Supporting Information

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SI Materials and Methods

Mice. C57BL/6J, B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II), and B6.B10ScN-*Tlr4*$^{lps\text{-}del}$/JthJ (*Tlr4*$^{-/-}$) mice were obtained from The Jackson Laboratory. *Acyloxyacyl hydrolase* (*Aoah*)$^{-/-}$ mice on the C57BL/6 background, a gift from R. Munford (National Institute of Allergy and Infectious Diseases, Bethesda, MD), were generated as described previously (1). *Aoah*$^{-/-}$ and *Toll-like receptor 4* (*Tlr4*)$^{-/-}$ mice were bred with WT C57BL/6J mice, and the heterozygous offspring were mated to generate WT and KO littermates. Littermates and offspring of littermates (littermate-derived) age 6–9 wk were used for all experiments. All mice were housed under specific pathogen-free conditions that included negative surveillance for *Norovirus* and *Helicobacter*.

Antibiotic Treatment. For depletion of intestinal Gram-negative bacteria, neomycin sulfate and metronidazole antibiotics (Fisher) were used at 1 g/L in sterile water. Antibiotics were added to the drinking water, which was replaced every week. The efficacy of antibiotic activity against Gram-negative bacteria was confirmed on feces by Gram stain analysis (Fisher) and culture on MacConkey agar media plates (Fisher) selective for Gram-negative bacteria.

Quantitative RT-PCR. PCR data were obtained using the Taqman Fast Cells-to-CT Kit (Life Technologies), including lysis reagents with genomic DNA removal, RT Enzyme Mix, RT Buffer, and TaqMan Fast Universal PCR Master Mix (all from Life Technologies). In brief, purified dendritic cells (DCs) and T cells were lysed to obtain RNA before performing an RT reaction to convert RNA into cDNA. Samples were run in triplicate on a 7500 Fast Real-Time PCR machine (Life Technologies) and normalized to the β-actin reference gene. Sample data from DC and T-cell populations was quantified according to the ddCt method (ddCt = $dCt_{AOAH} - dCt_{Actin}$) compared with control groups. Primer and probe sets (AOAH, Mm00600104_m1; β-actin, Mm00607939_s1) were obtained from Life Technologies.

AOAH Activity. Purified DCs isolated from the spleen, lymph nodes (LN), lungs, or lamina propria (LP) of 30 mice or derived from bone marrow precursors (BMDCs) were lysed in PBS 0.1% Triton X-1000. Lysed samples were used for protein quantification (Bio-Rad) and analyzed for AOAH activity as described previously (2). In brief, lysate supernatants were incubated with [^{3}H/^{14}C] LPS in AOAH reaction mix for 18 h at 37 °C. Intact LPS was then precipitated using ethanol, and the ^{3}H-fatty acids in the ethanol supernatant were quantitated using a Beckman Coulter LS6500 beta scintillation counter with corrections for spillover and quenching. Recombinant human AOAH (rhAOAH) was used to construct a standard curve for each assay. Results are expressed as picograms of rhAOAH activity per milligram of total lysate protein.

BMDC Generation. BMDCs were generated by culturing mouse bone marrow precursors for 6 d in complete [10% FBS (HyClone), 1× GlutaMax, 1× nonessential amino acids, 100 nM sodium pyruvate, 1× penicillin, and streptomycin antibiotics (Life Technologies)] RMPI 1640 medium (Life Technologies) supplemented with 1000 U/mL each of GM-CSF and IL-4 (R&D Systems). Fresh media and cytokines were replenished on day 3 of culturing. BMDCs were purified by sorting for cells expressing CD11c and I-A^{b} on a FACSAria cell sorter (BD Biosciences; purity ≥98%).

Isolation of LP Cells. Small and large intestines were excised and flushed with 10% FBS in Mg_2Cl_2- and $CaCl_2$-free HBSS (Life Technologies) to clear any remaining fecal material. Excess fat and Peyer's patches were then removed from the intestines. Next, the intestines were cut open via a longitudinal incision, then horizontally sliced into several smaller pieces. Samples were incubated in HBSS supplemented with 10% FBS and 50 mM EDTA (Cellgro) for 30 min at 37 °C with shaking at 100 rpm, followed by gentle scraping of the epithelial layer to expose the LP. The processed samples were minced into small pieces and incubated in HBSS with 20% FBS, 2% collegenase D (Roche), 1.5% dispase II (Roche), and 20 μg/mL DNase I (Roche) for 1 h at 37 °C with shaking at 185 rpm.

After incubation, tissue and supernatant from both samples were passed over 100-μM and 40-μM cell strainers (Fisher) to filter out debris. The single cell suspensions were then washed twice with cold PBS (Fisher) and prepared for FACS labeling of anti-mouse antibodies to identify DC and CD4^{+} T-cell populations. Cells were typically pooled from 5–30 mice unless indicated otherwise.

Isolation of Cells from Lungs. Lungs from 5–10 mice were perfused with HBSS containing 2% FBS and 1 mM EDTA. Draining LN were removed, and lung tissue was excised and collected. Lung tissue was chopped into small pieces then placed in HBSS supplemented with 10% FBS, 1% collagenase D, and 20 μg/mL DNase I for 30 min at 37 °C with shaking at 125 rpm. After incubation, cells were resuspended in ACK lysis buffer (Fisher) at 25 °C for 2 min before washing with cold PBS. After two washes, lung single-cell suspensions were prepared for FACS labeling of anti-mouse antibodies to identify DC populations.

Isolation of Cells from Secondary Lymphoid Organs. Spleens and LN were harvested for isolation of DCs. Spleens were perfused with HBSS containing 10% FBS and 1% collagenase D and cut into pieces before incubation for 30 min at 37 °C with shaking at 100 rpm. After incubation, cells were resuspended in ACK lysis buffer at 25 °C for 2 min, followed by resuspension in cold PBS. LN were excised and teased apart with sterile needles, and then incubated with HBSS containing 5% FBS, 1% collagenase D, and 1% dispase II for 30 min at 37 °C with shaking at 125 rpm. Spleen and LN samples were then mashed through a 70-μM cell strainer mesh with the end of a sterile syringe. Cold PBS was used to rinse the mesh and obtain single-cell suspensions. To isolate CD4^{+} T cells, spleens and LN were processed in a similar manner, but did not undergo tissue digestion with collagenase D and dispase II. The single-cell suspensions were then washed twice with cold PBS and prepared for FACS labeling of anti-mouse antibodies to identify DC and CD4^{+} T-cell populations. Cells were typically pooled from 5–30 mice unless indicated otherwise.

Purification of DC and CD4^{+} T-Cell Populations. For sorting DC and CD4^{+} T-cell populations from gut, lungs, and lymphoid tissues, single-cell suspensions from these tissues were resuspended in cold PBS containing 2% FBS and incubated for 10 min. To isolate DCs, combinations of anti-mouse FITC-conjugated CD45, Pacific Blue- or PE-CY5–conjugated I-A^{b}, APC-conjugated CD11c, Pacific Blue-conjugated CD11b, and PE-conjugated CD103 antibodies (ebioscience) were used to label cells for 30 min at 4 °C. ALDH activity in DC samples was detected with the FITC-labeled ALDEFLUOR reagent system kit (STEMCELL Technologies) before immune labeling with antibodies.

Total CD4$^+$ T cells were isolated from colonic LP (cLP), mesenteric LN (mLN), and spleens by labeling single-cell suspensions at 4 °C for 30 min with anti-mouse PerCP-Cy5–conjugated CD3, FITC-conjugated CD45, and PE-CY7–conjugated CD4 antibodies (eBioscience). Naïve CD4$^+$ T cells from the spleens of OT-II mice were identified with anti-mouse FITC-conjugated CD4, PE-conjugated CD62L, and APC-conjugated CD44 antibodies (eBioscience). All DC and CD4$^+$ T-cell populations were then washed twice with cold PBS and filtered (40 μM) before sorting on a FACSAria cell sorter (BD Biosciences). The purity of sorted DC and CD4$^+$ T-cell subsets from various tissues was verified as 89–99%.

Analysis of cLP DC Cytokine Secretion. Sorted cLP DCs (CD103$^+$ CD11b$^+$ALDH$^-$) were cultured in complete RPMI 1640 medium supplemented with or without 400 ng/mL LPS (List Biological Laboratories) at 37 °C for 12 h. ELISA (eBioscience) or Bioplex assays (Bio-Rad) were performed to quantify IL-6, IL-1β, TNF-α, IL-12p70, IL-12p40, IL-23p19, and TGF-β1 cytokine secretion in sample supernatants.

Analysis of cLP DC Phenotype. Single-cell suspensions from cLP of mice were resuspended in cold PBS containing 2% FBS for 10 min. Cells were then incubated at 37 °C for 20 min with the FITC-labeled ALDEFLUOR reagent system kit (STEMCELL Technologies) to detect ALDH activity. Cells were washed twice with cold PBS, then labeled with anti-mouse APC-conjugated CD11c, PE-CY5–conjugated I-A^b, PE-conjugated CD103, Pacific Blue-conjugated CD11b, and PE-CY7–conjugated TLR4 antibodies (eBioscience) for 30 min at 4 °C to detect TLR4 expression in cLP DC subsets. To assess the maturation state of cLP DC subsets, anti-mouse PE-CY5–conjugated CD86 or CD40 antibodies were used with anti-mouse PE-CY7–conjugated CD11c, Pacific-Blue-conjugated I-A^b, APC-conjugated CD103, and PE-conjugated CD11b antibodies (eBioscience). Some samples were stained with control anti-mouse PE-conjugated IgG2a or PE-CY5–conjugated IgG2b antibodies (eBioscience). Cells were then washed and filtered, followed by acquisition on an LSR Fortessa flow cytometer (BD Biosciences) and analysis with Flowjo software (Tree Star).

Analysis of CD4$^+$ T Cells. For assessment of polyclonal CD4$^+$ T-cell cytokine secretion, sorted CD4$^+$ T cells from cLP and mLN of mice were cultured with purified anti-mouse CD3 and CD28 antibodies (BD Biosciences) for 3 d at 37 °C in complete DMEM medium (Life Technologies). Bioplex and ELISA analyses were performed to quantify cytokines in supernatants.

To determine the frequency of cytokine-secreting CD4$^+$ T cells, nonsorted single-cell suspensions from cLP and mLN of mice were activated for 4 h with complete DMEM medium supplemented with 50 ng/mL PMA and 1 mM ionomycin (Sigma-Aldrich). GolgiPlug (BD Biosciences) was added for the final 3 h. Cells were then labeled with Aqua Live/Dead stain (Life Technologies), followed by anti-mouse FITC-conjugated CD45 and PE-CY7–conjugated CD4 antibodies at 4 °C for 30 min. Stained cells were fixed in 2% paraformaldehyde and permeabilized in Cytoperm buffer (BD Biosciences) at 4 °C for 30 min before incubation with anti-mouse APC-conjugated IFN-γ and PE-conjugated IL-17A antibodies (eBioscience) for 30 min at 4 °C.

To assess CD4$^+$ Treg frequency, nonsorted single-cell suspensions from cLP and mLN of mice were labeled with Aqua Live/Dead stain, followed by anti-mouse FITC-conjugated CD45, PE-CY7–conjugated CD4 and APC-conjugated CD25 antibodies (eBioscience) at 4 °C for 30 min. Cells were then fixed and permeablized using a transcription factor staining buffer kit with anti-mouse PE-conjugated Foxp3 antibody (eBioscience) for 30 min at 4 °C. All flow cytometry data were acquired on a LSR Fortessa flow cytometer and analyzed with Flowjo software.

1. Lu M, Varley AW, Ohta S, Hardwick J, Munford RS (2008) Host inactivation of bacterial lipopolysaccharide prevents prolonged tolerance following gram-negative bacterial infection. *Cell Host Microbe* 4(3):293–302.
2. Lu M, et al. (2003) Stimulus-dependent deacylation of bacterial lipopolysaccharide by dendritic cells. *J Exp Med* 197(12):1745–1754.

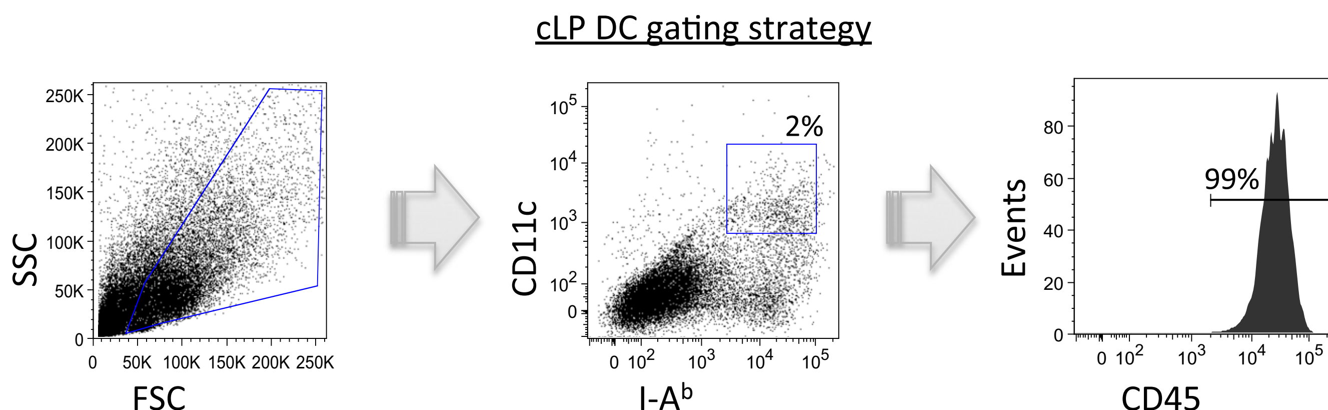

Fig. S1. Gating strategy for identifying and sorting cLP DCs. Flow cytometry analysis of DCs harvested from colonic tissues and sorted by CD45, CD11c, and I-A^b cell surface molecule expression. Data are representative of more than five independent experiments. FSC, forward scatter; SSC, side scatter.

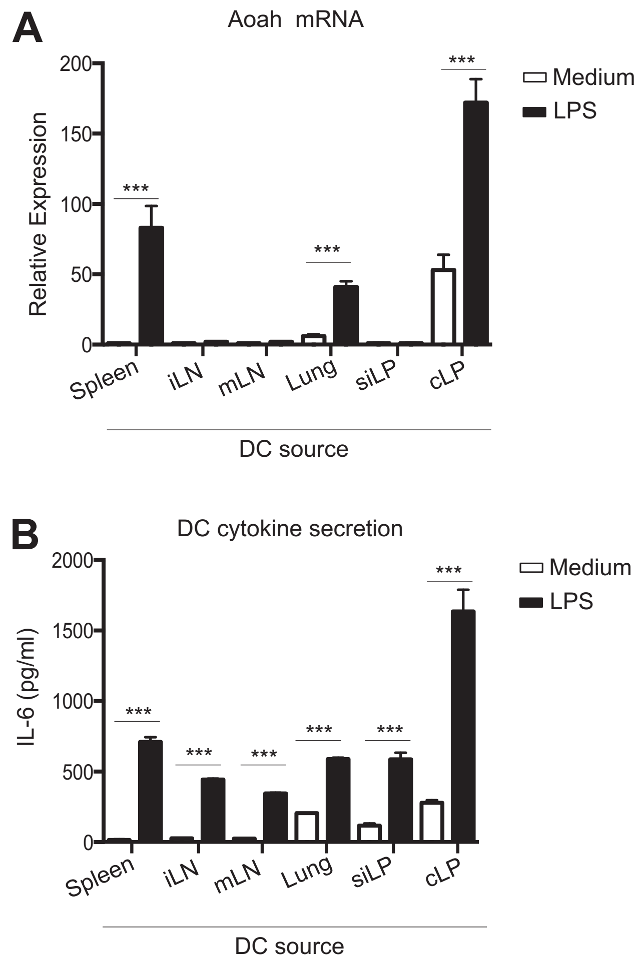

Fig. S2. LPS differentially regulates AOAH expression in DC populations. AOAH mRNA expression (*A*) and IL-6 cytokine secretion (*B*) from DCs isolated from spleen, inguinal LN (iLN), mLN, lungs, or cLP or LP from the small intestines (siLP) and then cultured overnight without (white bars) or with (dark bars) LPS (400 ng/mL). All sorted DC populations were viable $CD45^{+}CD11c^{+}I\text{-}A^{b+}$ cells. Data are mean ± SD of triplicate samples and are representative of three independent experiments.

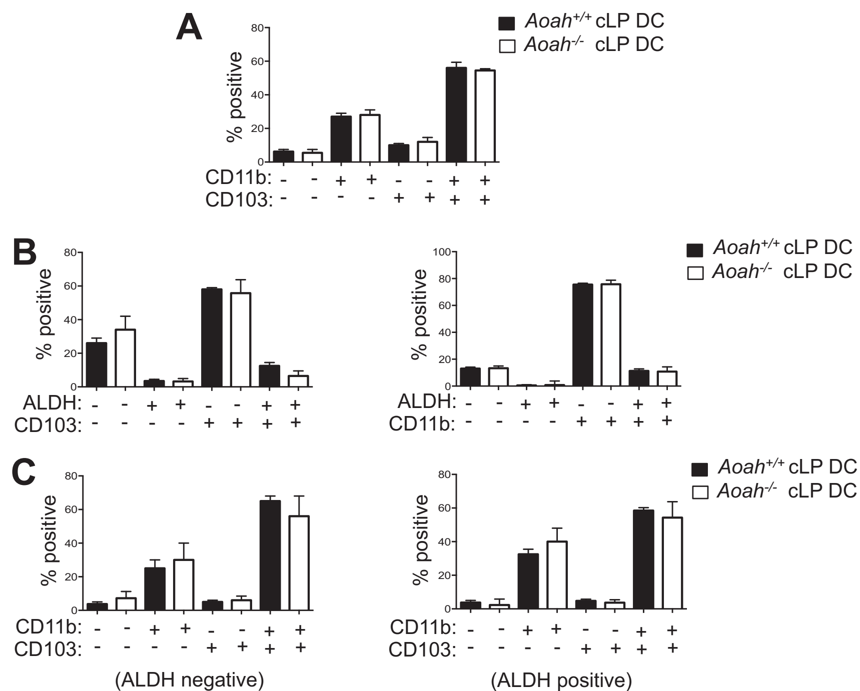

Fig. S3. AOAH deficiency does not alter the frequency of cLP DC subsets. Frequency of cLP DC subsets based on cell surface CD103 and CD11b expression (*A* and *C*) and ALDH activity (*B* and *C*) from $Aoah^{+/+}$ mice (dark bars) and $Aoah^{-/-}$ mice (white bars). All cLP DC subsets were gated on $CD45^{+}CD11c^{+}I\text{-}A^{b+}$ cells. Data are mean ± SD of samples from individual mice (*n* = 3) and are representative of three independent experiments.

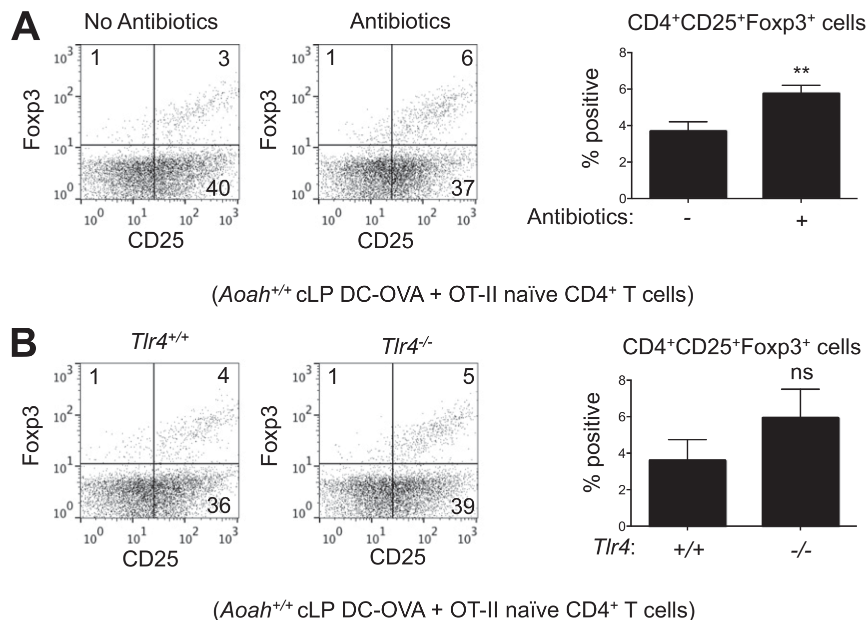

Fig. S4. Gram-negative microbiota suppress the ability of cLP DCs to induce Tregs. Flow cytometry analysis of viable $CD4^{+}$ T cells expressing cell surface CD25 and intracellular Foxp3 molecules from day 3 OT-II naïve $CD4^{+}$ T-cell cocultures with OVA-loaded DCs isolated from the cLP of mice receiving normal or antibiotic (neomycin and metronidazole)-treated water (*A*) or $Tlr4^{+/+}$ or $Tlr4^{-/-}$ mice (*B*). Data are shown as flow cytometry plots (*Left*) or mean ± SD of samples using cLP DCs from individual mice (*n* = 3, *Right*) and are representative of three independent experiments.

Fig. S5. cLP DC subsets differentially promote $CD4^+$ T-cell polarization. Supernatant cytokine (IFN-γ, IL-4, IL-5, and IL-13) levels from day 3 OT-II naïve $CD4^+$ T-cell cocultures with indicated $Aoah^{+/+}$ (dark bars) and $Aoah^{-/-}$ (white bars) cLP DC subsets loaded with OVA in the absence (*A*) or presence (*B*) of LPS (400 ng/mL). No exogenous cytokines were added to these cocultures. Data are mean ± SD of triplicate samples and are representative of three independent experiments.

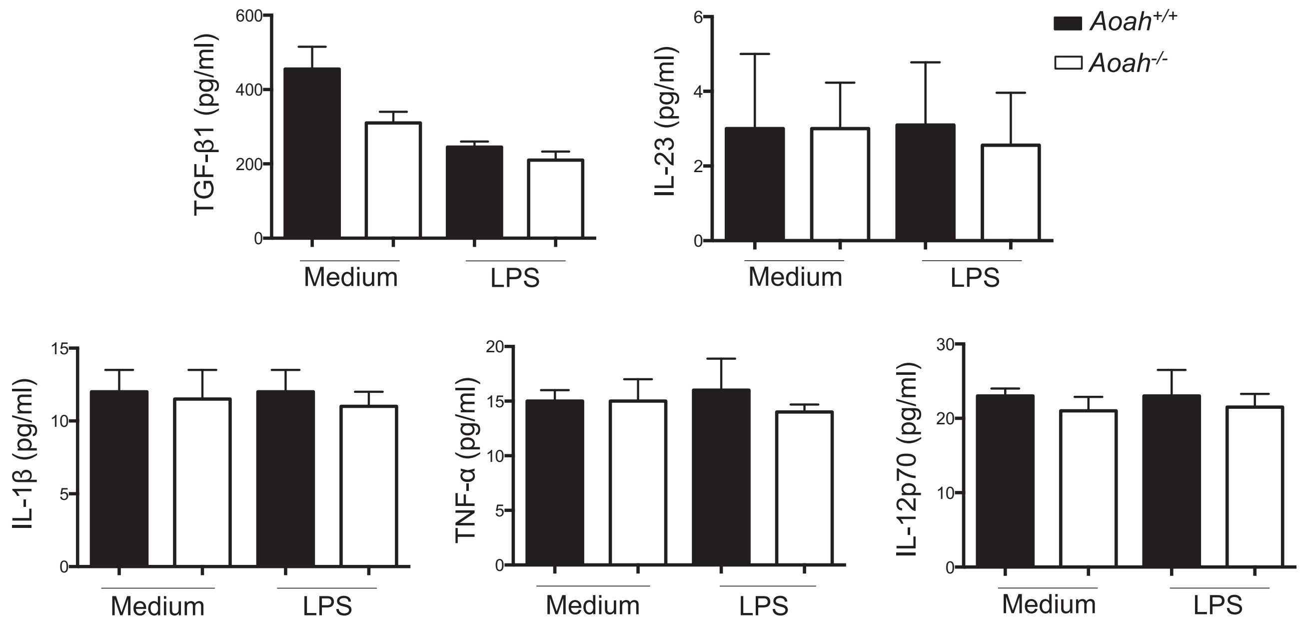

Fig. S6. AOAH deficiency does not alter cLP DC secretion of various Th17-promoting cytokines. Supernatant cytokine (TGF-β1, IL-23, IL-1β, TNF-α, and IL-12p70) secretion from cLP DCs derived from *Aoah*$^{+/+}$ mice (dark bars) and *Aoah*$^{-/-}$ mice (white bars) and cultured overnight in the absence or presence of LPS (400 ng/mL). Data are mean ± SD of triplicate samples and are representative of three independent experiments.

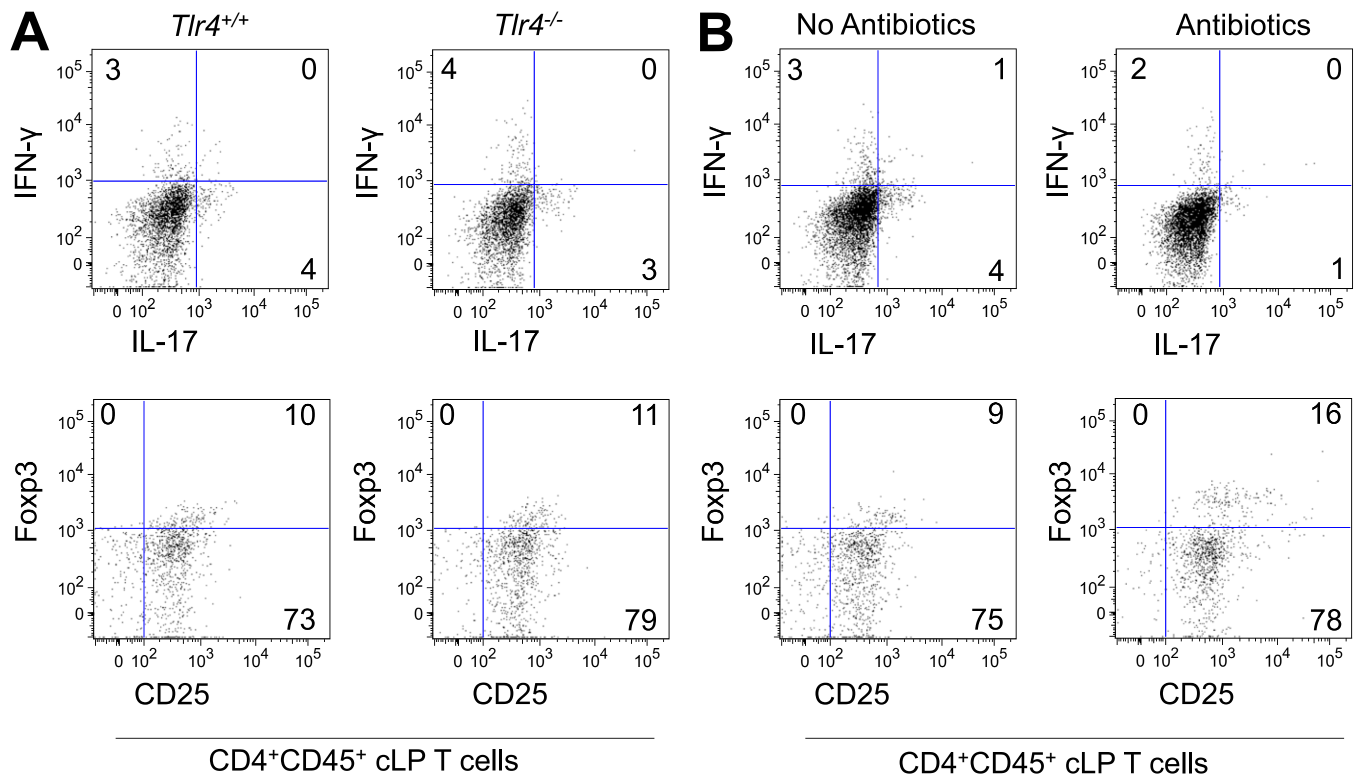

Fig. S7. Gram-negative microbiota modulate gut Th17 and Treg responses in vivo under homeostatic conditions. Flow cytometry analysis of PMA/ionomycin-activated cLP CD4^{+} T cells secreting IL-17 and IFN-γ (*Upper*) and nonstimulated CD4^{+} T cells expressing cell surface CD25 and intracellular Foxp3 molecules (*Lower*). Cells were isolated from *Tlr4*$^{+/+}$ or *Tlr4*$^{-/-}$ mice (*A*) or from *Aoah*$^{+/+}$ mice receiving normal or antibiotic (neomycin and metronidazole)-treated water (*B*) and gated on viable CD4^{+}CD45^{+} cLP cells. Data are displayed as flow cytometry plots and are representative of three independent experiments.

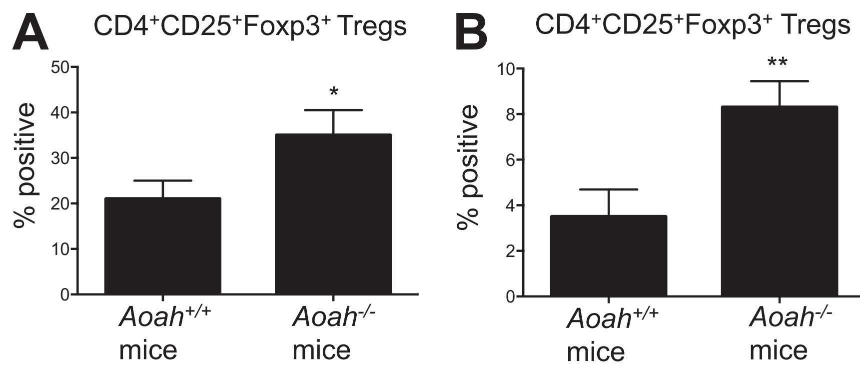

Fig. S8. AOAH deficiency enhances gut Treg responses in vivo under homeostatic conditions. Percentage of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs in unstimulated cells isolated from the cLP (*A*) and mLN (*B*) of *Aoah*$^{+/+}$ and *Aoah*$^{-/-}$ mice. Data are mean ± SD of samples from individual mice ($n = 3$) and are representative of three independent experiments.