Supplemental Data
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Specific Microbiota Direct the Differentiation of IL-17-Producing T-Helper Cells in the Mucosa of the Small Intestine

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Please find Supplemental Experimental Procedures, Supplemental References, and 12 supplemental figures in the following pages.

SUPPLEMENTAL METHODS:

Altered Schaedler's Flora (ASF)

ASF-only colonized Swiss-Webster mice were obtained from Taconic Farms. The ASF consists of a defined mix of 8 organisms (Dewhirst et al., 1999): four extremely oxygen sensitive (EOS) "fusiform-shaped" anaerobes, TAC:ASF numbers 356, 492 (*Eubacterium plexicaudatum*), 500 and 502, and four aerotolerant species, *Lactobacillus acidophilus* (TAC:ASF 360), *Lactobacillus murinus* (TAC:ASF 361), the *Bacteroides distasonis* (TAC:ASF 519) and recently named *Mucispirillum schaedleri* (TAC:ASF 457). The presence of the ASF in the analyzed mice was confirmed by macroscopic and culturing methods by Taconic Farms.

RNA isolation and real-time PCR

0.5 cm of the most distal part of the small intestine was dissected. Total RNA was extracted with TRIzol (Invitrogen) and cDNA was synthesized with Superscript II (Invitrogen) and random primers. The iQ SYBR Green Supermix (Bio-Rad) or QuantiTect Multiplex PCR mix (Qiagen) and the iCycler Sequence Detection System (Bio-Rad) were used for analysis of cDNA by real-time quantitative PCR in triplicate. The primers sets were: IL17A: as described (Ivanov et al., 2006), HPRT: 5'-AGGTTGCAAGCTTGCTGGT-3' and 5'-TGAAGTACTCATTATAGTCAAGGGCA-3'. The quantity of IL17A relative to HPRT in each sample was calculated using threshold cycle (Ct) values for each gene according to the formula "2^([Ct]HPRT - [Ct]IL17A)"

FISH probes:

From (Lupp et al., 2007)

EUB338 probe (5' GCT GCC TCC CGT AGG AGT 3') fluorescently labeled with Texas Red

CFB286 probe (5' TCC TCT CAG AAC CCC TAC 3') labeled with fluorescein

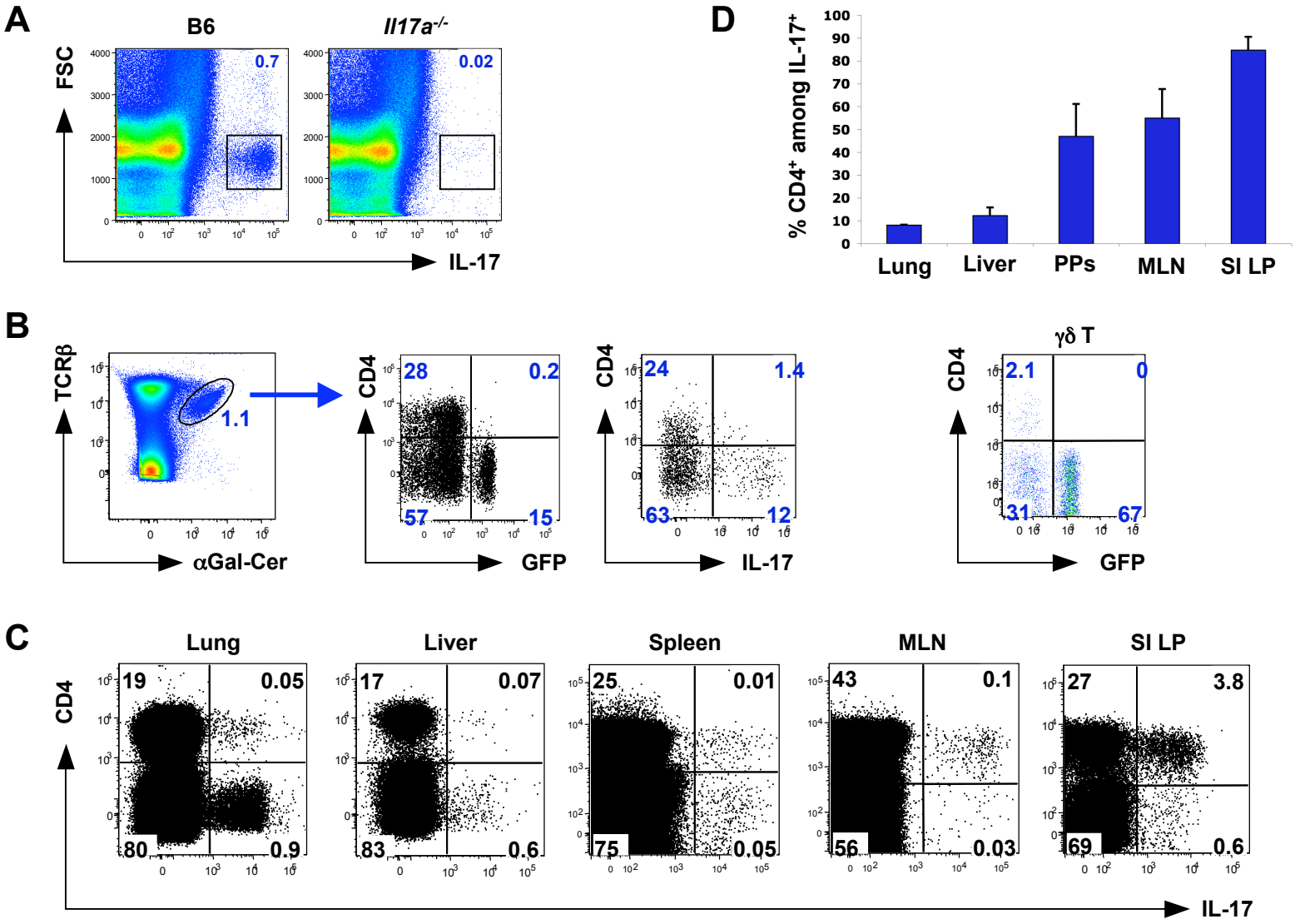
GAM42a probe (5' GCC TTC CCA CAT CGT TT 3') labeled with fluorescein

REFERENCES:

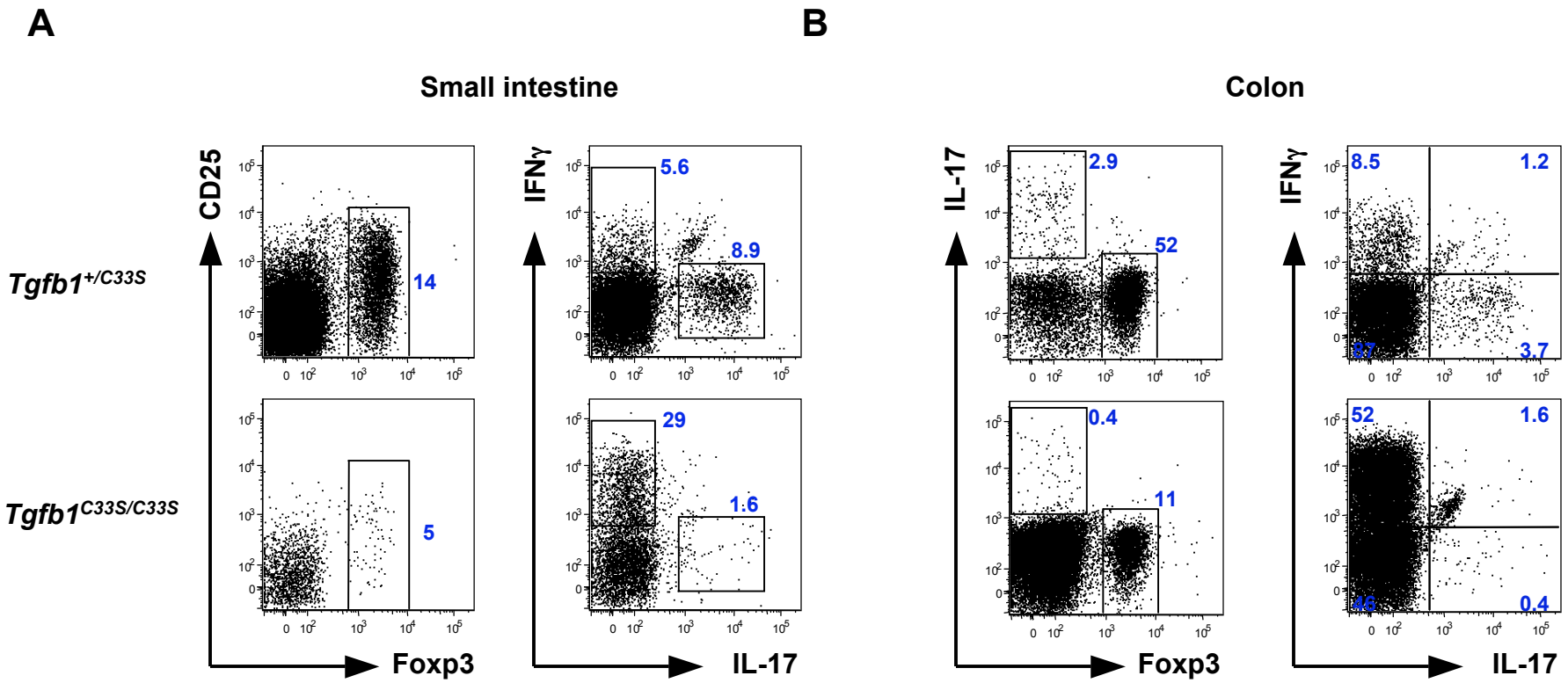
Dewhirst, F. E., Chien, C. C., Paster, B. J., Ericson, R. L., Orcutt, R. P., Schauer, D. B., and Fox, J. G. (1999). Phylogeny of the defined murine microbiota: altered Schaedler flora. Appl Environ Microbiol *65*, 3287-3292.

Ivanov, II, McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua, D. J., and Littman, D. R. (2006). The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell *126*, 1121-1133.

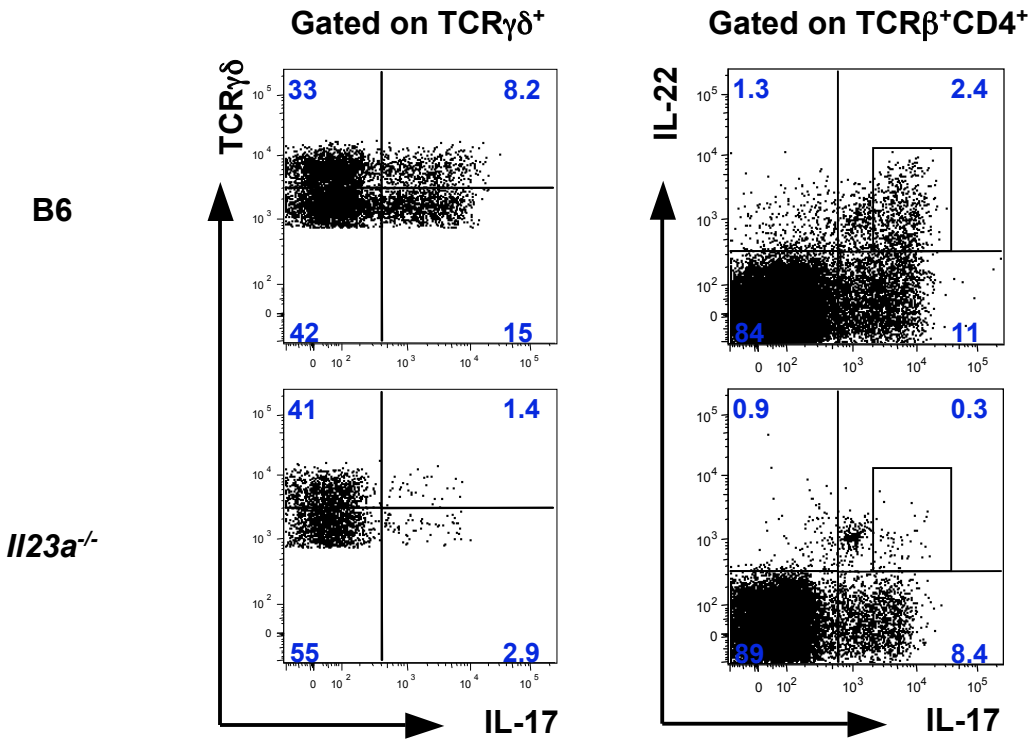
Lupp, C., Robertson, M. L., Wickham, M. E., Sekirov, I., Champion, O. L., Gaynor, E. C., and Finlay, B. B. (2007). Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe *2*, 119-129.



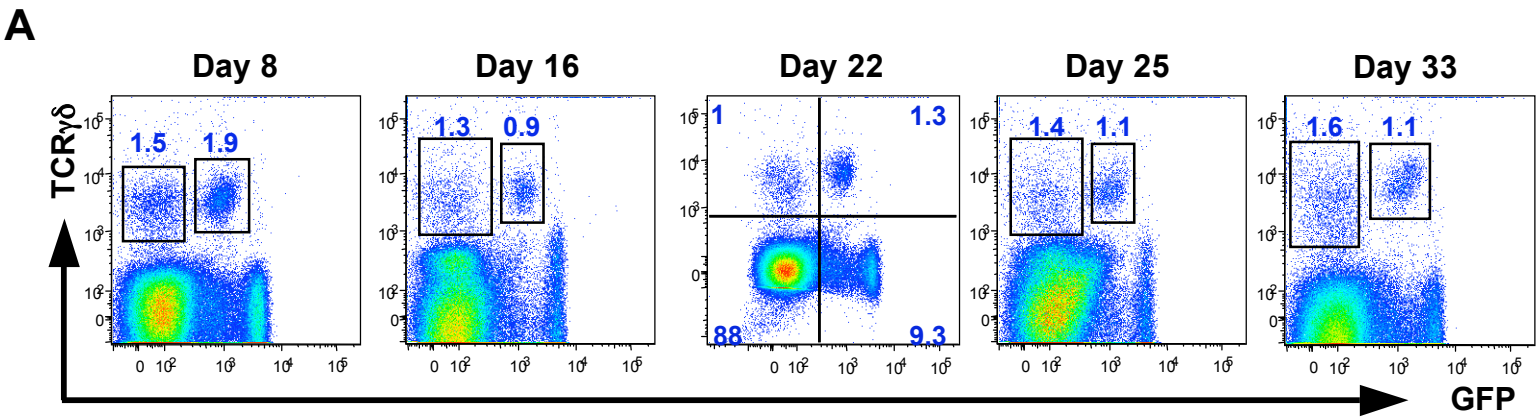
Suppl Fig 1. IL-17 expressing cell populations in different tissues. **(A)** IL-17 expression in ungated lung cells isolated as described in Methods from WT (B6) and *Il17a*$^{-/-}$ mice (to serve as control for the IL-17 staining) as assessed by flow cytometry. **(B)** RORγt(GFP) and IL-17 expression in lung iNKT cells (gated as shown) and RORγt(GFP) expression in lung TCRγδ T cells (lymphocytes gated on TCRγδ^{+}TCRβ^{-} cells) from *Rorγt* $^{gfp/+}$ mice. Plots were first gated on lymphocytes and represent individual mice. **(C)** CD4 vs IL-17 expression in cells isolated from different tissues of WT (B6) mice. Plots gated on lymphocytes and represent individual mice. **(D)** Representation of CD4^{+} cells among the IL-17 expressing cells in different tissues of WT (B6) mice. For IL-17 staining, cells were stimulated for 4 hours with PMA and Ionomycin as described in Methods.



Suppl Fig 2. Loss of Th17 cells and decrease in Treg cells in the LP in the absence of proper TGF-β activation. LPLs were isolated form small intestines **(A)** and colons **(B)** of 11-week-old *Tgfb1*$^{C33S/C33S}$ mice and heterozygous littermates and IL-17, IFNγ, and Foxp3 expression analyzed after 4 hour stimulation with PMA/Ionomycin. Data from one mouse of each genotype are shown and the plots were gated on TCRβ^{+}CD4^{+} lymphocytes. Data representative of 3 independent experiments.



Suppl Fig 3. IL-17 and IL-22 expression in T cell populations from SI LP of *Il23a*$^{-/-}$ mice. *Il23a*$^{-/-}$ and B6 mice from Jackson were cohoused with Taconic B6 mice for 17 days. LPLs were isolated from the SI LP as described in the methods and stimulated with PMA/Ionomycn for 4 hours before staining for surface and intracellular markers. One mouse of each genotype is shown. Plots on the left were gated on TCRγδ^{+}TCRβ^{-} lymphocytes. Two populations of IL-17 expressing cells could be identified based on the level of TCRγδ expression. Plots on the right were gated on TCRβ^{+}CD4^{+} lymphocytes. For cytokine staining, cells were stimulated for 4 hours with PMA and Ionomycin as described in Methods.



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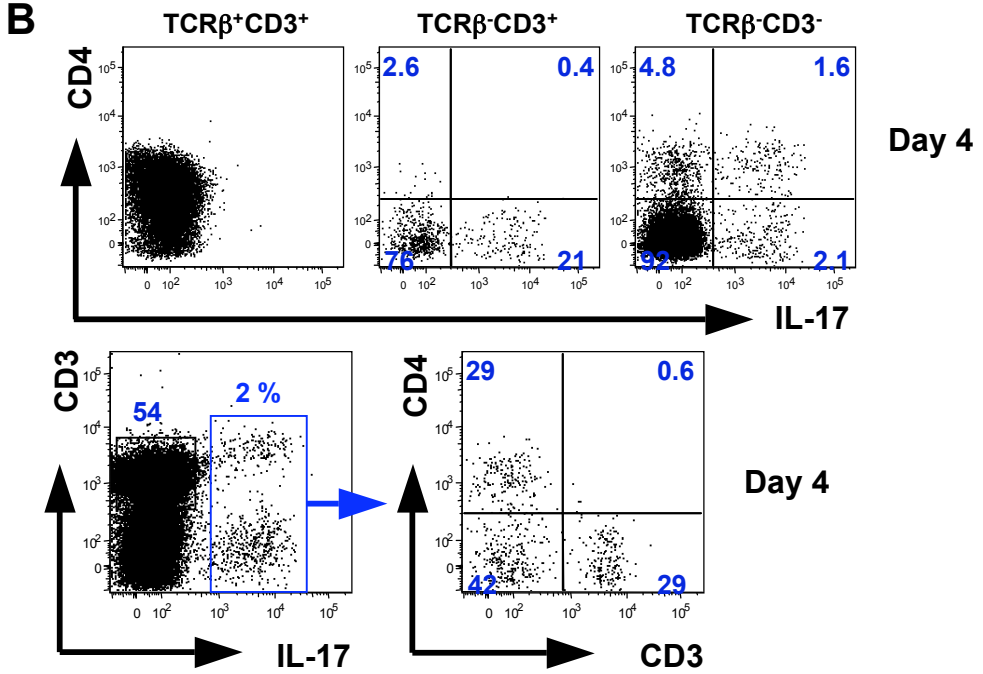
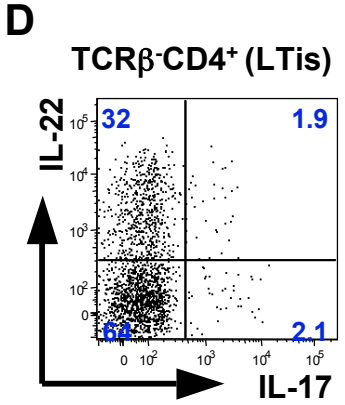
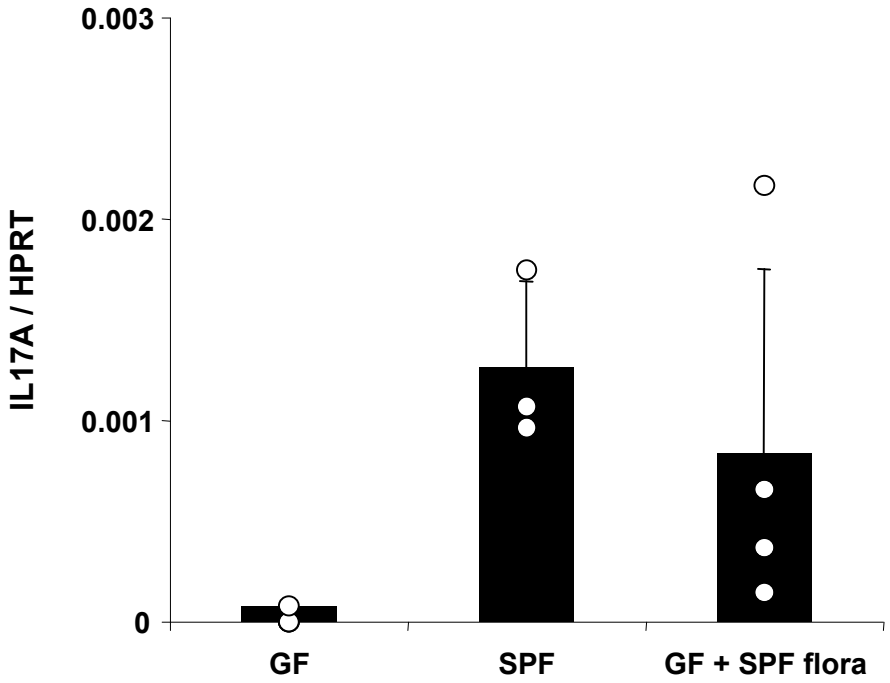
	Nursing	Solid food/feces	Cecum	Th17
Day 4	+++	-	-	-
Day 8	+++	-	-	-
Day 16	+++	-	-	-
Day 22	++	+	+	-
Day 25	+	++	++	+
Day 33	-	+++	+++	+++

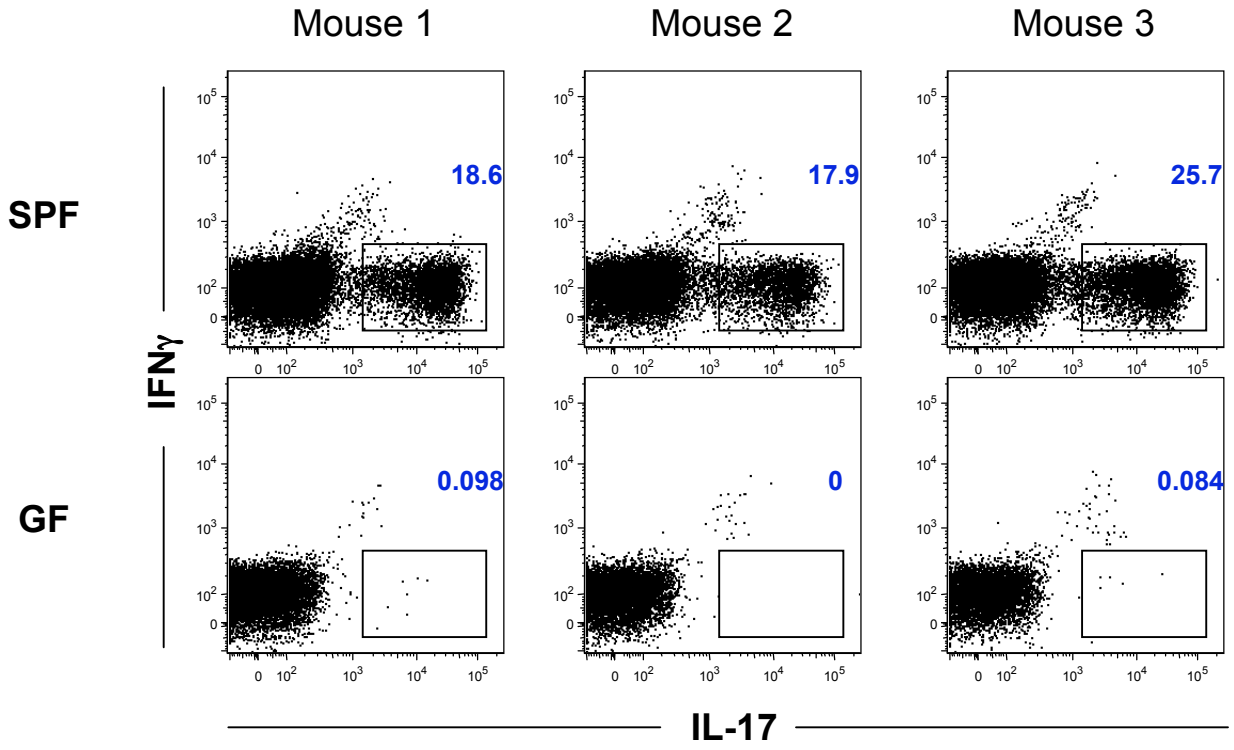




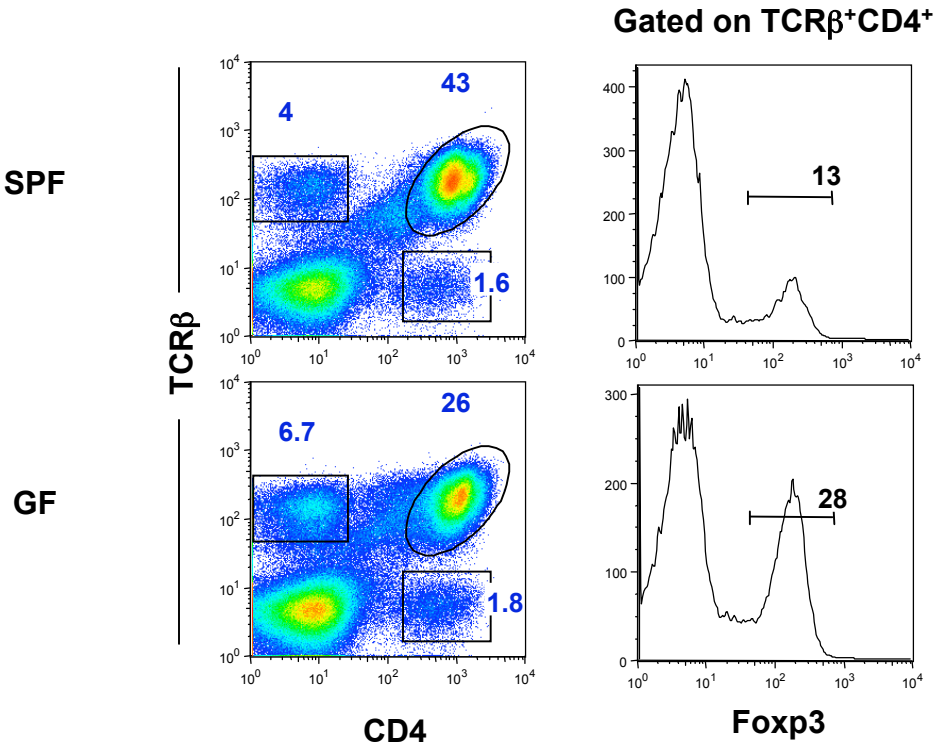
Suppl Fig 4. RORγt and IL-17 expression during ontogeny. **(A)** RORγt(GFP) and TCRγδ expression in LPLs isolated from SI LP of *Rorγt* $^{gfp/+}$ mice of different ages. Plots represent individual samples (see Methods) and were gated on lymphocytes. **(B)** IL-17 expression in TCRβ (TCRβ$^+$CD3$^+$), TCRγδ (TCRβ$^-$CD3$^+$) and LTi-like (CD4$^+$CD3$^-$) cells in the SI LP of 4 day old mice. Top panels: plots were gated first on lymphocytes and then on the corresponding population. Bottom panels: left, CD3/IL-17 staining in the lymphocyte gate; right, CD4/CD3 staining in the IL-17$^+$ lymphocytes. **(C)** Th17 cell development and physiologic characteristics. "-" not observed, "+" some, but few/very small, "++" intermediate (size for the cecum), "+++" – adult proportions/size. **(D)** IL-17 and IL-22 expression in TCRβ$^-$CD4$^+$ LTi-like cells of 7-week old B6 mice. For IL-17 and IL-22 stainings, cells were stimulated for 4 hours with PMA and Ionomycin as described in Methods.



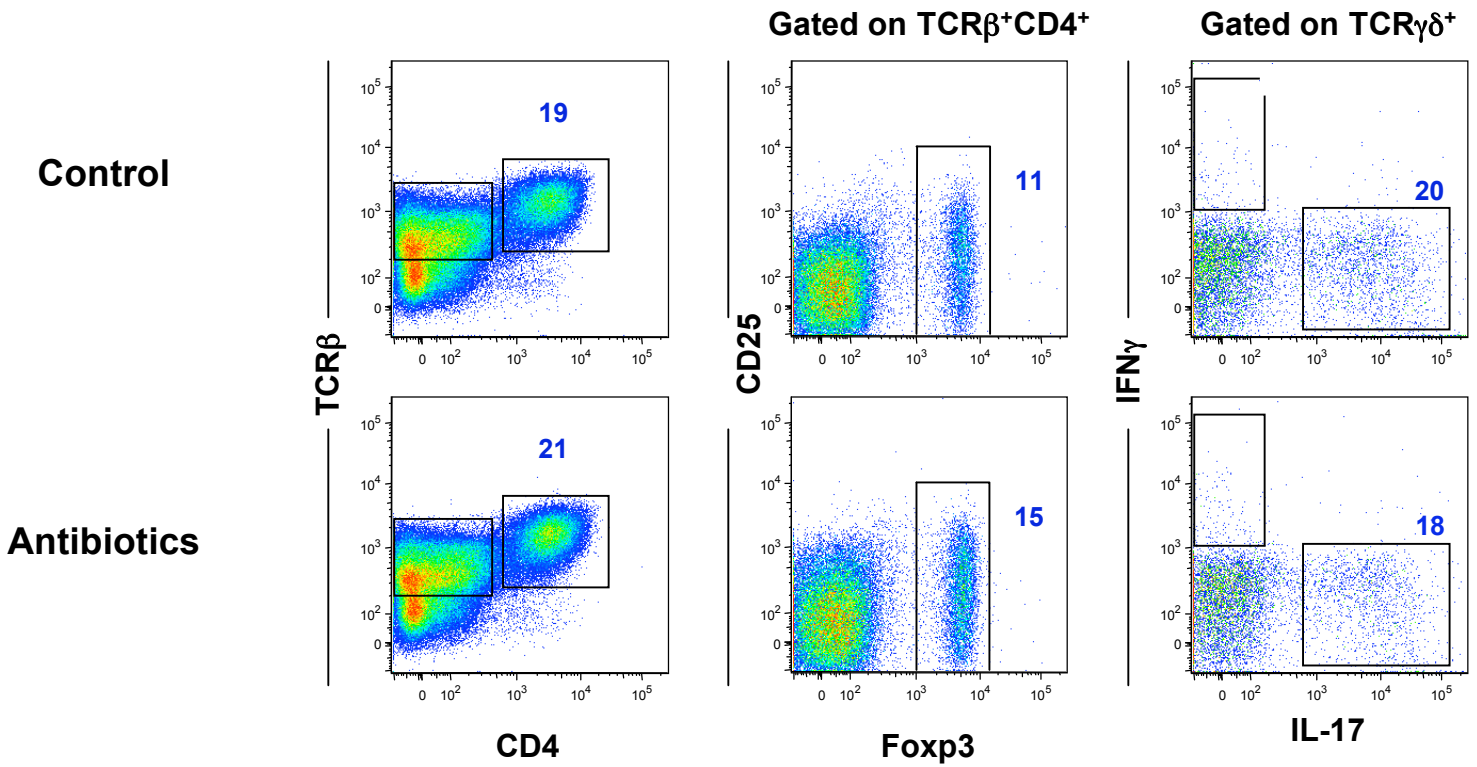
Suppl Fig 5. Lamina propria Th17 cell differentiation requires intestinal commensal bacteria. Real-time PCR analysis of IL-17 mRNA expression in total RNA from terminal ileum of C57BL/6 germ-free (GF), conventionally raised (SPF) mice, and GF mice conventionalized with SPF flora for 6 weeks. Data represented as mean of 3-4 mice per group. Error bars, standard deviation of the mean. Each data point is a triplicate analysis of a single animal. Data analysis was done by the Ct method as described in Methods



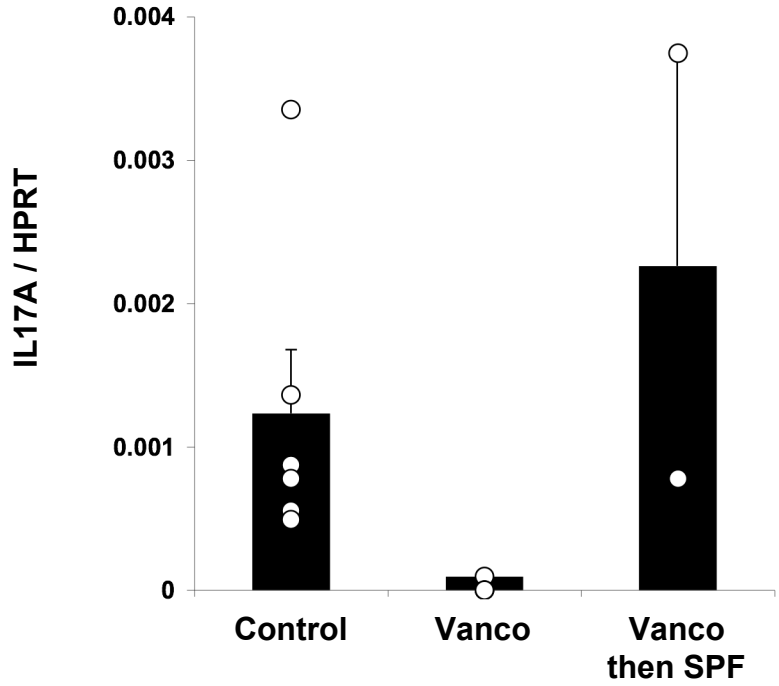
Suppl Fig 6. Lamina propria Th17 cell differentiation requires intestinal commensal bacteria. Th17 cells in SI LP of 6-week old germfree (GF) and conventionally raised (SPF) Swiss-Webster mice from Taconic Farms. SI LPLs were isolated as described in Methods and stimulated for 4 hours with PMA and Ionomycin before intracellular staining for IL-17. Plots represent individual mice and were gated on $TCR\beta^{+}CD4^{+}$ lymphocytes. Data from one out of four experiments with identical results.



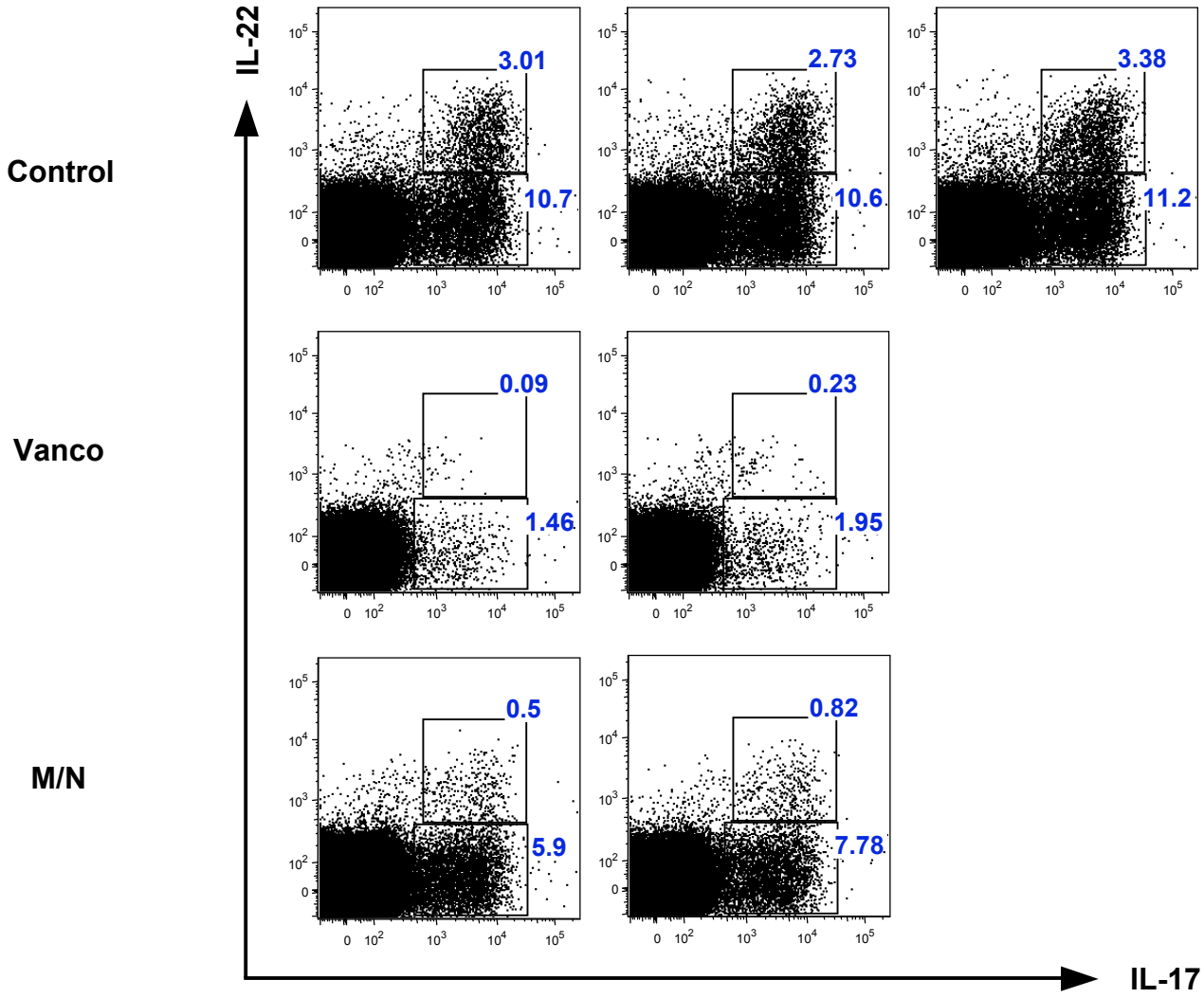
Suppl Fig 7. Increased Foxp3$^+$ T cell proportions in germfree mice. Representation of Foxp3$^+$ cells within the TCRβ$^+$CD4$^+$ lymphocytes isolated from the SI LP of 6-month-old C57BL6 germfree (GF) and conventionally raised (SPF) mice. SI LPLs were isolated as described in Methods and stimulated for 4 hours with PMA and Ionomycin before intracellular/nuclear stainng for Foxp3. One mouse of each group is shown. Plots on the left gated on lymphocytes. Plots on the right gated on TCRβ$^+$CD4$^+$ lymphocytes.



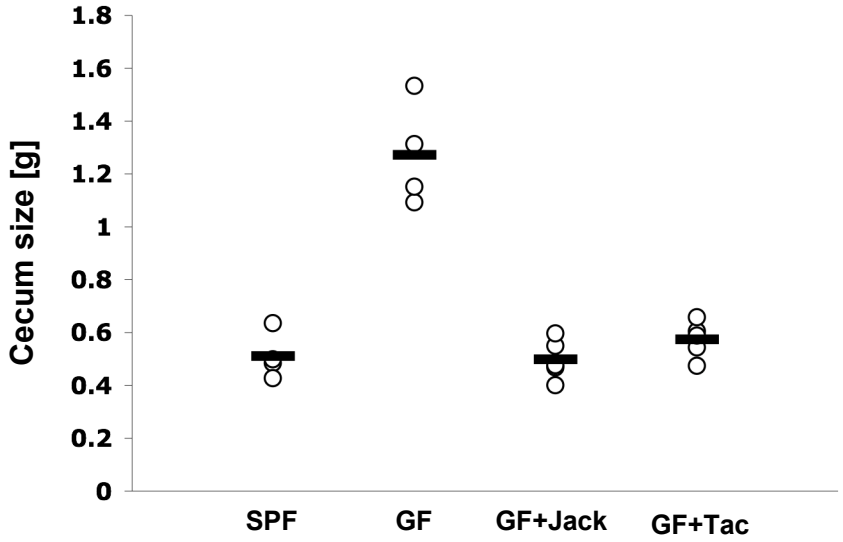
Suppl Fig 8. Effect of antibiotic treatment on SI LP lymphocytes. 12 week old Taconic C57BL/6 mice were treated with an antibiotic cocktail as described in Methods for 4 weeks. SI LPLs from treated (Antibiotics) and untreated (Control) animals were isolated as described in Methods and stimulated for 4 hours with PMA and Ionomycin before intracellular/nuclear stainng for Foxp3 and IL-17. One mouse of each group is shown. Left, plots gated on lymphocytes. Middle, plots gated on $TCR\beta^{+}CD4^{+}$ lymphocytes. Right, plots gated on $TCR\gamma\delta^{+}TCR\beta^{-}$ lymphocytes.



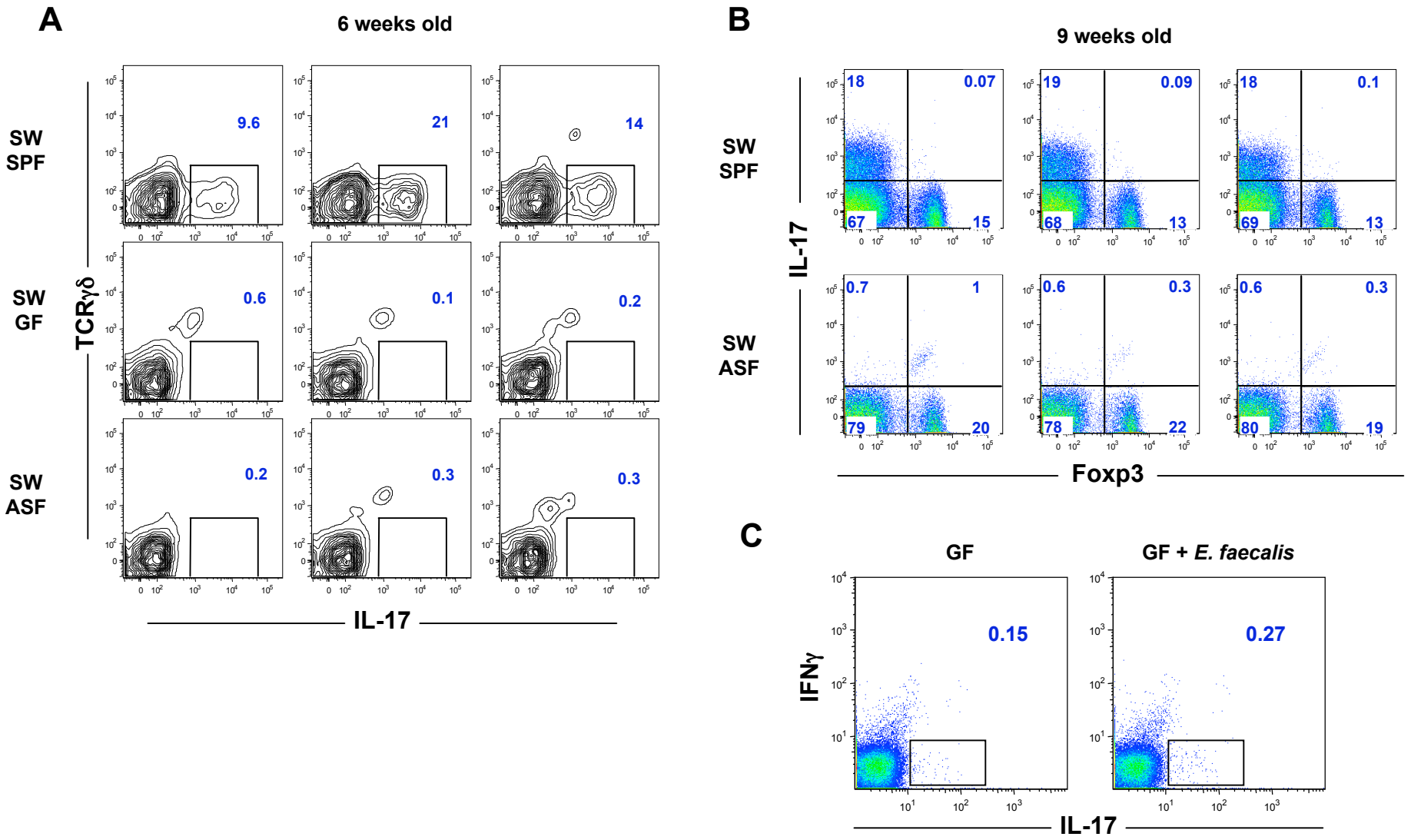
Suppl Fig 9. Effect of antibiotic treatment on IL-17 mRNA. Real-time PCR analysis of IL-17 mRNA expression in total RNA from terminal ileum of control-untreated, 11-week old mice treated with vancomycin from birth, and 11 week old mice treated with vancomycin from birth for 6.5 weeks and then re-colonized with SPF flora for 4.5 weeks. Data represented as mean of 2-6 mice per group. Error bars, standard deviation of the mean. Each data point is a triplicate analysis of a single animal. Data analysis was done by the Ct method as described in Methods



Suppl Fig 10. Effects of antibiotic treatment on IL-22$^+$ SI LP Th17 cells. SI LPLs were isolated from 7-week old C57BL/6 mice treated with different antibiotic combinations from birth as described in Methods and stimulated for 4 hours with PMA and Ionomycin before intracellular/nuclear staining for IL-17 and IL-22. Each plot represents a separate animal. All plots were gated on TCRβ$^+$CD4$^+$ lymphocytes. Control, untreated; Vanco, 0.5 g/L vancomycin; M/N, 1 g/L each metronidazole and neomycin sulfate.



Suppl Fig 11. Colonization of germfree mice with different microbiota.Cecum size of 6-week old germfree Swiss-Webster mice (GF) compared to GF mice colonized with cecal contents from Jackson B6 (GF+Jack) or Taconic B6 (GF+Tac) mice for 10 or 12 days (data combined from the two timepoints) and to mice raised in conventional conditions (SPF). N = 4 (SPF, GF) and 5 (GF+Jack, GF+Tac). Circles, individual mice. Horizontal line, mean.



Suppl Fig 12. Altered Schaedler's Flora or *Enterococcus faecalis* do not induce SI LP Th17 cell differentiation. SI LPLs were isolated as described in Methods and stimulated for 4 hours with PMA and Ionomycin before intracellular stainng for IL-17 and Foxp3. Plots represent individual mice and were gated on TCRβ$^+$CD4$^+$ lymphocytes. **(A, B)** Th17 cells and Foxp3$^+$ cells in SI LP of Taconic Swiss-Webster mice raised under conventional conditions (SPF), germfree (GF), or from gnotobiotic isolators with defined altered Schaedler's flora (ASF) (see Methods for ASF composition). **(C)** Th17 cells in the SI LP of 129S6/Sv germfree mice (GF) and the same mice monocolonized with *E. faecalis* for 11+ weeks. Data representative of two independent experiments.