Supplemental Table. Sources of Primary Antibodies Used in Study

Antibody (Flow cytometry)	Clone number	Conjugated	Brand
LIVE/DEAD Fixable Dead Cell Stain Kit	-N/A	Near-IR	Molecular Probes
anti-mouse B220	RA3-6B2	Pacific Blue	Invitrogen
anti-mouse CD11b	M1/70	PE-Cy7	BD Pharmingen
anti-mouse CD11c	HL3	Alexa Fluor 700	BD Pharmingen
anti-mouse CD16/CD32	2.4G2	-	BD Pharmingen
anti-mouse CD19	1D3	PerCP-Cy5.5, APC	BD Pharmingen
anti-mouse CD19	6D5	Brilliant Violet 605	BioLegend
anti-mouse CD1d	1B1	PE	eBioscience
anti-mouse CD21	7G6	Brilliant Violet 650	BD Pharmingen
anti-mouse CD210 (IL-10R)	1B1.3a	functional grade	BD Pharmingen
anti-mouse CD23	B3B4	PE	eBioscience
anti-mouse CD24	M1/69	APC	BD Pharmingen
anti-mouse CD25	PC61.5	PE	eBioscience
anti-mouse CD282 (TLR2)	QA16A01	functional grade	BioLegend
anti-mouse CD3	145-2C11	eFluor 450, PE-Cy5	eBioscience
anti-mouse CD3	17A2	functional grade	R&D systems
anti-mouse CD4	GK1.5	PE-Cy7	BD Pharmingen
anti-mouse CD4	RM4-5	APC	BioLegend
anti-mouse CD45	30-F11	Pacific Orange	Invitrogen
anti-mouse CD49b	DX5	PE-Cy7	eBioscience
anti-mouse CD5	53-7.3	PE-Cy5	BD Pharmingen
anti-mouse CD8a	53-6.7	PE-Cy7	eBioscience
anti-mouse F4/80	BM8	Alexa Fluor 647	Invitrogen
anti-mouse F4/80	BM8	PE	eBioscience
anti-mouse Foxp3	FJK-16s	eFluor 660	eBioscience
anti-mouse IL-10	JES5-16E3	APC	BD Pharmingen
anti-mouse IgD	11-26c.2a	APC	BD Pharmingen
anti-mouse IgM	II/41	PE-Cy7	eBioscience
anti-mouse MHC-II	M5/114.15.2	Brilliant Violet 650	BioLegend
anti-mouse RORgt	Q31-378	Alexa Fluor 647	BD Pharmingen
hamster IgG1 λ	G235-2356	PerCP-Cy5.5	BD Pharmingen
hamster IgG1 λ	A19-3	APC	BD Pharmingen

mouse IgG1 κ	MOPC-21	functional grade	BioLegend
mouse IgG2a κ	G155-178	Alexa Fluor 647	BD Pharmingen
rat IgG1 κ	eBRG1	PE	eBioscience
rat IgG1 κ	R3-34	functional grade	BD Pharmingen
rat IgG1 λ	G0114F7	PE	BioLegend
rat IgG2a κ	R35-95	PE	BD Pharmingen
rat IgG2a κ	RTK2758	Brilliant Violet 605	BioLegend
rat IgG2a κ	eBR2a	eFluor 660	eBioscience
rat IgG2b κ	A95-1	PE-Cy7, PE-Cy5, APC	BD Pharmingen
rat IgG2b κ	R35-38	Brilliant Violet 650	BD Pharmingen
Antibodies (Immunohistochemistry)	**Clone number**	**Catalog number**	**Brand**
anti-GFP	-	A-11122	Invitrogen
anti-mouse B220	RA3-6B2	550286	BD Pharmingen
anti-mouse CD3	145-2C11	550275	BD Pharmingen
anti-rabbit IgG (unconjugated)	-	31210	Invitrogen
Antibodies (Western blots)	**Clone number**	**Catalog number**	**Brand**
anti-p-STAT3 (Y705)	D3A7	9131	Cell Signaling Technology
anti-STAT3	79D7	4904	Cell Signaling Technology
anti-p-Akt (Ser473)	D9E	4060	Cell Signaling Technology
anti-Akt	C67E7	4691	Cell Signaling Technology
anti-pERK1/2 (T202/Y204)	D13.14.4E	4370	Cell Signaling Technology
anti-ERK1/2	C-16	sc-093	Santa Cruz Biotechnology
anti-pp38 (T180/Y182)	28B10	9216	Cell Signaling Technology
anti-p38	-	9212	Cell Signaling Technology
anti-p-p85 (Y458/Y199)	-	4228	Cell Signaling Technology
anti-p85	19H8	4257	Cell Signaling Technology
anti-p-PDK1 (Ser241)	C49H2	3438	Cell Signaling Technology
anti-PDK1	-	3062	Cell Signaling Technology
anti-p-GSK3β (Ser9)	D85E12	5558	Cell Signaling Technology
anti-GSK3α/β	D75D3	5676	Cell Signaling Technology
anti-actin	C4	Sc-47778	Santa Cruz Biotechnology

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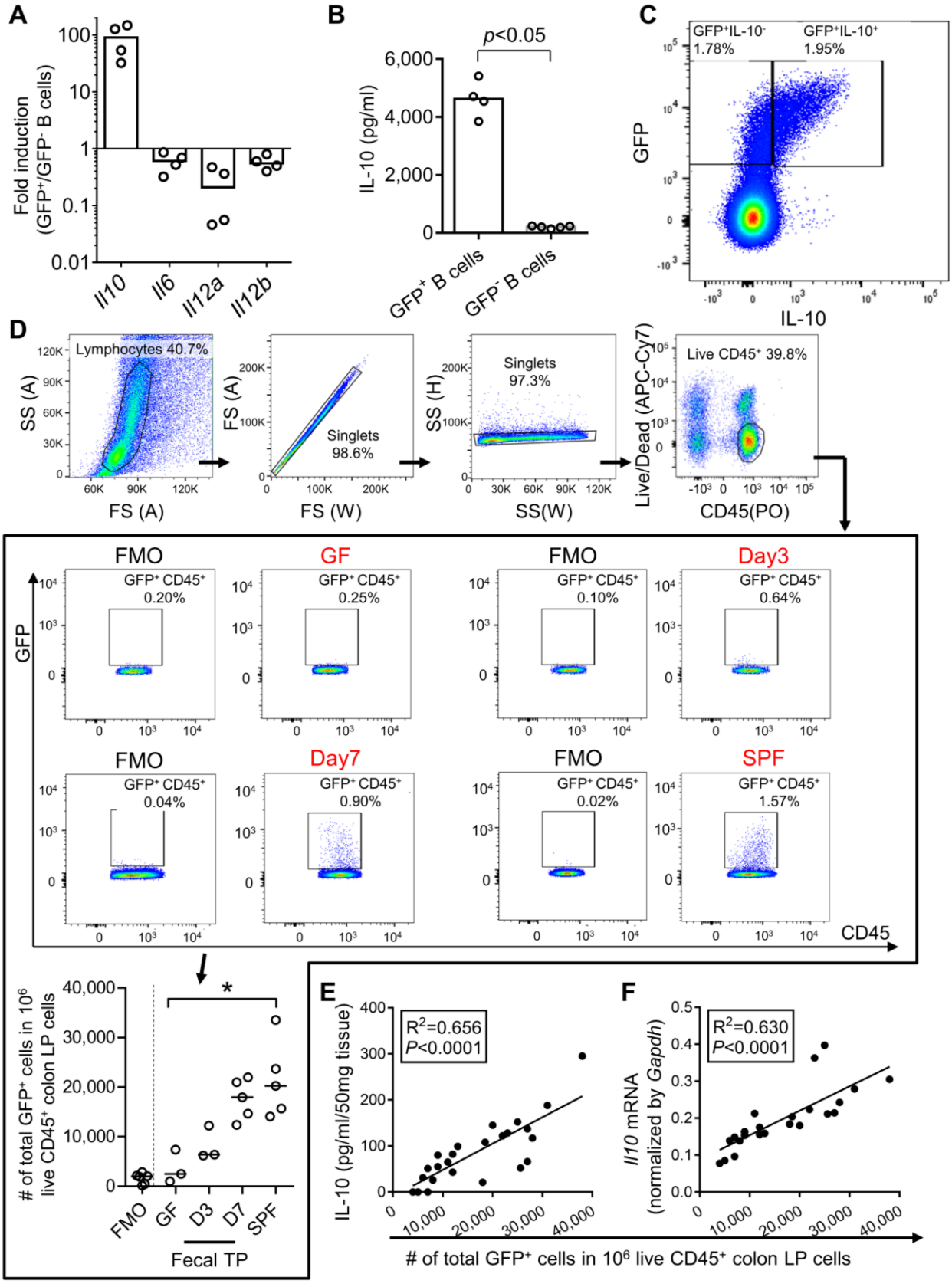
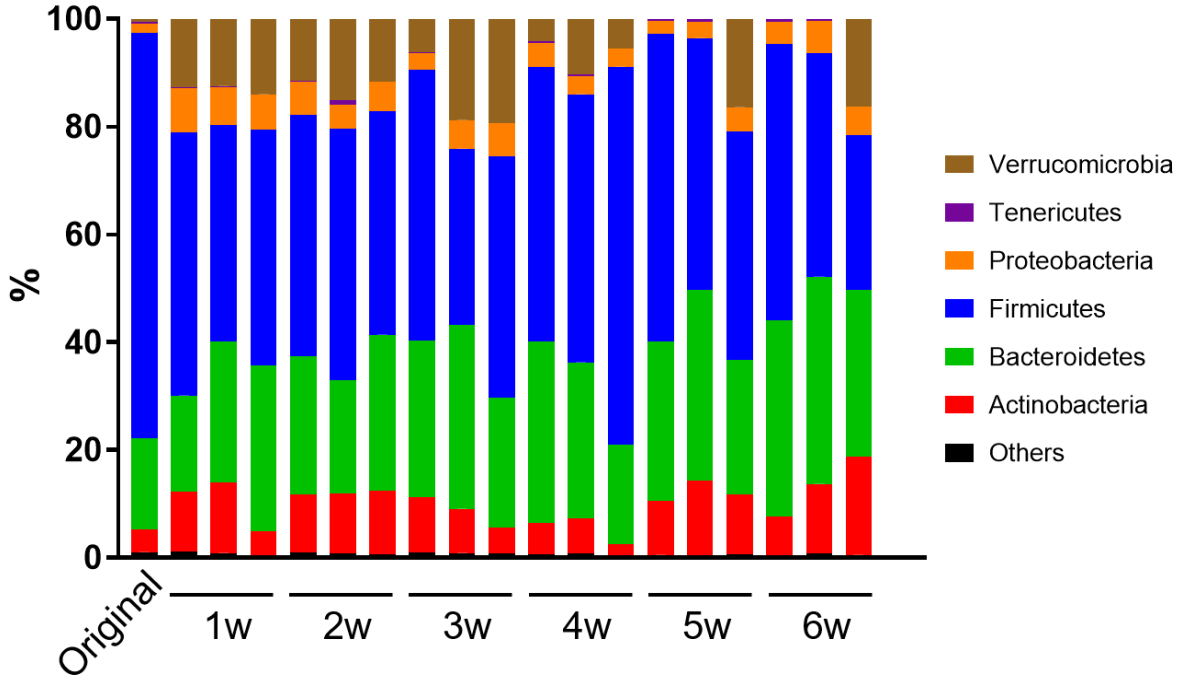
3 **Supplemental Figures**

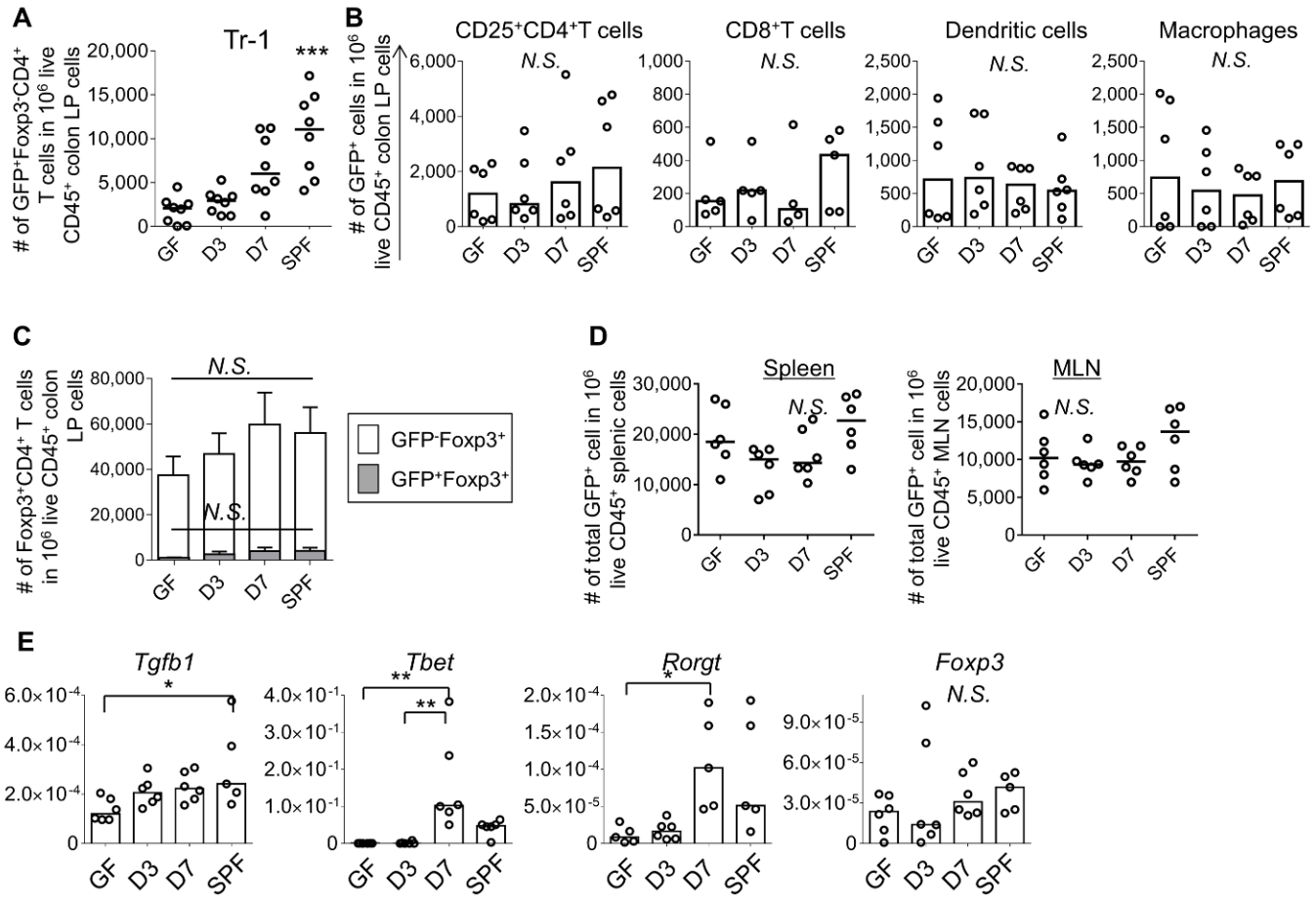


Supplemental Figure 1. GFP$^+$ B cells exhibit higher *Il10* mRNA expression and produce more IL-10 protein than GFP^{neg} B cells. Splenic B cells from specific pathogen-free (SPF) *Il10*$^{+/EGFP}$ reporter mice were cultured with 1nM CpG-DNA for 48 hours and separated into GFP$^+$ (96.2% pure) and GFP^{neg} (GFP$^-$) (98.7% pure) B cells by flow cytometry sorting. **(A)** mRNA expression in sorted GFP$^+$ and GFP$^-$ B cells were analyzed by real time PCR. Gene fold induction of GFP$^+$ over GFP$^-$ cells are shown. **(B)** Sorted B cells were further cultured without stimulation for 24 hours and supernatant level of IL-10 was measured by ELISA. N=4-5. Data are presented as median, Mann-Whitney U test. **(C)** Splenic B cells from SPF-raised *Il10*$^{+/EGFP}$ reporter mice or WT (GFP$^-$) mice were cultured *in vitro* with 1nM CpG for 24 hours, intracellularly stained with anti-IL-10 antibody or isotype control antibody followed by CD19, B220, CD45 and LIVE/DEAD™ Fixable Dead Cell Stain Kit cell surface staining, and analyzed with flow cytometry as described in Methods, Flow cytometry section. As a negative control for GFP, WT cells were stained with the same antibodies except GFP/FITC. Representative dot plots in B cell gate (live CD45$^+$CD19$^+$B220$^+$) are shown. **(D)** The gating strategy targeting GFP$^+$ cells is demonstrated. When IL-10$^{+/EGFP}$ reporter cells were used, SPF-raised C57BL/6 WT (GFP$^-$) cells were utilized. WT cells were stained with the same antibodies as target samples to determine a basal intensity level of GFP and set a GFP gate, as a Fluorescence minus one (FMO) control. Representative dot plots for GFP$^+$ CD45$^+$ cells (LIVE/DEAD™ Fixable Dead Cell Stain Kit (APC-Cy7)^{neg} CD45 (Pacific Orange)$^+$GFP$^+$) related to **Figure 1A, middle** are shown for demonstration.

N=3-5/group. Each sample was pooled from 2-3 mice. Data are presented as median, *p<0.05, Kruskal-Wallis test with Dunn's posttest. **(E and F)** Association between **(E)** GFP^+ cells and IL-10 protein concentrations and **(F)** GFP^+ cells and *Il10* mRNA expression *in vivo* are analyzed by using the raw data in **Figure 1A**. Correlation between GFP^+ cells and IL-10 protein or mRNA levels was determined by Pearson's Correlation Coefficient.



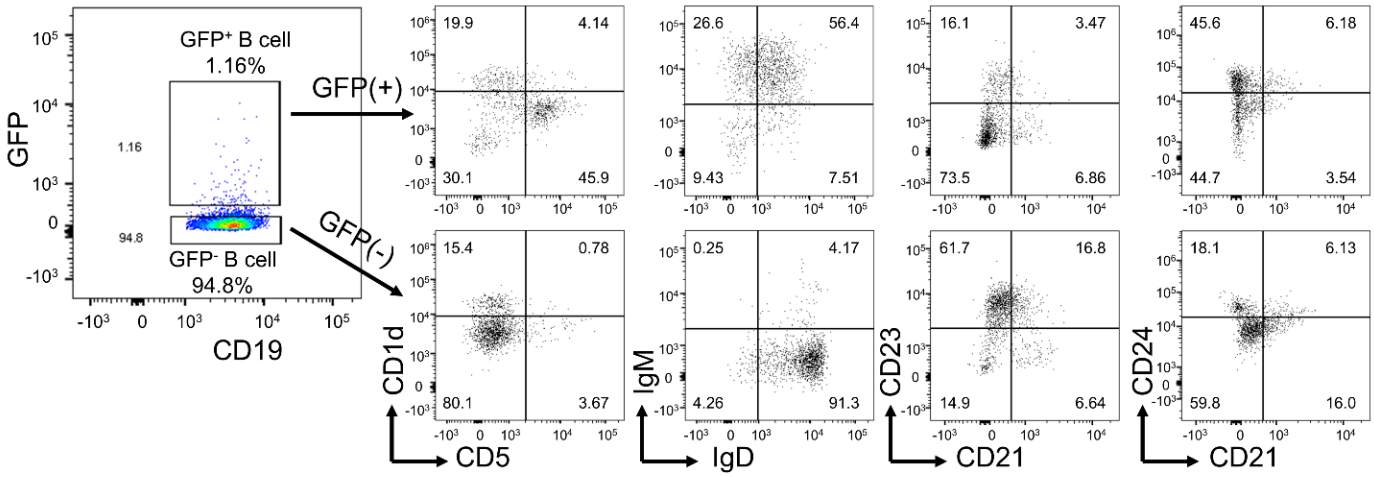
Supplemental Figure 2. SPF bacteria were stably colonized in ex-GF mice. Feces collected from SPF-born *Il10*$^{+/EGFP}$ reporter (Vert-X) mice were inoculated into 8-12-week-old germ-free (GF) Vert-X mice. After inoculation, fecal samples were collected weekly and the bacterial populations were evaluated by 16S rRNA gene sequencing by the Illumina MiSeq platform and analyzed by QIIME. The relative abundance ratios of fecal bacterial taxa sequentially over 6 weeks are shown with baseline (original) values from the SPF mouse feces used to colonize the GF mouse. N=3/group.



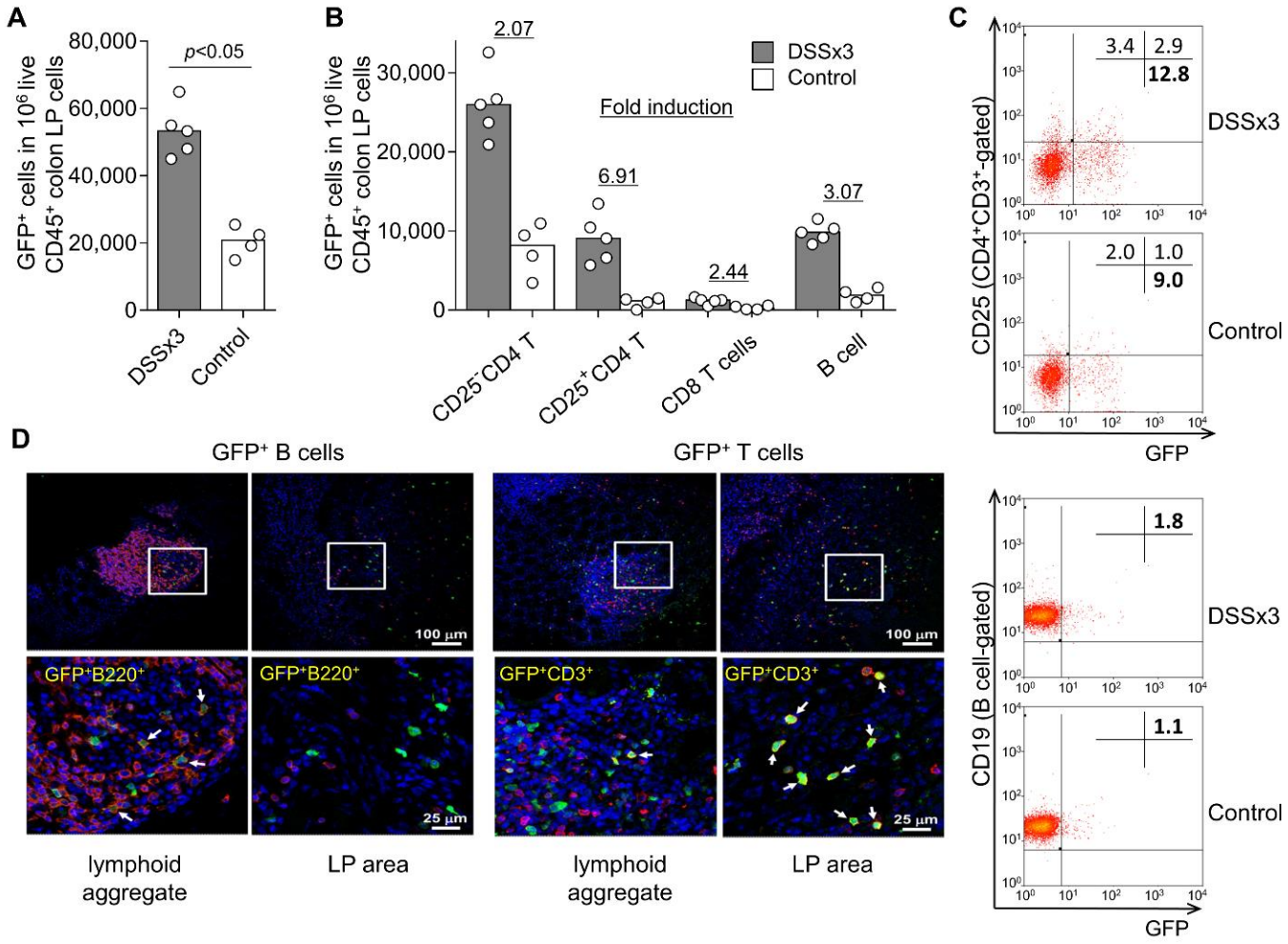
Supplemental Figure 3. Colonization of resident bacteria induces variable subsets of CD4⁺ T cells in the colon LP, but not MLN or spleen and stimulates colonic immune gene expression.

(A) IL-10-producing GFP⁺ colonic Foxp3^{neg}CD4⁺ T cells (Tr1) cells, **(B)** GFP⁺CD25⁺CD4⁺ T cells (CD25⁺CD4⁺CD3⁺), GFP⁺CD8⁺ T cells (CD8⁺CD3⁺), GFP⁺ macrophages (F4/80⁺CD11b⁺), GFP⁺ dendritic cells (CD11c⁺CD11b⁺), and **(C)** GFP⁺ or GFP⁻ Foxp3⁺CD4⁺ T cells in the colonic lamina propria (LP) were assessed by flow cytometry from GF, SPF-raised (SPF) or conventionalized ex-GF [3 days (D3) and 7 days (D7) after colonization with SPF feces] *Il10*$^{+/EGFP}$ reporter mice. All cells were stained with Foxp3

eFlour660 and also stained with CD45 and LIVE/DEAD™ Fixable Dead Cell Stain Kit. Colon LP cells from WT mice stained with the same antibodies as sample cells were used as negative controls for GFP. **(D)** Colonization of resident bacteria does not induce GFP^+ cells in the MLN or spleen. GFP^+ spleen and MLN cells from the three groups of mice described above were analyzed by flow cytometry as shown above and live $CD45^+$ cells were analyzed. MLN cells or splenocytes from WT mice were used as negative controls for GFP. **(E)** Stimulation of immune regulatory gene expression in colonic mucosa by bacterial colonization. Kinetic expression of selected immune-related genes in the distal colons from the three groups of mice described above was determined by real-time PCR. Each gene was normalized by *Actb*. N=5-8/group, combined from 2-3 independent experiments. All data are presented as median except **Figure C** (Mean ± standard error), **p*<0.05, ***p*<0.01, ****p*<0.001, *N.S.*: not significant, Kruskal-Wallis test with Dunn's posttest.



Supplemental Figure 4. Phenotypic characteristics of colonic GFP$^+$ B cells. Colon LP cells were freshly isolated from SPF-raised *Il10*$^{+/EGFP}$ reporter mice. GFP$^+$ or GFP$^-$ live B cell populations (B220 (Pacific blue)$^+$CD19 (BV605)$^+$CD45 (Pacific orange)$^+$LIVE/DEAD™ Fixable Dead Cell Stain kit (Near-IR)^{neg}) were further analyzed by flow cytometry with the following phenotypic markers: IgM (PE-Cy7), IgD (APC), CD5 (PE-Cy5), CD1d (PE), MHC-II (Qdot655), CD21 (Qdot655), CD23 (PE), and CD24 (APC). As negative control for GFP (FMO control), colon LP cells from WT mice stained with the same antibodies as sample cells were used for setting the GFP-gate and compensation. Representative dot plots of GFP$^+$ and GFP$^-$ cells gated on B cells (CD19$^+$B220$^+$) are shown. N=4, each sample was pooled from 3 mice. 2 independent experiments.



63

64 **Supplemental Figure 5. Chronic colonic inflammation increases the frequency of colonic**

65 **GFP$^+$IL-10-producing T and B cells in the presence of resident bacteria.** Colonic LP cells were

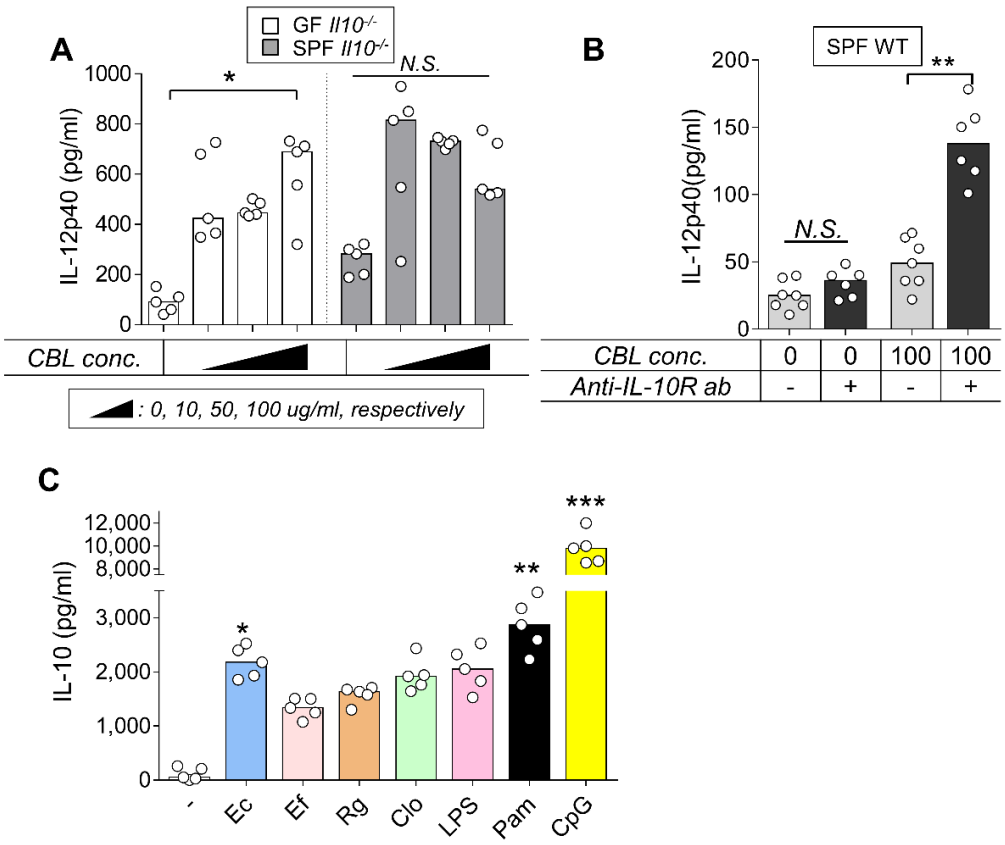
66 isolated from SPF-raised *Il10$^{+/EGFP}$* mice without (control) or following treatment with 3 cycles of 1.5%

67 dextran sulfate sodium (DSS) administration (DSSx3). **(A)** Total live CD45$^+$GFP$^+$ cells in colon LP cells,

68 and **(B)** three subpopulations of lymphoid cells (CD25$^-$CD4$^+$CD3$^+$, CD25$^+$CD4$^+$CD3$^+$ and CD8$^+$CD3$^+$ T

69 cells and CD19$^+$B220$^+$ B cells) were characterized by flow cytometry as described in the Methods. SPF

WT mice were used as a negative control for GFP. N=4-5/group, combined from 2 independent experiments. Data are presented as median, Mann-Whitney U test. **(C)** Representative dot blots for LP GFP^+CD4^+ T cells ($CD4^+CD3^+$-gated) and GFP^+ B cells ($CD19^+B220^+$-gated) in SPF untreated (control) and with 3 cycles of DSS-treated (DSSx3) mice are shown**.** **(D)** The distribution of GFP^+ B cells and GFP^+ T cells in lymphoid aggregates and the LP of the distal colon from DSS-treated mice was analyzed by immunohistochemistry. GFP^+ cells are shown in green and B cells ($B220^+$) and T cells ($CD3^+$) are shown in red. Tissue sections were counterstained with DAPI. Bottom panel is a higher magnification image of the rectangular area indicated in the top panel.



78

79 **Supplemental Figure 6. CBL is unable to suppress colonic LP cell IL-12p40 production in the**

80 **absence of IL-10 or IL-10 signaling and bacterial products stimulate IL-10 secretion *ex vivo*. (A)**

81 Unfractionated colonic LP cells from GF or SPF-raised *Il10*$^{-/-}$ mice were cultured with 0-100μg/ml of cecal

82 bacterial lysate (CBL) from SPF WT mice for 2 days. **(B)** In parallel, unfractionated colonic LP cells

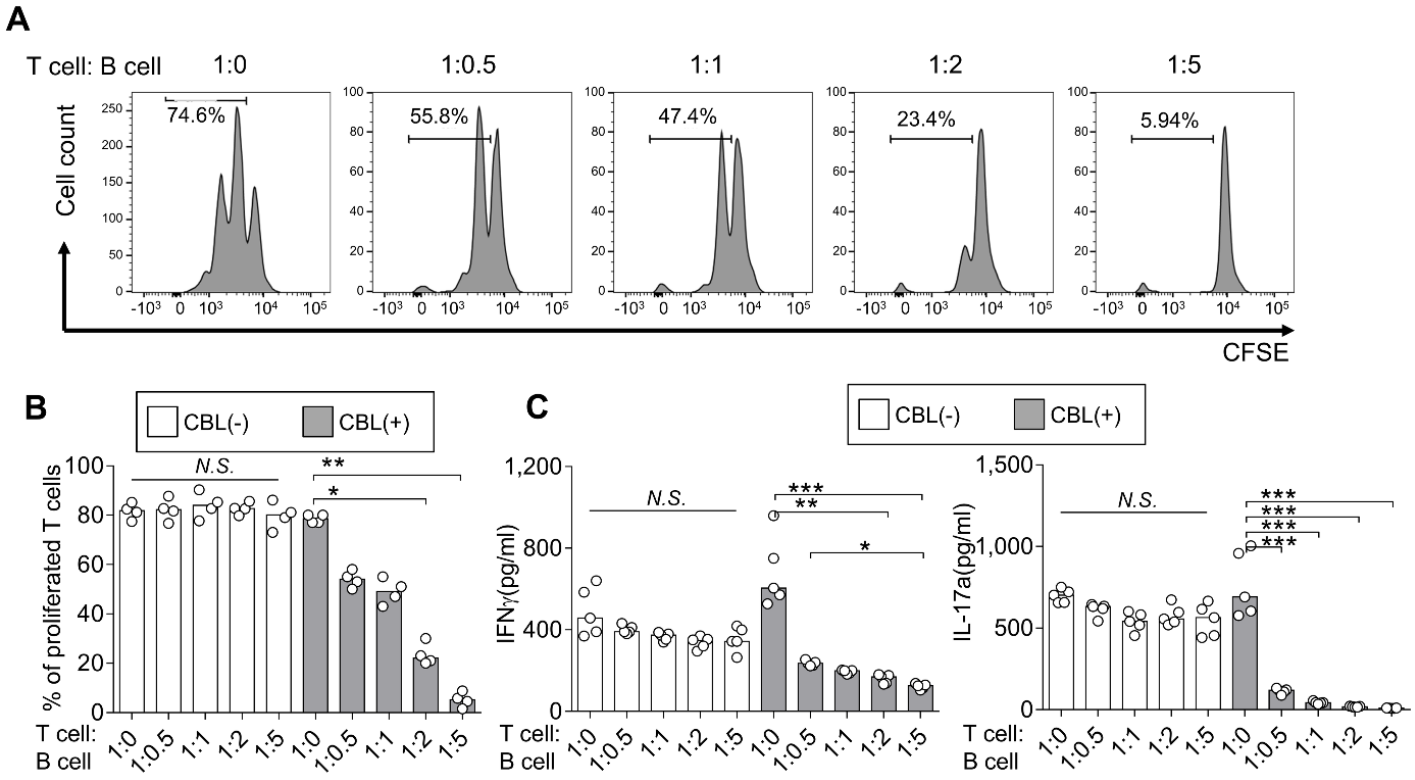
83 from SPF-raised WT mice were cultured with and without 100μg/ml CBL in the presence or absence of

84 10μg/ml anti-IL-10R antibodies for 2 days. IL-12p40 levels in culture supernatants were measured by

85 ELISA. Data are presented as median of 4-5 separate cell cultures with cells in each culture pooled

86 from 2-4 mice, *p<0.05, *N.S.*: not significant, Kruskal-Wallis test with Dunn's posttest. **(C)** Bacterial

products stimulate IL-10 secretion in colonic cells, *ex vivo*. Unfractionated colonic LP cells from GF *Il10*$^{+/EGFP}$ reporter mice were cultured without (-, media only) or with 200ng/ml LPS, 50ng/ml Pam3csk (Pam), 1nM CpG-DNA (CpG), 10µg/ml of lysates of *E. coli LF82 (Ec), Enterococcus faecalis (Ef), Ruminococcus gnavus (Rg)* or a mixture of 17 *Clostridia* strains *(Clo)* for 2 days. IL-10 levels in culture supernatants were measured by ELISA. Data are presented as the median of 4 separate cell cultures with cells in each culture pooled from 2-4 mice, **p*<0.05, ***p*<0.01, ****p*<0.001, vs. non-stimulation, Kruskal-Wallis test with Dunn's posttest.



94

95 **Supplemental Figure 7. Colonic B cells suppress T cell proliferation and differentiation in the**

96 **presence of CBL stimulation.** CFSE-labeled colonic $CD4^+$ T cells ($5x10^5$) were cultured with or

97 without different numbers of colonic B cells (0, $2.5x10^5$, $5.0x10^5$, $1.0x10^6$, $2.5x10^6$) from SPF mice on

98 CD3-coated plates for 24 hours in the presence or absence of CBL stimulation. T cell proliferation was

99 determined by the loss of intensity of CFSE staining in live $CD45^+$ $CD4^+CD3^+$ T cells using flow cytometry.

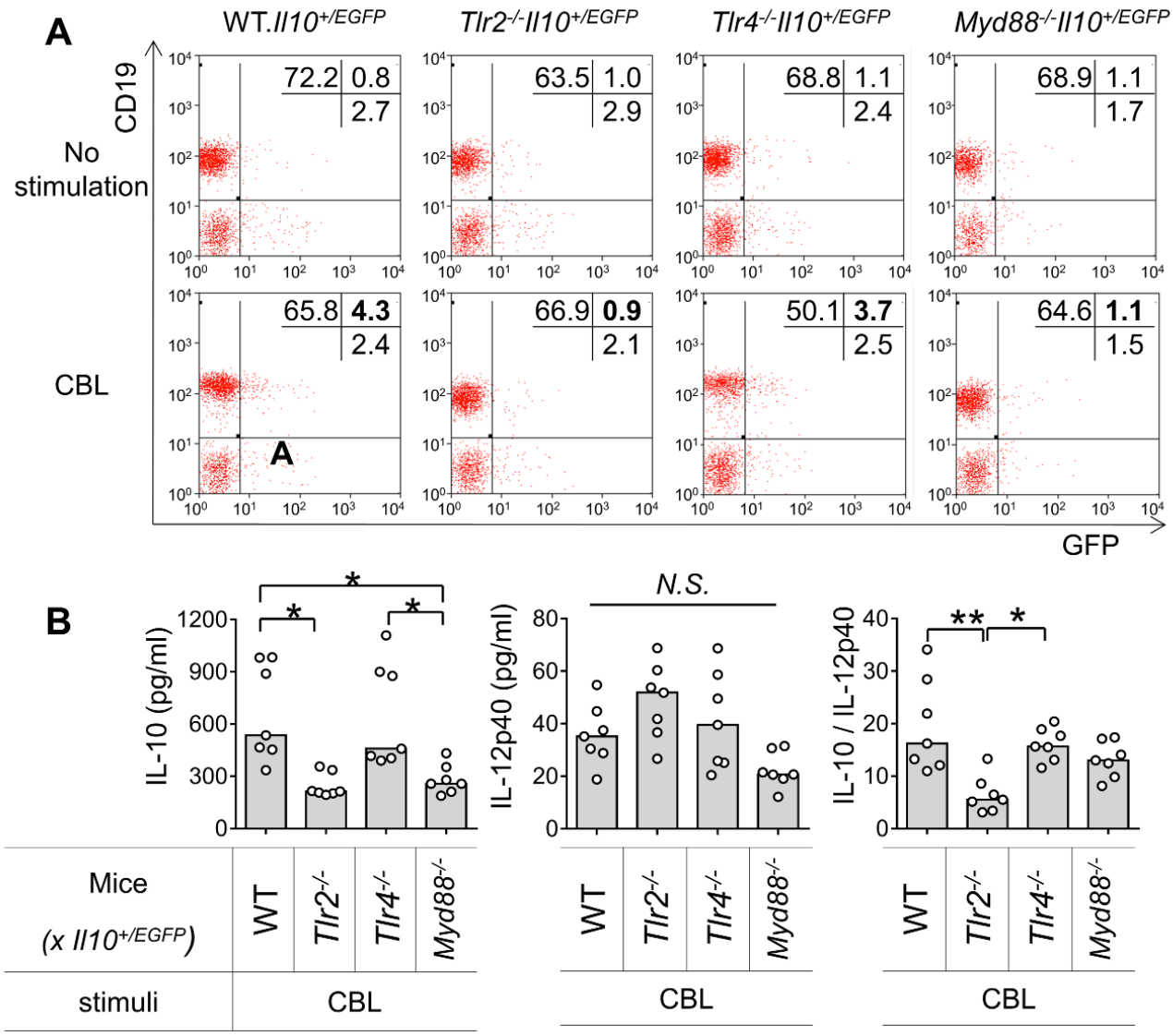
100 **(A)** Representative histograms of T cell proliferation in CBL-treated co-cultures with the degree of

101 proliferation noted. **(B)** Quantified T cell proliferation. **(C)** Supernatant levels of IFNγ and IL-17a

102 measured by ELISA. Data are presented as the median of 4-5 separate cell cultures with cells in each

culture pooled from 2-4 mice. *p<0.05, **p<0.01, ***p<0.001, Kruskal-Wallis test with Dunn's posttest.

N.S.: not significant.



105

106 **Supplemental Figure 8. Lack of TLR2/MyD88-signaling negates resident bacteria-stimulated**

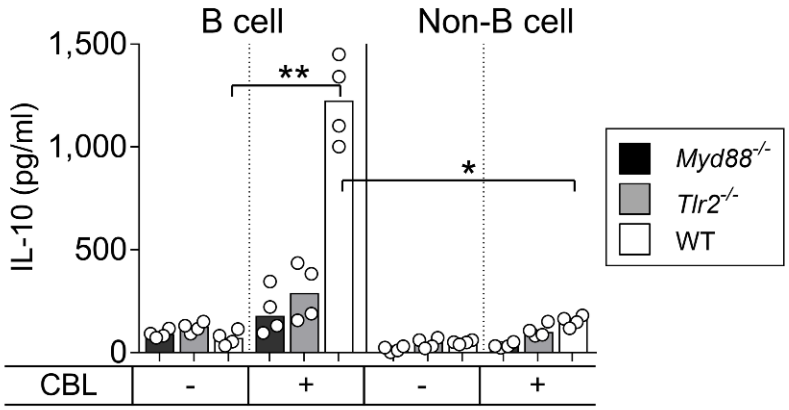
107 **development of inducible, but not native, mucosal IL-10-producing GFP⁺ B cells, but has no**

108 **effect on IL-12 production.** Unfractionated colon LP cells from 8-10-week old WT*Il10*$^{+/EGFP}$,

109 *Tlr2*$^{-/-}$*Il10*$^{+/EGFP}$, *Tlr4*$^{-/-}$*Il10*$^{+/EGFP}$ and *Myd88*$^{-/-}$*Il10*$^{+/EGFP}$ mice were cultured with or without *ex vivo* CBL

110 (10μg/ml)-stimulation for 24 hours, then GFP^{+} cell populations were analyzed by flow cytometry and

supernatant IL-10 and IL-12p40 measured by ELISA. **(A)** Representative dot plots for LP GFP^+ B cells ($CD19^+B220^+$). **(B)** CBL-stimulated IL-12p40 production is TLR-independent. IL-10 and IL-12p40 production by colonic tissue explants, and ratios of IL-10/IL-12p40 are shown. Data are presented as median. N=7/group, combined from 2 independent experiments. *$p<0.05$, Kruskal-Wallis test with Dunn's posttest.



116

117 **Supplemental Figure 9. B cells are the primary source of bacteria-stimulated**

118 **TLR2/MyD88-dependent IL-10-production in colon.** Freshly isolated unfractionated colonic LP cells

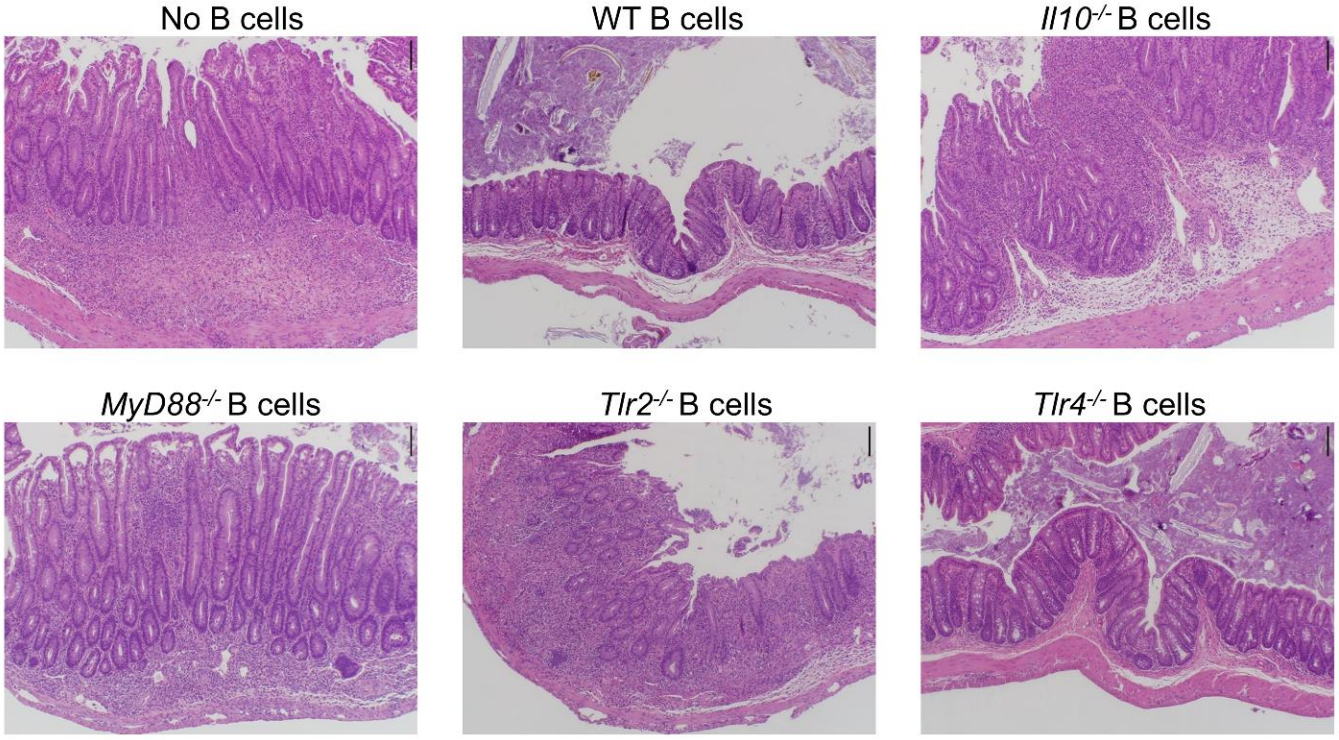
119 from *Myd88*$^{-/-}$, *Tlr2*$^{-/-}$ and WT mice were magnetically separated into B cell and non-B cell populations.

120 B cells or non-B cells ($5x10^5$) were independently cultured with or without 10µg/ml CBL for 24 hours,

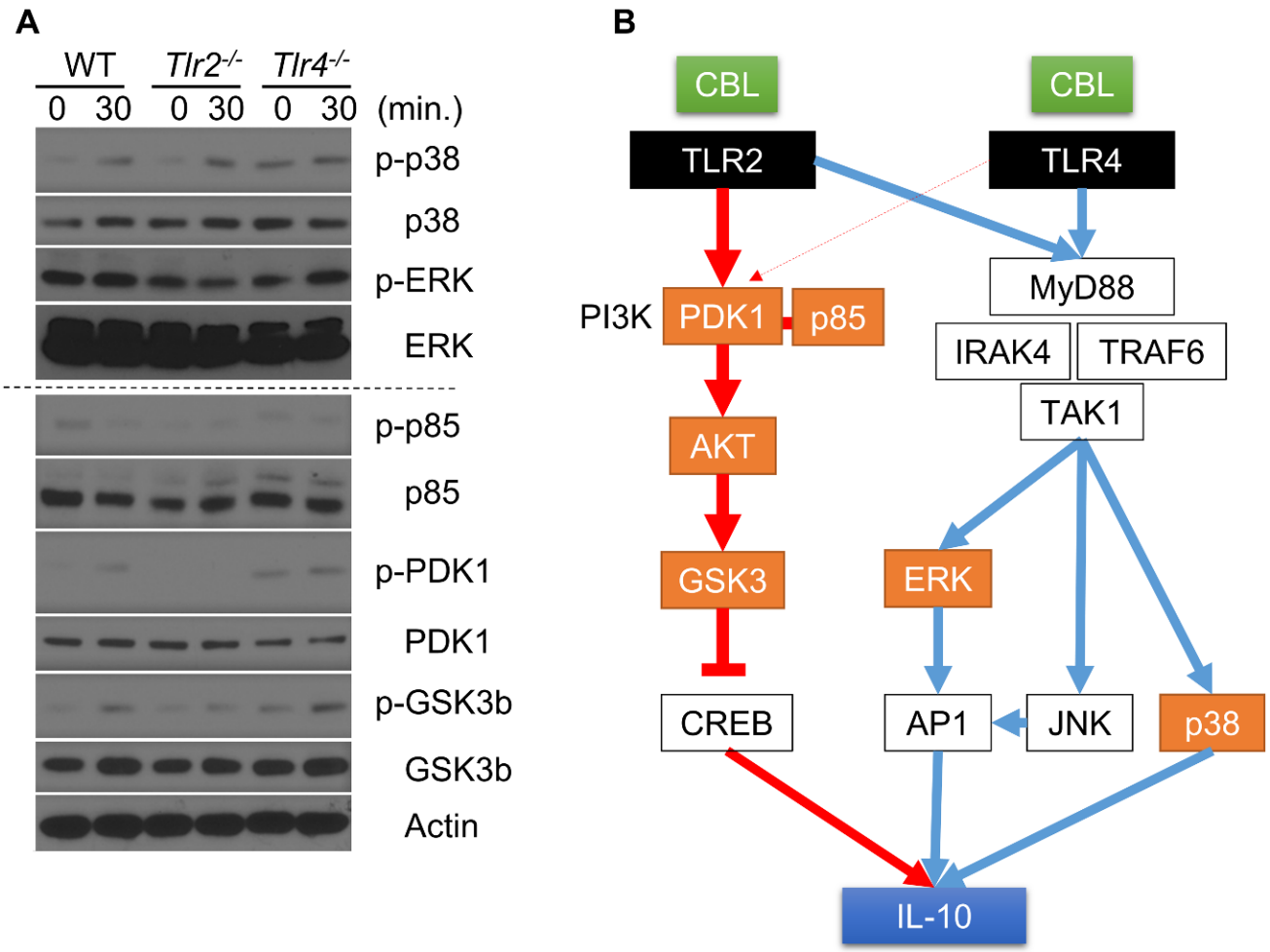
121 then supernatant levels of IL-10 were measured by ELISA. Data are presented as median of 4

122 separate cell cultures with cells in each culture pooled from 2-4 mice, *p<0.05, **p<0.01, Kruskal-Wallis

123 test with Dunn's posttest.



Supplemental Figure 10. B cells ameliorate experimental T cell-mediated colitis in IL-10 and TLR2/MyD88-dependent manners, *in vivo*. Splenic B cells (1×10^6) from SPF-raised WT, *Il10*$^{-/-}$, *Myd88*$^{-/-}$, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice were co-transferred with WT naïve CD4$^+$ T cells (5×10^5) into SPF *Rag2*$^{-/-}$*Il10*$^{-/-}$ recipients. Six weeks after cell transfer, mice were evaluated for severity of colitis by histology with hematoxylin and eosin staining. Representative photomicrographs of the cecum associated with **Figure 7C** are shown. Magnification: 100x.



132 **Supplemental Figure 11. TLR2 signaling activates the PI3K/AKT/GSK3B pathway in**

133 **CBL-stimulated B cells.** **(A)** Splenic B cells from 8-10-week-old SPF WT, *Tlr2*$^{-/-}$ and *Tlr4*$^{-/-}$ mice were

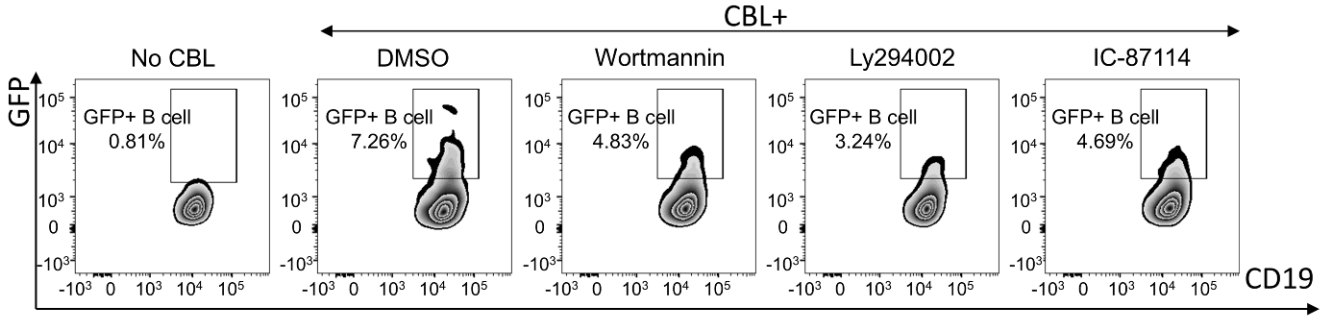
134 cultured with or without 10μg/ml CBL. Cells were harvested 0 and 30 minutes after CBL stimulation

135 and phosphorylation of the indicated protein was analyzed by Western blots using antibody to signaling

136 pathway components. **(B)** Schematic pathways of TLR2-dependent activation of IL-10 signaling in

137 CBL-stimulated B cells. The molecules indicated in orange were assessed by Western blots in this

138 study.



139

140 **Supplemental Figure 12. The PI3K pathway is partially involved in induction of GFP$^+$ B cell by**

141 **CBL.** Splenic B cells (1x10^6/well) from 8-10-week old SPF *Il10$^{+/EGFP}$* mice were cultured with 1uM

142 pan-PI3K inhibitors; Wortmannin or Ly294002, 1uM PI3Kp110δ-specifc inhibitor; IC-87114, or DMSO

143 alone with stimulation of 10μg/ml CBL for 48hours. GFP$^+$ B cells (live CD45$^+$CD19$^+$B220$^+$) were

144 analyzed with flow cytometry in reference to WT (GFP$^-$) control cells stained with the same antibodies as

145 target samples. Representative flow cytometry plots related to **Figure 8D**

Supplemental methods

Flow cytometric cell sorting

Freshly isolated splenic B cells were cultured with 1nM CpG for 24 hours and then GFP^+ or $GFP^-CD19^+B220^+$ B cells were sorted by BD FACS Aria III (BD Biosciences, San Jose, CA). Purity: GFP^+B cells 96.2%, GFP^-B cells 98.7%. As a negative control for GFP, colon LP cells from WT mice stained with the same antibodies used to stain the sample cells that were used for setting the GFP-gate and compensation.

Isolation of fecal microbial DNA and sequencing of 16S rRNA gene

Bacterial DNA was extracted from fecal pellets with a phenol-chloroform DNA extraction method with physical agitation to rupture bacterial envelopes, as previously described (1). The resulting DNA was cleaned using a DNA clean-up kit (Qiagen DNeasy Blood & Tissue Kit. Valencia, CA). The bacterial composition of fecal samples was determined by amplification and sequencing of the V4 region of the 16S rRNA gene. The V4 region was amplified with two distinct polymerase chain reactions. The first reaction employed validated 16S rRNA V4 primers (forward 515 and reverse 806), using the KAPA2G Robust PCR kit. Thermocycling conditions for the first reaction were 95°C/3m; 10

cycles of 95°C/30s, 50°C/30s and 72°C/30s, respectively; and 72°C/5m. For the second reaction, 5 µL of cleaned product from the first reaction was amplified with the KAPA HiFi HotStart ReadyMix PCR kit (Roche Diagnostics, Indianapolis, IN) using Illumina MiSeq adapter primers with a 12-base Golay barcode appended to the reverse primer (2). Thermocycling conditions for the second reaction were 95°C/3m; 22 cycles of 95°C/30s, 50°C/30s and 72°C/30s, respectively; and 72°C/5m. After each PCR step, the amplicons were purified with the HighPrep PCR Clean Up Kit (MagBio, Gaithersburg, MD). The final purified amplicons were quantified, equimolar pooled, and sequenced on a desktop Illumina MiSeq (2x250) at the High-Throughput Sequencing Facility at University of North Carolina at Chapel Hill. Sequence data analysis was performed with QIIME (3). OTUs were picked with a 97% similarity threshold and taxonomy was assigned using the Greengenes database.

Chronic DSS-induced colitis

Chronic intestinal inflammation was induced by repetitive administration of 1.5% w/v dextran sulfate sodium (DSS; ICN Biomedicals, Aurora, OH) drinking water solution in the SPF-raised *Il10*$^{+/EGFP}$ mice. The control group was given normal water lacking DSS. Each cycle consisted of 7 days with DSS solution followed by 14 days without DSS solution, which continued for 3 cycles.

T cell suppression assay

Colonic LP cells were freshly isolated as described in the Cell isolation section of Methods from SPF WT mice. $CD4^+$ T cells and B cells were independently further isolated by CD4 and CD19 microbeads, respectively (Miltenyi Biotec, Auburn, CA). $CD4^+$ T cells were labeled with CFSE (Sigma, St. Louis, MO) as per the manufacturer's protocol. $5x10^5$ $CD4^+$ T cells were cultured on 48-well plates coated with 10ug/ml anti-CD3 antibody (BD Biosciences, San Jose, CA), with 0-2.5 x 10^6 colonic LP B cells in the presence or absence of 10ug/ml of CBL for 72 hours. Cells were harvested and proliferation of T cells was assessed by flow cytometry. Supernatant levels of IL-17a and IFNγ were measured by ELISA.

Western blots

Western blots were performed as described in the Western blots session of Method. Primary antibodies used in Supplemental Figure 11 were described in the Supplemental Table. All primary antibodies were used at 1:1,000.

Supplemental references

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2. Werner JJ et al. Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys.. *ISME J.* 2012;6(1):94–103.

3. Caporaso JG et al. QIIME allows analysis of high-throughput community sequencing data.. *Nat. Methods* 2010;7(5):335–6.