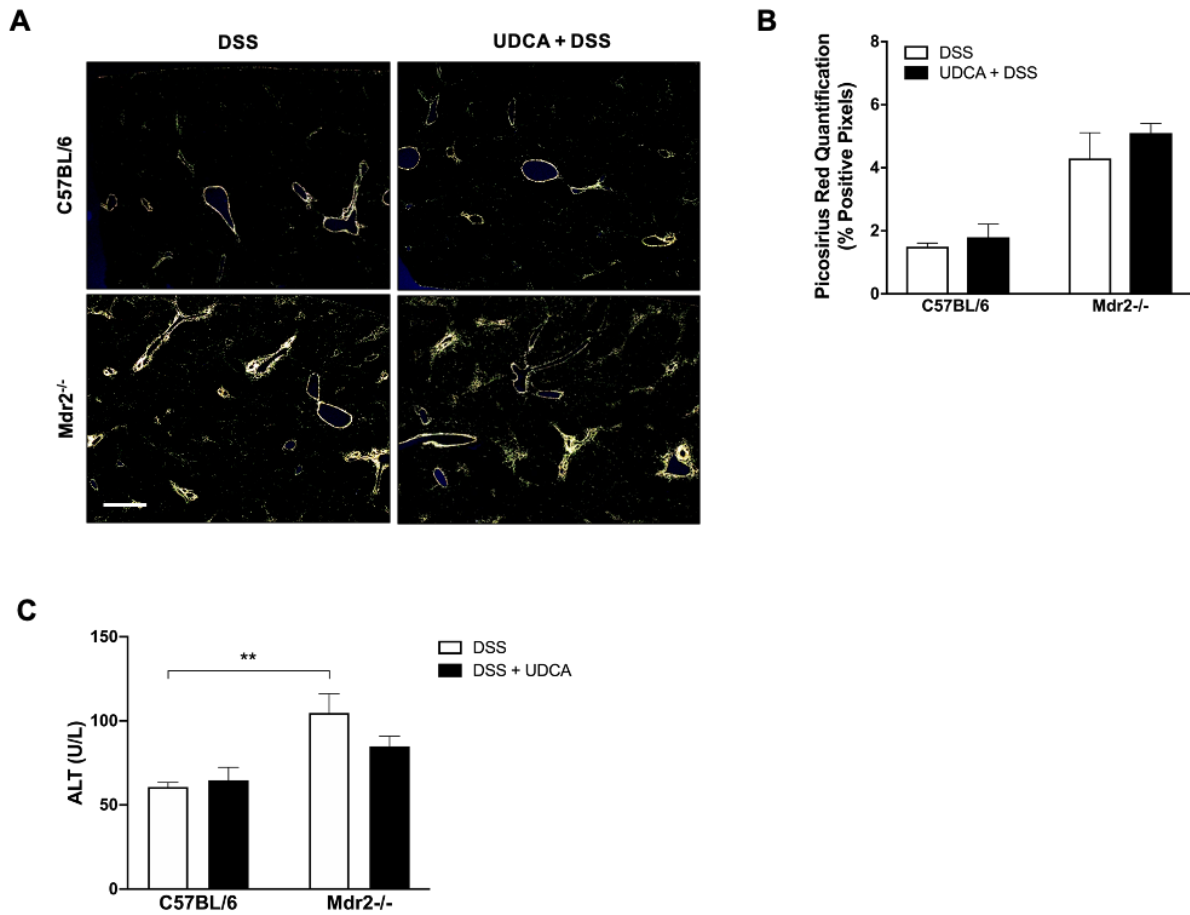
* $P < 0.01$, *** $P < 0.001$, Student's *t*-test.

Supplementary 6**Supplementary Fig. S6. Fecal UDCA levels with dietary supplementation (A)** Fecal

UDCA levels following dietary UDCA supplementation. Fecal bile acids were measured using mass spectrometry. n = 5 mice per group. Data are expressed as means \pm SEM.

P<0.01, *P<0.0001, two-way ANOVA.

Supplementary 7



Supplementary Fig. S7. Liver fibrosis assessment following dietary UDCA supplementation. Mice were treated with UDCA and DSS as described in Fig. 5 (A, B) Liver fibrosis quantified by picosirius red stain (PSR; 200X) seen under polarized light. Quantitation of picosirius red staining is shown. (C) Serum alanine transaminase (ALT). $n \geq 5$ mice per group. Data are expressed as means \pm SEM. ** $P < 0.01$, two-way ANOVA.

Supplementary 8

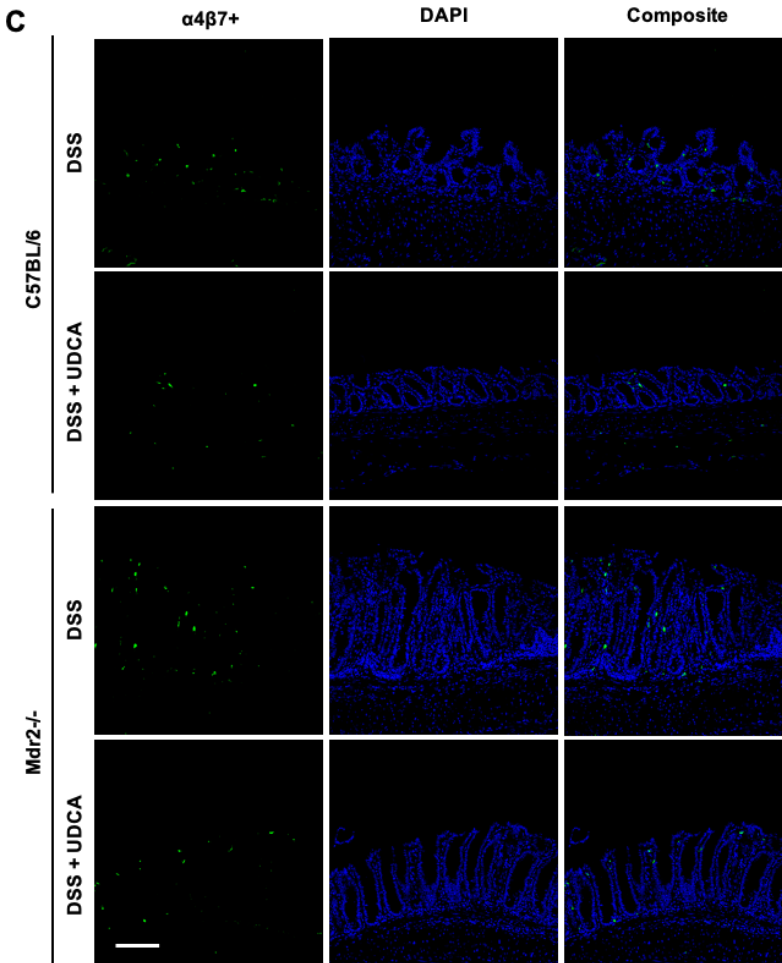
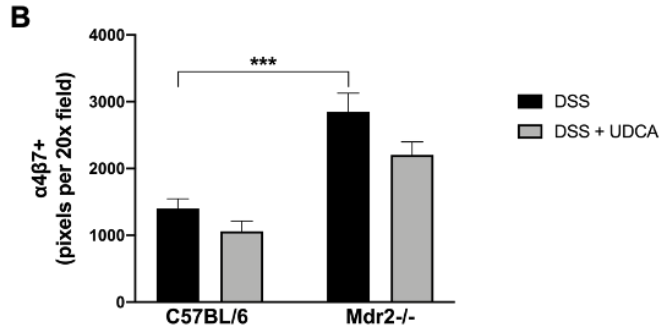
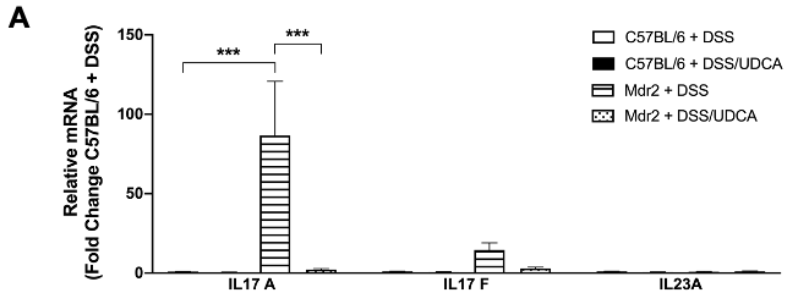


Fig. S8. Effects of dietary UDCA supplementation on colonic adaptive immune response. WT and Mdr2^{-/-} mice were treated as described in Fig. 5. **(A)** Colon tissue was homogenized, and colonic cytokines were measured by RT-qPCR. **(B, C)** Mice were treated with UDCA and DSS as described in Fig. 5. Colon tissue was stained with an $\alpha 4\beta 7$ antibody, the density of $\alpha 4\beta 7^+$ cells was quantified. $n \geq 8$ mice per group. Data are expressed as means \pm SEM. ** $P < 0.01$, *** $P < 0.0001$, two-way ANOVA.