Immunity

Supplemental Information

MyD88-Dependent Microbial Sensing by Regulatory T cells Promotes Mucosal Tolerance and Enforces Commensalism

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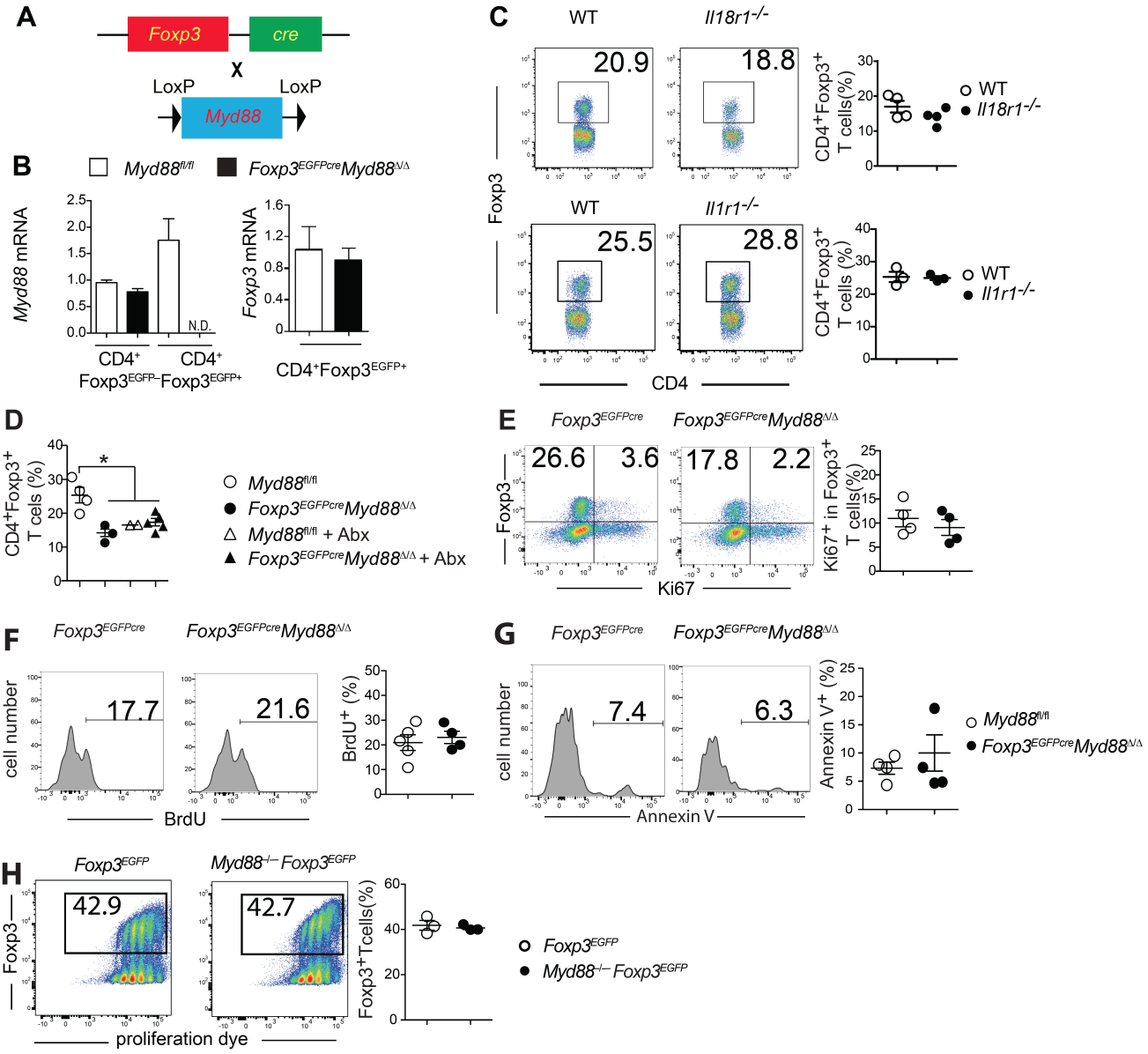

Figure S1 (Related to Figure 1). Generation of Treg cell-specific *Myd88*-deficient mice. A. Scheme for the generation of *Foxp3*^{EGFPcre}*Myd88*$^{Δ/Δ}$ mice. **B.** Real time PCR analysis of *Myd88* transcripts in cell-sorted CD4^{+}EGFP$^{–}$ (Tconv) and CD4^{+}EGFP^{+} Treg cells (left panel) and *Foxp3* transcripts CD4^{+}EGFP^{+} Treg cells (right panel) isolated from *Foxp3*^{EGFPcre}*Myd88*$^{Δ/Δ}$ and *Myd88*$^{fl/fl}$ mice. **C**. Flow cytometric analysis and frequencies

of cLP CD4$^+$Foxp3$^+$ cells isolated from *Il18r1*$^{-/-}$ and *Il1r1*$^{-/-}$ mice. **D**. Frequencies of cLP CD4$^+$Foxp3$^+$Treg cells in *Myd88*$^{fl/fl}$ and *Foxp3*^{EGFPcre}*Myd88*$^{\Delta/\Delta}$ mice treated with an antibiotic cocktail of ampicillin, neomycin, vancomycin, and metronidazole at 200 µg each in a final volume of 200 µl PBS administered by oral gavage 3 times/week for 3 weeks. **E-G**. Flow cytometric analysis and frequencies of Ki67$^+$ (**E**), BrDU (**F**) and AnnexinV staining (**G**) in cLP CD4$^+$Foxp3$^+$Treg cells of *Myd88*$^{fl/fl}$ and *Foxp3*^{EGFPcre}*Myd88*$^{\Delta/\Delta}$ mice. BrDU staining was done following treatment of mice with BrdU in drinking water for 9 days. **H**. Flow cytometric analysis and frequencies of iTreg cells present in cultures of splenic naïve CD4$^+$ T cells of WT *Foxp3*^{EGFP} or *Myd88*$^{-/-}$ *Foxp3*^{EGFP} mice treated with plate-bound anti-CD3 and soluble anti-CD28 mAbs and TGF-β for 5 days. Results representative of at least 3 independent experiments. Numbers in quadrants of flow cytometric plots indicate percentages of cells. N=3-5 mice/group. *p<0.05; **p<0.01; ***p<0.001 by Student's unpaired two tailed *t* test and One-way ANOVA.

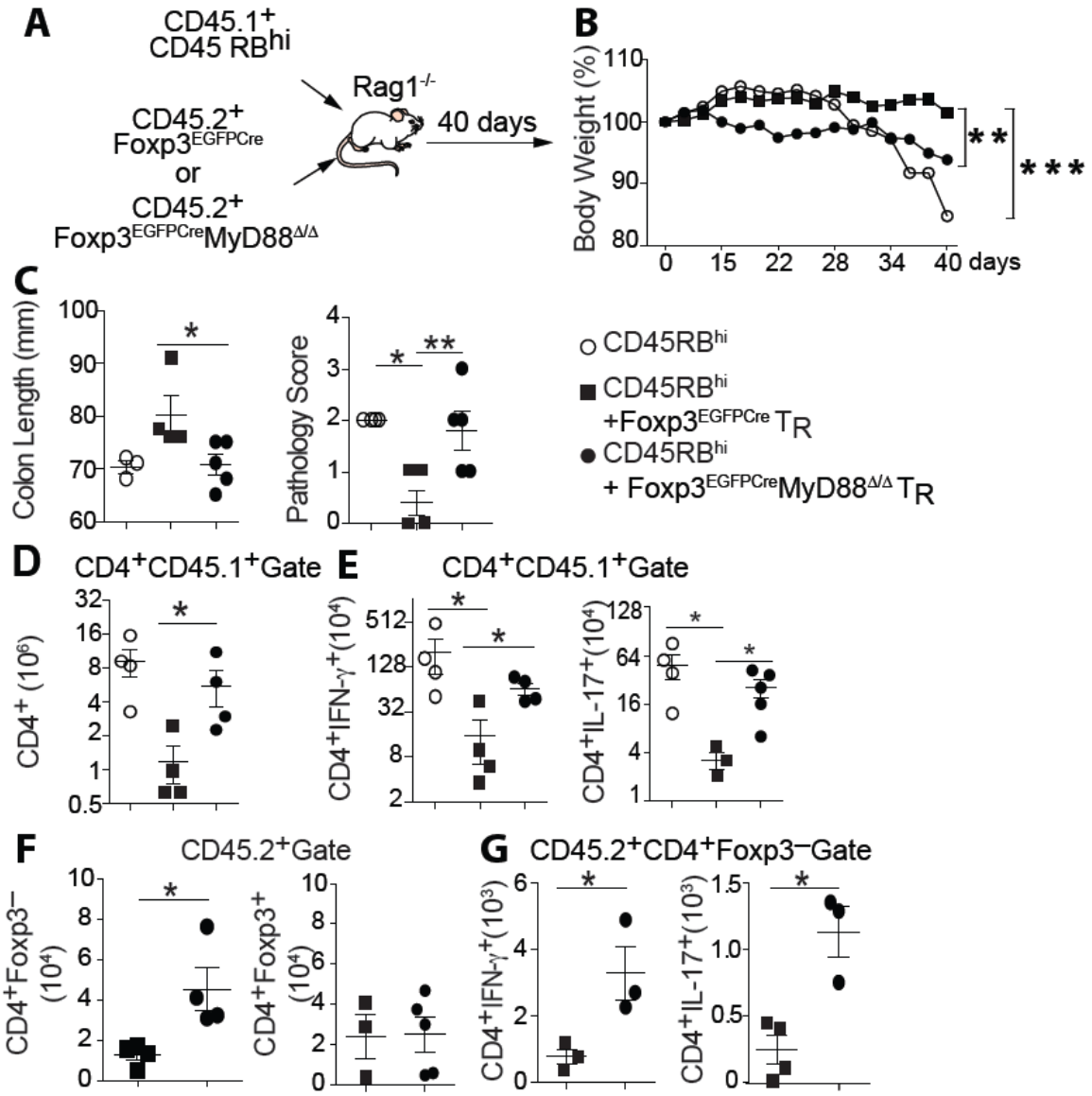

Figure S2 (Related to Figure 3). **Treg cell-specific *Myd88* deficiency aggravates the severity of a lymphopenia-induced colitis model. A**. Schematic representation of the experimental design. *Rag1*$^{-/-}$ mice received congenic wild type CD45.1^{+}CD4^{+}CD45RB^{hi} T cells alone or combination with CD45.2^{+}CD4^{+}Foxp3^{+} Treg

cells from either *Foxp3*^{EGFPcre} (*Myd88*-sufficient) or *Foxp3*^{EGFPcre}*Myd88*$^{\Delta/\Delta}$ (*Myd88*-deficient) mice. **B.** Body weight change of the mouse groups described in (**A**) following adoptive transfer. **C.** Colon length and pathology score of the mouse groups described in panel (**A**). **D, E.** Total number (**D**) and IL-17^{+} and IFN-γ^{+} donor CD45.1^{+}CD4^{+} T cells (**E**) in the cLP of the mouse groups in (**A**) at day 40 post-adoptive transfer. **F, G.** Frequencies and numbers of donor CD45.2^{+}CD4^{+}Foxp3^{+} Treg cells (**F**) and IL-17^{+} and IFN-γ^{+} CD45.2^{+}CD4^{+}Foxp3^{-} ex-Treg cells (**G**) in the cLP of the mouse groups in (**A**) at day 40 post-adoptive transfer. Results are representative of 3 independent experiments. *p<0.05; **p<0.01; ***p<0.001 by Student's unpaired two tailed *t* test and One- and two-way ANOVA. N=3-5 mice/group.

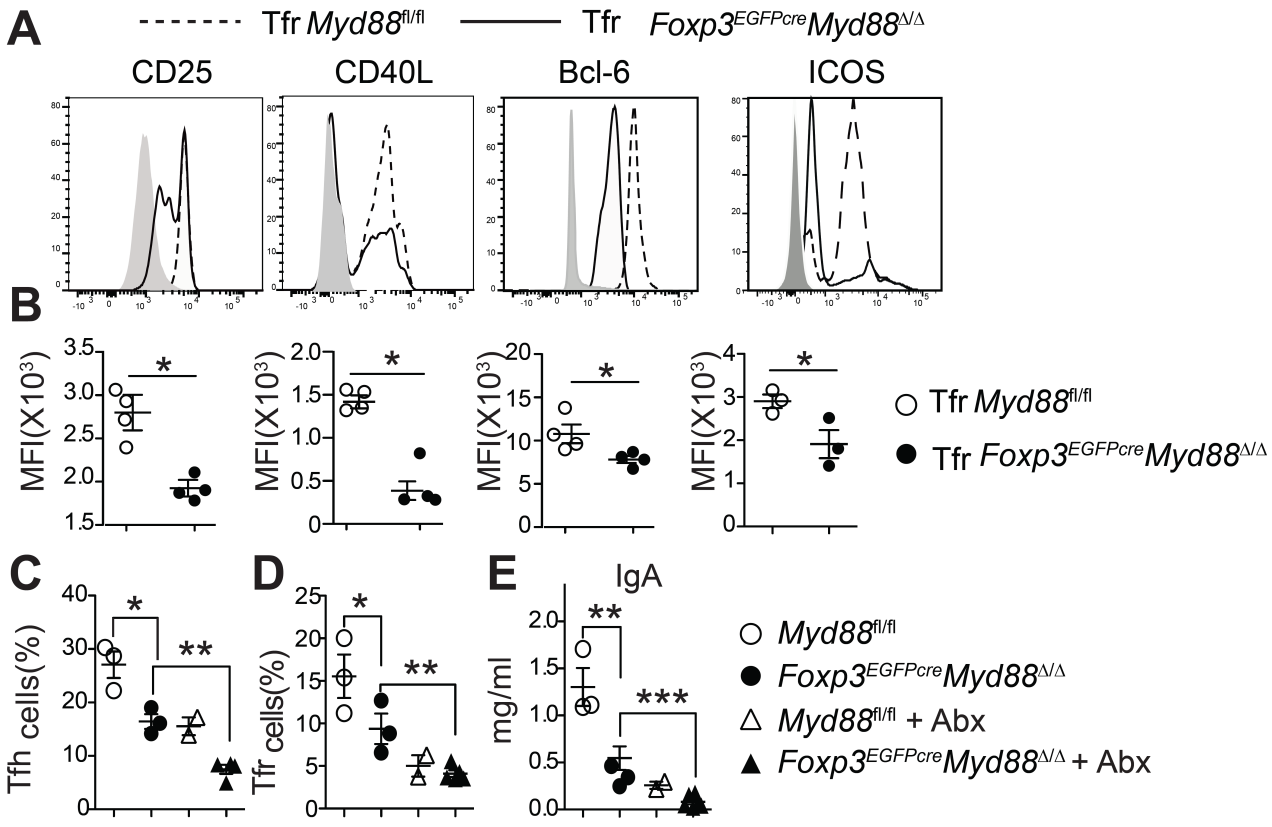

Figure S3 (Related to Figure 4). **Impaired PP Tfr cell differentiation in *Foxp3^{EGFPcre}Myd88$^{Δ/Δ}$* and antibiotic treated mice. A, B**. Flow cytometric analysis (**A**) and MFI **(B)** of the canonical Tfr cell markers CD25, CD40-L, Bcl6 and ICOS, in Tfr cell isolated from the PP of *Myd88$^{fl/fl}$* and *Foxp3^{EGFPcre}Myd88$^{Δ/Δ}$* mice. **C, D.** Frequencies of PP Tfh and Tfr cells in untreated and antibiotic-treated *Myd88$^{fl/fl}$* and *Foxp3^{EGFPcre}Myd88$^{Δ/Δ}$* mice. **E**. Luminal IgA concentrations in mice shown in (**C, D**). *p<0.05; **p<0.01; ***p<0.001 by Student's unpaired two tailed *t* test and One-way ANOVA. N=3-5 mice/group.

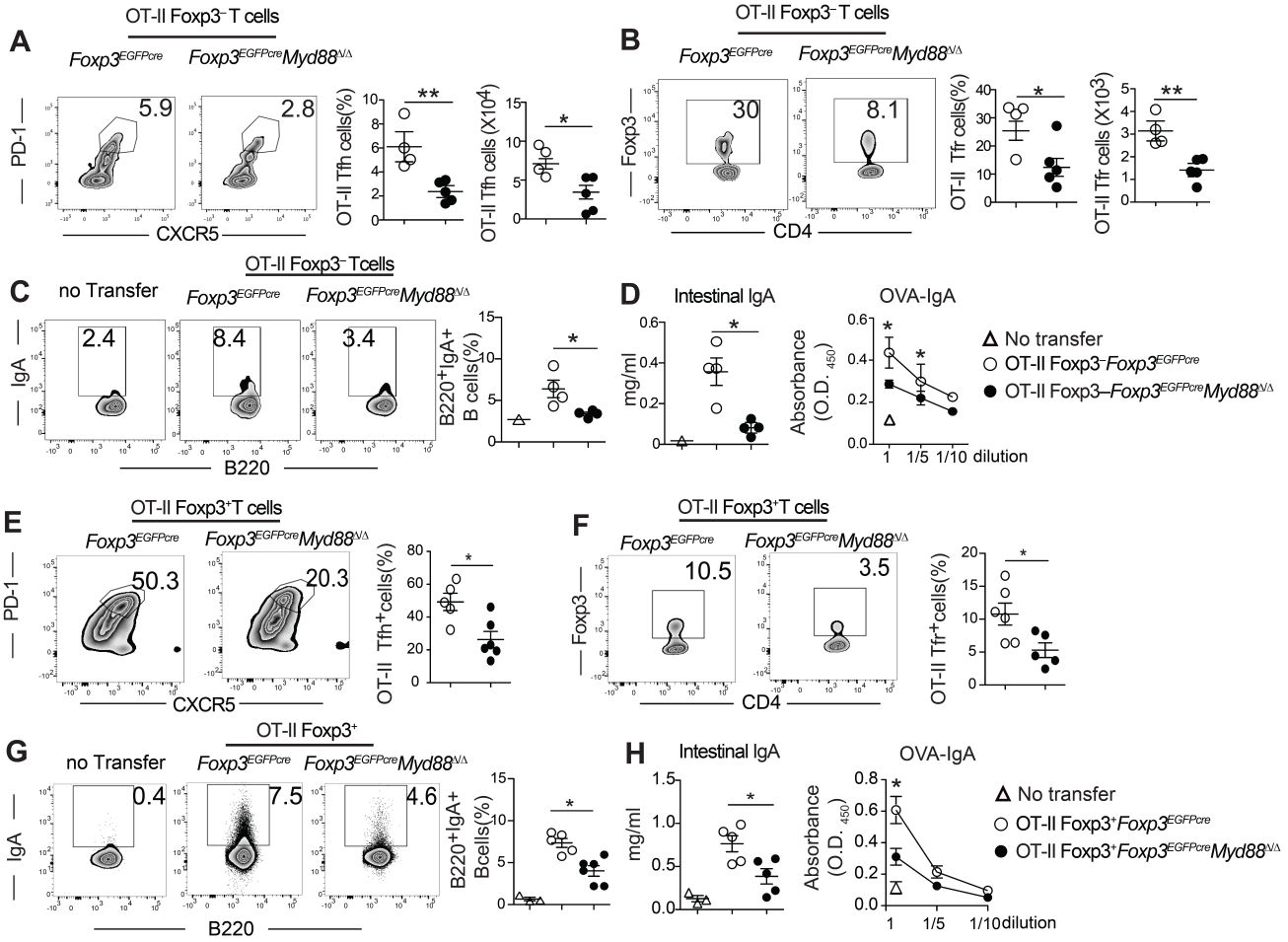

Figure S4 (Related to Figure 4). **Treg cell-specific *Myd88* deficiency blocks the differentiation of antigen-specific Tfr and Tfh cells in the PP. A-B.** Flow cytometric analysis and frequencies and numbers of Tfh cell (**A**) and Tfr (**B**) cells in *Tcra*$^{-/-}$ mice that received naïve OT-II^{+}CD4^{+}Foxp3^{-} Tconv cells from either *OT-II*$^{+}$*Foxp3*^{EGFPcre} or *OT-II*$^{+}$*Foxp3*^{EGFPcre}*Myd88*$^{\Delta/\Delta}$ and were then fed 1% OVA in the drinking water for 2 weeks. **C.** Flow cytometric analysis and frequencies of IgA^{+}B220^{+} in the PP of the mouse groups shown in (**A**). **D.** Luminal concentration of IgA and OVA-specific IgA in the mouse groups from panel (**A**). **E-F.** Flow cytometric analysis and frequencies and numbers of Tfh cell (**E**) and Tfr (**F**) cells in *Tcra*$^{-/-}$ mice that received splenic Treg cells

from either *OT-II*$^{+}$*Foxp3*^{EGFPcre} or *OT-II*$^{+}$*Foxp3*^{EGFPcre}*Myd88*$^{\Delta/\Delta}$ mice then fed 1% OVA in the drinking water for 2 weeks. **G.** Flow cytometric analysis and frequencies of IgA^{+}B220^{+} in the PP of the mouse groups from panels (**E**). **H.** Luminal concentration of IgA and OVA-specific IgA in the mouse groups from panel (**E**). Data are represented as mean ± SEM. Similar results were obtained in 3 independent experiments. *p<0.05; **p<0.01; ***p<0.001 by Student's unpaired two tailed *t* test and One-way ANOVA. N=3-5 mice/group.

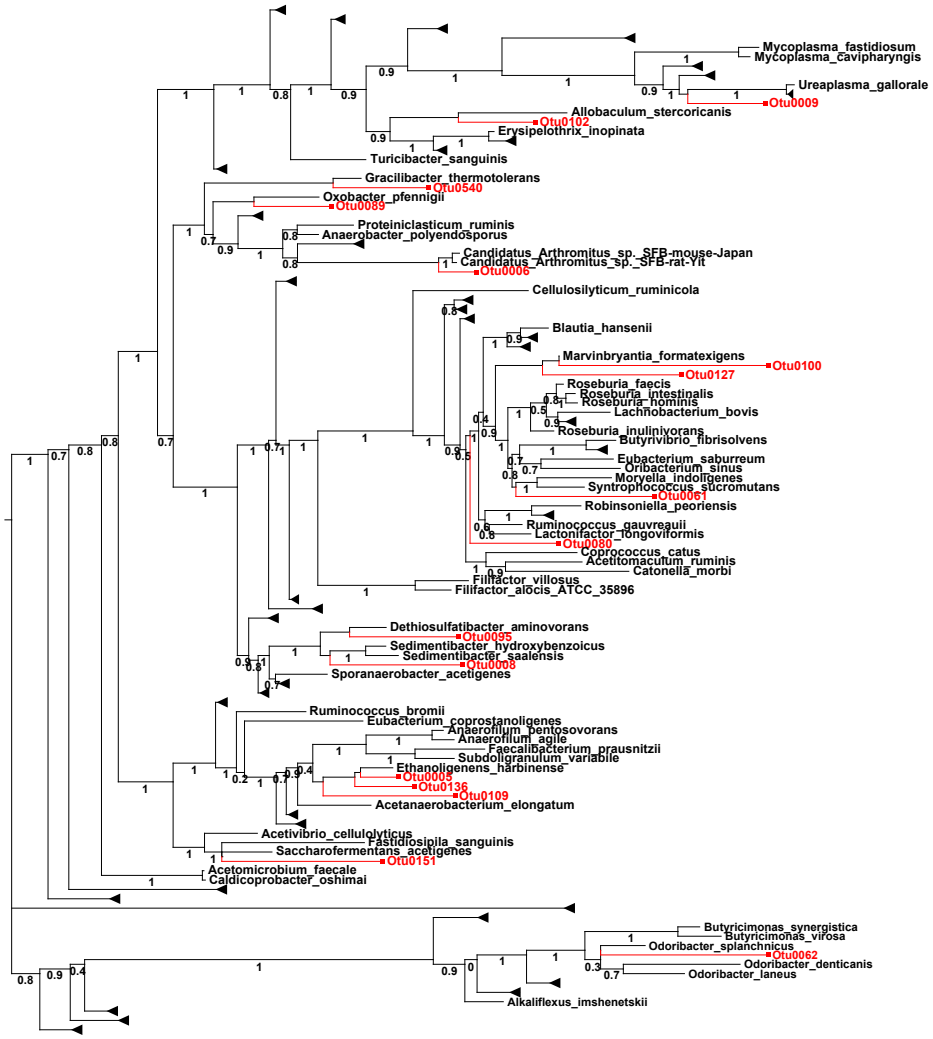

Figure S5 (Related to Figure 5). **OTUs placed on 16S rRNA reference tree**. Red branches represent placed OTUs, black branches represent 16S rRNA of reference tree, and partial tree of mouse gut microbiome.

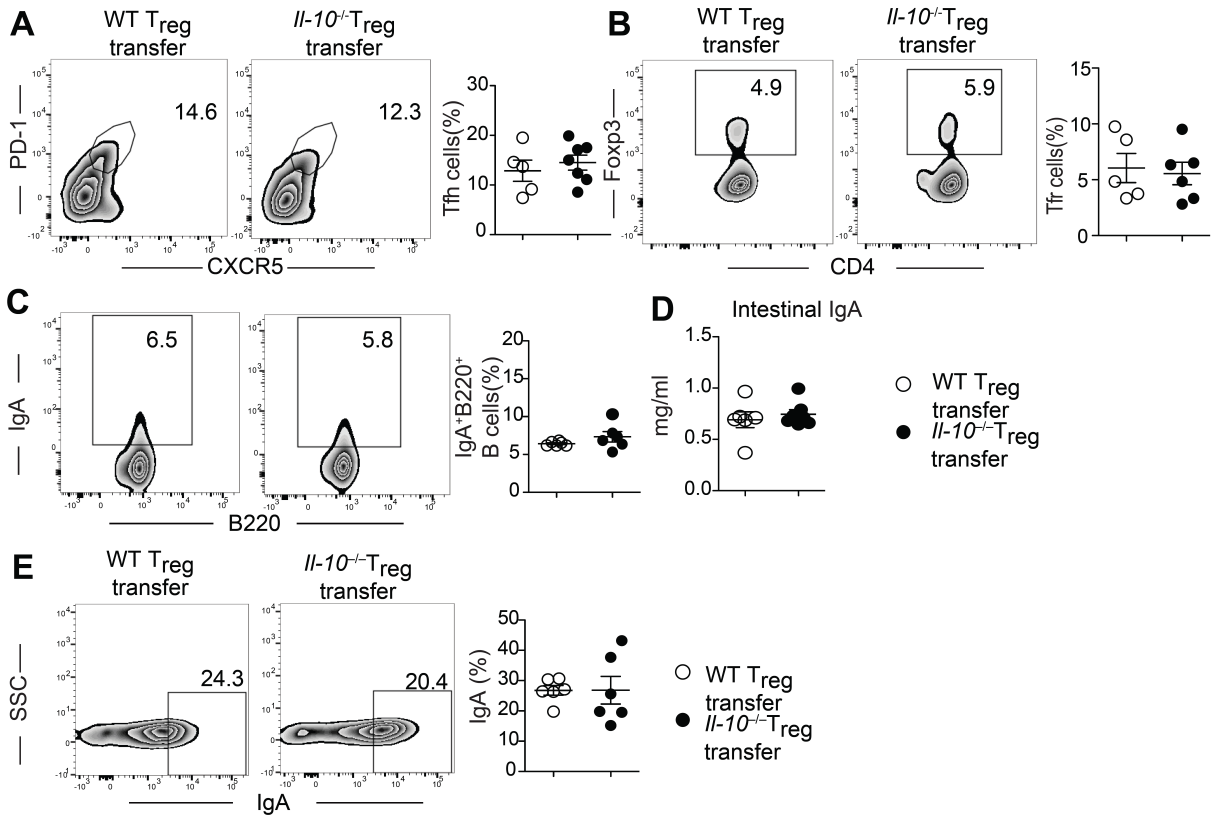

Figure S6 (Related to Figure 6). *IL-10*-deficient Treg cells support PP Tfr and Tfh cell differentiation and intestinal IgA production in *Tcra*$^{-/-}$ mice. A-C. Flow cytometric analysis, and frequencies of Tfh(**A**), Tfr (**B**) and IgA^{+}B220^{+} (**C**) cells isolated from the PP of *Tcra*$^{-/-}$ mice 2 weeks following the transfer of CD4^{+}CD25^{+}Treg cells isolated from either wide type or *Il10*$^{-/-}$ mice. **D**. Luminal concentration of IgA in the mouse groups from panels (**A-C**). **E**. Flow cytometric analysis and frequencies of IgA-coated bacteria in the fecal pellets of mouse groups from panels (**A-C**). Results are representative of 3 independent experiments. N=3-5 mice/group. *P<0.05; **P<0.01, ***P<0.001 by One-way ANOVA with post-test analysis.

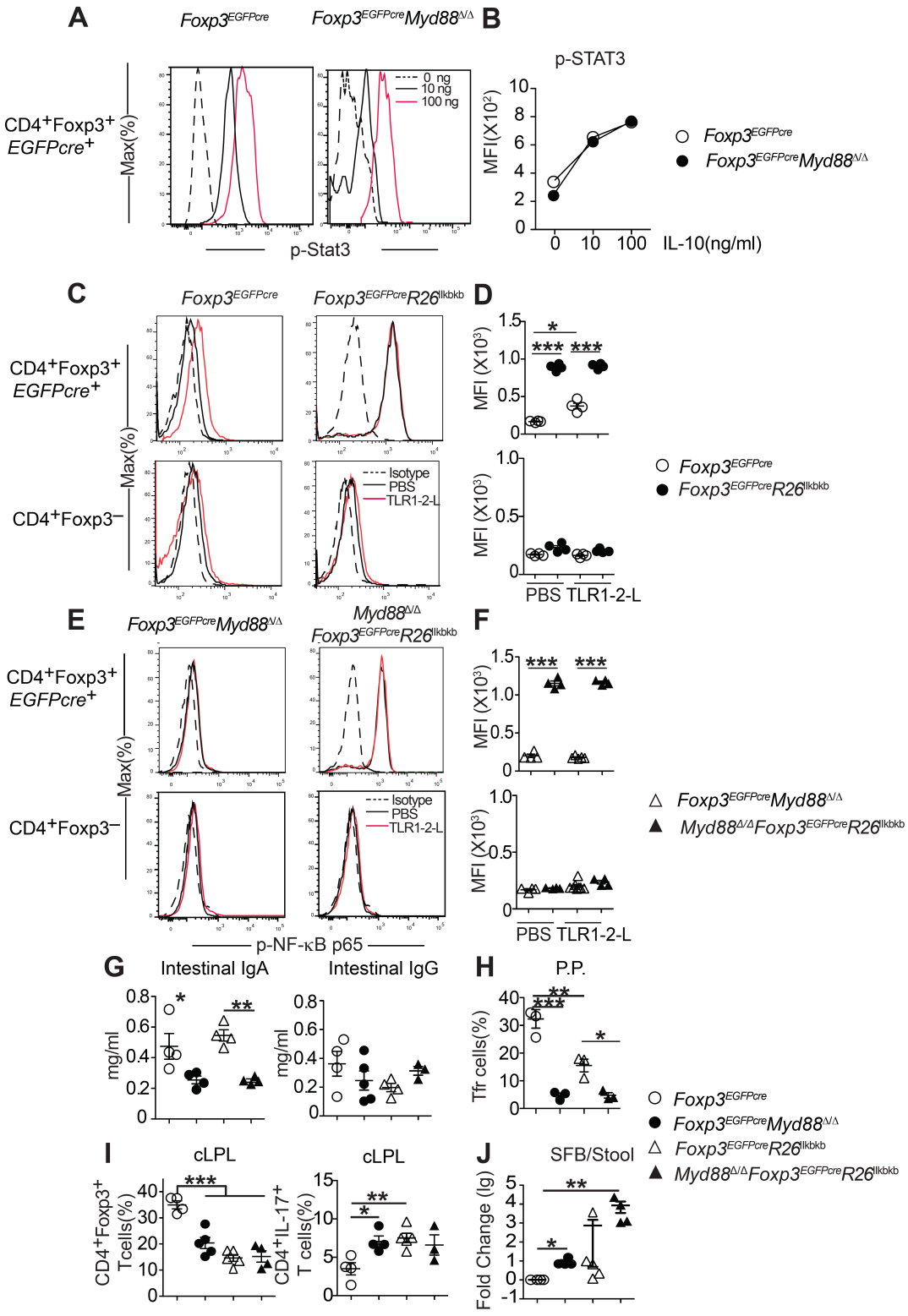
A
Foxp3EGFPcre
Foxp3EGFPcreMyd88Δ/Δ
CD4+Foxp3+ EGFPcre+
Max(%)
0 ng
10 ng
100 ng
p-Stat3
B
p-STAT3
MFI(X10²)
IL-10(ng/ml)
Foxp3EGFPcre
Foxp3EGFPcreMyd88Δ/Δ
C
Foxp3EGFPcre
Foxp3EGFPcreR26Ilkbkb
CD4+Foxp3+ EGFPcre+
CD4+Foxp3−
Isotype
PBS
TLR1-2-L
D
MFI (X10³)
PBS TLR1-2-L
Foxp3EGFPcre
Foxp3EGFPcreR26Ilkbkb
E
Foxp3EGFPcreMyd88Δ/Δ
Myd88Δ/Δ
Foxp3EGFPcreR26Ilkbkb
p-NF-κB p65
F
Foxp3EGFPcreMyd88Δ/Δ
Myd88Δ/Δ Foxp3EGFPcreR26Ilkbkb
G
Intestinal IgA
Intestinal IgG
mg/ml
H
P.P.
Tfr cells(%)
I
cLPL
CD4+Foxp3+ Tcells(%)
CD4+IL-17+ T cells(%)
J
SFB/Stool
Fold Change (Ig)

Figure S7 (Related to Figure 7). A gain of function *Ikkb* transgene does not rescue Treg cell-specific *Myd88* deficiency. **A-B**. Flow cytometric analysis (**A**) and MFI (**B**) of p-STAT-3 in cLP $CD4^+Foxp3^+$Treg treated with graded concentrations of IL-10. **C-F**. Flow cytometric analysis MFI of p-p65-NF-κB in cLP $CD4^+Foxp3^+$Treg cells and $CD4^+Foxp3^-$Tconv cells of $Foxp3^{EGFPcre}$ and $Foxp3^{EGFPcre}R26^{Ilkbkb}$ mice (**C, D**), and $Foxp3^{EGFPcre}Myd88^{\Delta/\Delta}$ and $Foxp3^{EGFPcre}Myd88^{\Delta/\Delta}R26^{Ilkbkb}$ mice (**E, F**) treated with either PBS or the TLR1-2 ligand (TLR1-2-L) Pam3SCK4. **G**. Intestinal lavage fluid IgA and IgG concentrations in $Foxp3^{EGFPcre}$, $Foxp3^{EGFPcre}Myd88^{\Delta/\Delta}$, $Foxp3^{EGFPcre}R26^{Ilkbkb}$ and $Foxp3^{EGFPcre}Myd88^{\Delta/\Delta}R26^{Ilkbkb}$ mice. **H, I**. Frequencies of PP Tfr cells (**H**) and cLP $CD4^+Foxp3^+$ Treg and Th17 cells (**I**) in indicated mouse strains. **J**. Real time PCR analysis of SFB 16S rRNA of stool of indicated mice. Data are representative of 3 independent experiments. *P<0.05; **P<0.01, ***P<0.001 by One-way ANOVA.

Taxonomic level	MYD88$^{fl/fl}$	Foxp3^{EGFPCre} MyD88$^{\Delta/\Delta}$	q-value
Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Odoribacter	0	0.001096	0.01
Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Clostridium_XlVa	0	0.001271	0.01
Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Clostridium_IV	0.000045	0.146123	0.01
Firmicutes;Clostridia;Clostridiales;unclassified;unclassified	0.000453	0.089211	0.01
*Firmicutes;unclassified;unclassified;unclassified;unclassified**	0.000075	0.211234	0.01
Tenericutes;Mollicutes;Mycoplasmatales;Mycoplasmataceae;Ureaplasma	0.054417	0.004587	0.01

Table S1 (Related to Figure 5). Selected taxa at genus level that are significantly different between *Myd88*$^{fl/fl}$ and *Foxp3*^{EGFPcre}*Myd88*$^{\Delta/\Delta}$ mice. Table depicts the mean relative abundances of taxon with significant group differences. Relative abundance is higher in specific group marked as red. *Indicates genus includes three OTUs. OTU0006, found present in the sequenced samples of *Foxp3*^{EGFPcre}*Myd88*$^{\Delta/\Delta}$ mice, was one of them, and has been identified as SFB (*Candidatus Arthromitus sp. SFB-mouse-Japan*) using blastn (identify 97.6%). The relative abundances of OTU0006 are 0.0% (*Myd88*$^{fl/fl}$), 20.2% (*Foxp3*^{EGFPcre}*Myd88*$^{\Delta/\Delta}$).

#OTU ID	taxonomy
Otu0062	Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Odoribacter
Otu0080	Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Clostridium_XlVa
Otu0005	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Clostridium_IV
Otu0109	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Clostridium_IV
Otu0136	Bacteria; Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; Clostridium_IV
Otu0008	Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified; unclassified
Otu0061	Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified; unclassified
Otu0095	Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified; unclassified
Otu0100	Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified; unclassified
Otu0127	Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified; unclassified
Otu0151	Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified; unclassified
Otu0540	Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified; unclassified
Otu0006	Bacteria; Firmicutes; unclassified; unclassified; unclassified; unclassified
Otu0089	Bacteria; Firmicutes; unclassified; unclassified; unclassified; unclassified
Otu0102	Bacteria; Firmicutes; unclassified; unclassified; unclassified; unclassified
Otu0009	Bacteria; Tenericutes; Mollicutes; Mycoplasmatales; Mycoplasmataceae; Ureaplasma

Table S2 (Related to Figure 5). OTUs included in the taxa at genus level that discriminate between *Myd88*$^{fl/fl}$ and *Foxp3*^{EGFPcre}*Myd88*$^{\Delta/\Delta}$ mice.

Supplemental Experimental Procedures

Antibodies and reagents. The following antibodies against the respective murine antigens were used: Foxp3 (clone FJK-16S), T-bet (eBio4B10), RORγ-t (B2D), IgA (mA-6E1), CD62L ((Mel-14), polyclonal IgG (13-40130-85), IgG1 (M1-14D12), B220 (RA3-6B2), IFN-γ (XMG1.2), IL-10 (JES5-16E3), GATA-3 (TWAJ), IRF-4 (3E4), ICOS (C398.4A), Helios (22F6), Ki67 (SolA15), CD25 (PC61.5), ICOS (7E.17.9), CTLA-4 (UC10-4B9), CXCR5 (SPRCL5), Vα2 (B20.1) (eBioscience), CD4 (RM4-5), CD3 (145-2C11), PD-1 (27F1A12), CD40L (MR1), CD25 (PC61), CD45.1 (A20), CD45.2 (104) IL-17a (TC11-18H10.1) (Biolegend), CD103 (M290), Bcl6 (K112-91), phospho-Stat-3 (4/P-Stat3), phospho-Stat-5 (47/Stat5(pY694)) (BD Biosciences), phospho-NF-κB p65 (93H1) (Cell signaling) and Nrp1 (R&D Systems). Mouse anti-murine IL-17 mAb (clone 17F3) and mouse IgG1 control mAb (Clone MOPC-21) were from BioXcell. TLR1-2 ligand (TLR1-2-L) Pam3SCK4 was from Invivogen. Recombinant murine IL-2 and IL-10 were from Peprotech.

Flow cytometry. Antibodies used in flow cytometric studies are described in the Supplemental Methods section. Intracellular cytokine staining was performed as previously described [38]. Dead cells were routinely excluded from the analysis based on the staining of eFluor 780 Fixable viability dye (eBioscience). Stained cells were analyzed on an LSRFortessa (BD Biosciences) and data were processed using FlowJo (Tree Star Inc.).

Bacterial Flow cytometry and magnetic sorting. Flow cytometric analysis of immunoglobulin-bound bacteria was performed as described (van der Waaij et al., 1996). Bacteria were stained with PE-labeled rat IgG1 F(ab)2 anti-mouse IgA mAb, or

with PE-labeled rat anti-mouse IgG1 antibody. Bacteria were resuspended in 4 μg/ml propidium iodide in PBS and analyzed by flow cytometry. All events that stained with propidium iodide were regarded as bacteria. For enrichment of IgA-coated bacteria, the latter were coated with PE-labeled rat IgG1 F(ab)2 anti-mouse IgA mAb then enriched using anti-PE MicroBeads (Miltenyi Biotec) and positive selection with MACS Separation Columns (Miltenyi Biotec) according manufacture protocol. The purity of IgA-coated bacteria was ≥90% after selection.

BrdU incorporation assay. Mice were injected intraperitoneally with 100 μl BrdU (Sigma-Aldrich) dissolved in PBS (10 mg/ml) at the onset of experiments. Thereafter, mice were fed water that contained 0.8 mg/ml BrdU and 5% glucose. Nine days later, cLPL cells were isolated and assessed for the BrdU incorporation by flow cytometric analysis using the BrdU Staining Kit for Flow Cytometry APC (eBioscience), following the manufacturer's instructions.

Apoptosis assay. Freshly isolated cLPLs were stained with AnnexinV staining kit (ebioscience) following the manufacturer's instructions. Dead cells were excluded from the analysis based on staining with eFluor 780 Fixable viability dye (eBioscience).

Real time PCR analysis. Tissue and cellular samples were snap-frozen in liquid nitrogen and homogenized in Trizol (Life Technologies). RNA was extracted by using the RNeasy kit (Qiagen). Reverse transcription was performed using Superscript III and oligo dT (Invitrogen). Taqman gene probes were used with Taqman Universal Fast Master Mix (Applied Biosystems) and ran on a Step-One-Plus^{TM} Real-Time PCR system machine (Applied Biosystems). GAPDH was used as an endogenous control (Applied Biosystems) and wild type (WT) mice RNA as the exogenous control. Samples were run

in triplicates and the relative expression was calculated using the ΔΔCt method.

For SFB analysis, bacterial genomic DNA was isolated from fecal pellets using ZR Fecal DNA MiniPrep kit following manufacturer's instructions. (ZYMO Research). Quantitative PCR analysis was carried out using SYBR Green Real-time PCR kit (Bio-Rad). Primer sequences for SFB and bacterial 16S rRNA genes as well as PCR conditions were as described (Barman et al., 2008). For SFB, relative quantity was calculated by the ΔΔCt method and normalized for total bacterial 16S rRNA abundance. Samples that were negative after 40 cycles of amplification were considered "not detected" (n.d.).

Methylation analysis. The methylation status of *Foxp3* CNS region in Foxp3^{+} Treg cells were analyzed as described previously (Haribhai et al., 2011; Schmitt et al., 2012).

ELISAs. Serum and luminal IgA and IgG1 concentrations and OVA-specific luminal IgA and IgG1 concentrations were measured by ELISAs according manufacture protocol (Bethyl, Inc).

ChIP assay. ChIP was performed with the Pierce Agarose ChIP Kit, per the manufacturer's instructions (Thermo Scientific). In brief, cells were fixed with 1% formaldehyde at room temperature for 10 min and lysed in the lysis buffer. DNA was then fragmented by sonication. After preclearance for 1 hr at 4^{0}C with salmon sperm DNA-saturated protein A-agarose, chromatin solutions were immunoprecipitated overnight at 4 ^{0}C with 1 mg of rabbit antibodies to NF-kBp65 (E1Z1T), Stat-3 (#9131), Stat-5 (#9350), or control rabbit IgG (DA1E; Cell Signaling). Input and immunoprecipitated chromatins were incubated for 4 hr at 65^{0}C to reverse cross-links. After proteinase K digestion, DNA was extracted with phenol-chloroform and

precipitated with ethanol. ChIP DNA was then analyzed by SYBR Green Real-time PCR kit (Bio-Rad) with the following primer sets: Foxp3 CNS2 promote forward: 5' – CCCTCTGGCATCCAAGAAAG-3'; Foxp3 CNS2 promote reverse: 5'-GGGTGCTAGCGGATGTGGTA -3'.

In vitro generation of iT_R cells. Naïve $CD4^+CD62L^+Foxp3^-(EGFP^-)CD44^{low}$ T cells were isolated from spleens of *Foxp3*^{EGFP} and *Myd88*$^{-/-}$*Foxp3*^{EGFP} mice by cell sorting using BD FACSAria III (BD Biosciences) (purity > 97%). Cells were loaded with CellTrace Violet Proliferation dye (Life Technologies), then cultured in 48-well plates with plate-bound anti-CD3 mAB (5 μg/ml) and soluble anti-CD28 mAb (3 μg/ml) and recombinant mouse TGF-β1 (2ng/ml) (R&D systems) for 5 days. RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 10 mM HEPES and 10% heat-inactivated FCS was used for all cultures. Foxp3 expression, reflected by EGFP expression, was determined by flow cytometry.

Isolation of cLP T cells. Excised colons were flushed to remove contents, opened longitudinally, and washed thoroughly in Ca and Mg free Dulbecco's Phosphate-Buffered Saline (DPBS) (GIBCO). They were then cut into 3–5 mm pieces that were incubated in 25 ml DPBS solution containing 5 mM EDTA and 20 mM HEPES buffer at 37^0C for 20 min to remove the epithelial layer. Intestinal pieces were collagenase-digested for 40 min at 37^0C in 25 ml RPMI 1640 (Caisson Labs) containing 0.5 mg/ml collagenase type VIII (Worthington), 50 U DNaseI (Roche), and 0.01 M HEPES (GIBCO). The crude cell suspension was loaded onto a 40%/ 60% Percoll (GE Healthcare) gradient and centrifuged at 2000 rpm for 30 min at room temperature with acceleration and brake turned off. Cells were collected from the 40%/60% interphase

(Kirkland et al., 2012).

Adoptive transfer studies into *Tcr a*$^{-/-}$ mice. Naïve OT-II^{+}(Vα2^{+}) CD4^{+}Foxp3^{-}(EGFP^{-}) Tconv cells and OT-II^{+}CD4^{+}Foxp3^{+}(EGFP^{+}) Treg cells were isolated from *OT-II^{+}Foxp3^{EGFPcre}* or *OT-II^{+}Foxp3^{EGFPcre}Myd88$^{\Delta/\Delta}$* mice and transferred into *Tcra*$^{-/-}$ mice by retro-orbital injection at 5x10^{5} cells/mouse. The mice were fed 1% OVA in the drinking water ad libitum for 2 weeks, then analyzed for T and B cell numbers and phenotypes in the PP and other lymphoid tissues of the recipient mice.

IL-10-deficient naïve CD4^{+}CD25^{+} cells were isolated from spleen of WT or *Il10*$^{-/-}$ mice by cell sorting using BD FACSAria III (BD Biosciences) (purity >95%). Of the isolated cells, 85-90% expressed Foxp3 as revealed by intracellular staining. The isolated cells were adoptively transferred (4x10^{5}) by retro-orbital injection into *Tcr a*$^{-/-}$ mice. After 2 weeks, the different organs were collected and analyzed for their immune cell phenotypes and numbers by flow cytometry.

Bacterial Cultures. Frozen tissue samples were homogenized in PBS under sterile conditions and plated at serial dilutions. Aerobic bacterial colonies were grown on LB agar. Media for recovery of obligate anaerobes included pre-reduced Brucella base blood agar containing 5% sheep blood, hemin and vitamin K (BMB) and Brucella base blood agar containing 5% laked sheep blood, 100 ug/mL of kanamycin, and 7.5 ug/mL vancomycin (LKV). Facultative organisms were recovered using Tryptic Soy base with 5% sheep blood agar (TSA) and MacConkey's agar (MAC). Media for the isolation of anaerobes (BMB and LKV) were incubated at 37^{o} in the anaerobic chamber with an atmosphere of 10% hydrogen, 10% carbon dioxide and 80% nitrogen (Coy Laboratory Products, Grass Lake, MI) for a minimum of 96 hours before enumeration. TSA and

MAC were incubated at 37^{o} in ambient air for a minimum of 24 hours before enumeration. All quantitative counts were recorded as CFU/gram of specimen. Bacterial identifications for the aerobic and facultative aerobic bacteria were performed using established criteria and Enterobacteriaceae were identified using API 20E strip (BioMerieux, Inc. Durham, NC) and/or long chain fatty acid analysis using the Microbial Identification System (MIS) (MIDI Inc., Newark, DE). Bacterial identifications for the anaerobic bacteria were performed using the MIS and/or RapID ANA II System (Remel Inc., Norcross, GA).

Preparation of DNA and pyrosequencing. DNA was extracted from mouse Illeum tissue on the standard protocol with PowerSoil DNA Isolation Kit (MoBio) per Human Microbiome Project (HMP) modifications (Barbara A. Methé et al., 2012; Curtis Huttenhower et al., 2012). The V3-V5 regions of the 16S rRNA gene were amplified by PCR using bar-coded universal primers 354F and 926R (V3-V5) containing the A and B sequencing adaptors (454 Life Sciences,Branford, CT) obtained from Invitrogen. Primer sequences are as follows: B-354F (59-cctatcccctgtgtgccttggcagtctcaGCCTACGGGAGGCAGCAG-39; B adaptor in lowercase letters); A-926R (59-ccatctcatccctgcgtgtctccgactcagNNNNNCCGTCAATTCMTTTRAGT; A adaptor in lowercase letters, and N represents a bar code that is unique for each sample). DNA pool emulsion PCR amplification and 454 sequencing were performed at the BCM Human Genome Sequencing Center (HGSC) in Houston, TX. Sequencing was performed using the 454/Roche B sequencing primer kit in the Roche Genome Sequencer GS-FLX Titanium platform.

16S rRNA Data preprocessing. Raw sequencing reads were processed using the mothur software package (v.1.3.33) (Schloss et al., 2009), which performs demultiplexing and denoising, quality filtering, alignment against the ARB Silva reference database of 16S rDNA gene sequences, and clustering into Operational Taxonomic Units (OTUs) at 97% identity. OTUs numbers were assigned by the mothur pipeline, which orders OTUs in descending order by the sum of the sequencing reads across all samples assigned to the OTU. In total, 482 OTUs were generated in mothur. After filtering OTUs that failed to ≥ 1/ mean # reads per sample in at least one cohort, 118 OTUs were available for further analysis.

16S rRNA data analysis. A measure of overall microbial community diversity, the Shannon entropy (Weaver and Shannon, 1963), was calculated for the samples. Shannon's index shows diversity index, taking into account the number of reads as well as number of OTUs. Rarefaction plots were also generated to assess whether adequate coverage of the microbial communities (number of sequencing reads) was obtained to adequately estimate ecological diversity. To visualize differences in overall microbial community structure, the unweighted and weighted Unifrac measure (Lozupone and Knight, 2005) was calculated between all pairs of samples, and Principal Coordinates Analysis (PCoA) plots were generated by custom R scripts. To access the differences between groups of overall microbial community using Analysis of Molecular Variance (AMOVA), it is widely used in population genetics to test the hypothesis that genetic diversity within two populations is not significantly different from that which would result from pooling the two populations (Excoffier et al., 1992). The relative abundances of the

phyla were analyzed using MetaStats and the BH method to correct for multiple hypothesis testing with a FDR<0.05 considered significant.

To assess differences in the microbiota between groups at the level of genus, the Metastats (White et al., 2009) software package was employed. Metastats is tailored for detecting differentially abundant OTUs in microbiome data, and gives *q-value* by using multiple hypothesis testing correction. Then pplacer algorithm (Matsen et al., 2010) was applied to more accurately identify the selected organisms and their phylogenetic relationships. Pplacer uses likelihood-based phylogenetics methodology to place short reads to 16S rRNA reference tree. Reference tree was constructed using full-length or near full-length 16S rDNA sequences from Ribosomal Database Project (RDP)(Cole et al., 2014) with curating process for mouse gut microbiota community by custom Python scripts. To further confirm OTUs placed to SFB clade, blast (Altschul et al., 1990) (ncbi-blast-2.2.27+) were applied with default parameters.

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