Supporting Information

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SI Materials and Methods

Mice. P14 T-cell receptor (TCR) transgenic mice expressing the TCR specific for the lymphocytic choriomeningitis virus (LCMV) gp33-41 peptide were a gift from S. Kaech (Yale University, New Haven, CT). Age- and sex-matched C57BL/6 mice were purchased from The Jackson Laboratories (National Cancer Institute, Frederick, MD) and housed in an specific pathogen-free (SPF) facility. The generation of mice deficient in ASC and caspase-1 has been reported previously (1, 2). All KO mice have been backcrossed at least nine generations onto the C57BL/6 background. All procedures used in this study complied with federal guidelines and were approved by the Yale Animal Care and Use Committee.

Respiratory Tract Infection with *Legionella pneumophila*. *L. pneumophila* JR32 strain (3) (a gift from R. Medzhitov and C. Roy, Yale University) from frozen stock was grown on agar plates for 3 d at 37 °C in 5% CO_2. A single colony of *L. pneumophila* was grown on buffered charcoal yeast extract (BCYE) agar for 2 d. A day before mouse infection, *L. pneumophila* were harvested with sterile water and adjusted to a concentration of 10^9 cells/mL by measuring the optical density at 600 nm with a spectrophotometer. Desired dilutions of *L. pneumophila* JR32 was cultured in BYCE broth overnight. For in vivo infection, mice were infected intranasally with 1×10^6 cfu of *L. pneumophila* JR32 in 50 μL of PBS as previously described (4). CD4 and CD8 T cells were isolated from the mediastinal lymph node and the spleen of *L. pneumophila*-infected mice using anti-CD4 microbeads or anti-CD8 microbeads (Miltenyi Biotec). For restimulation of CD8 T cells, *L. pneumophila* infected bone marrow-derived dendritic cells (BMDCs) were used as antigen-presenting cells. BMDCs were plated at a density of 5×10^5 in 96-well flat bottom tissue culture dishes and infected at the indicated multiplicity of infection (MOI) with *L. pneumophila*. After addition of bacteria, plates were centrifuged at 150 *g* for 5 min to enhance cell contact with the bacteria. Plates were incubated for 4 h before extracellular bacteria were removed by washing wells three times with PBS. CD8 T cells (2×10^5) were cultured with the infected BMDCs for 48 h at 37 °C in culture medium [RPMI 1640 containing 10% FBS (Invitrogen), 50 μM 2-mercaptoethanol (Sigma-Aldrich), and 100 units/mL penicillin and streptomycin (Invitrogen)]. For restimulation of CD4 T cells, 2×10^5 CD4 T cells were cultured with BMDCs pulsed with the indicated amounts of heat-inactivated *L. pneumophila* (80 °C for 45 min) for 48 h at 37 °C in culture medium. IFN-γ production in supernatants was measured by ELISA.

Flow Cytometry. The single-cell suspensions of lung samples were prepared as previously described (5) and stained with anti-MHC class II, anti-CD11c, anti-CD44, anti-CD45.2, anti-CD4, anti-CD25, or anti-FoxP3 antibodies. Leukocytes were gated on the basis of forward and side scatter properties, and live cells were gated on the basis of 7-aminoactinomycin D exclusion. Acquisition of samples was performed on a cytometer (LSR II; BD Biosciences). For tetramer staining, cells were incubated with APC-labeled tetramer specific for H2D^b complexed with peptides from the viral acid polymerase (SSLENFRAYV) in 0.1 mL of 1% FBS in PBS for 30 min on ice. After washing, samples were resuspended in 1% paraformaldehyde in PBS. The frequency of tetramer positive CD8+ T cells in the lung was quantified by determining the total number of gated CD44+ and

CD8+ T cells. The final analysis and graphical output were performed using FlowJo software (Tree Star).

CD4 and CD8 T-Cell Responses. CD4+ T cells and CD8+ T cells were isolated from the spleen of mice infected with 10 or 100 pfu of A/PR8 or 10^6 pfu of HSV-2 at 14 d postinfection using anti-CD4 microbeads or anti-CD8 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. A total of 10^5 CD4+ T and CD8+ T cells were restimulated with various amounts of heat-inactivated influenza or HSV-2 virion-, influenza virus nucleoprotein (NP) peptide (ARSALILRGSVAHKSCLPACVYGP, I-A^b or ASNENMETM, H-2D^b)-, or HSV glycoprotein B (gB) peptide-pulsed antigen presenting cells (APCs) for 72 h at 37 °C, respectively, and cultured in RPMI 1640 containing 10% FBS (Invitrogen), 50 μM 2-mercaptoethanol (Sigma-Aldrich), and 100 units/mL penicillin and streptomycin (Invitrogen) in 96-well *U*-bottom plates (BD Biosciences). IFN-γ production in supernatants was measured by ELISA in triplicates.

Measurement of Virus Titers and Anti-PR8 Antibodies. Serum, nasal swab, and BAL were collected for measurement of virus titer and antibodies against A/PR8 virus from mice as described previously (5). The levels of IgG, IgA, and IgM antibodies against A/PR8 viruses were determined by ELISA as described previously (5). Endpoint titers were considered positive for dilutions with OD values that were twofold higher than the background level (nonimmune serum).

P14 CD8+ T-Cell Proliferation. Naïve P14 CD8+ T cells were isolated from the spleens of P14 transgenic mice using a CD8 T-cell isolation kit according to the manufacturer's instructions. Purified P14 CD8 T cells were labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) for 10 min at 37 °C and washed three times in cold PBS, and either 10^5 or 10^6 cells were injected i.v. into water-fed or antibiotic-treated C57BL/6 mice on the day before PR8-GP33 virus infection. Mediastinal lymph nodes (LNs) were collected at 5 d p.i. and single-cell suspensions were stained with anti–CD8α-APC and anti-CD45.1 Pacific Blue. CD8α+ CD45.1+ T cells were analyzed for proliferation by flow cytometry.

Immunization with ovalbumin (OVA) in Complete Freund's Adjuvant (CFA). Water-fed and antibiotic-treated mice were immunized in hind footpads with 50 μg per mouse of OVA (Sigma) emulsified in CFA (Sigma). CD4+ T cells and CD8+ T cells were isolated from popliteal LN at 7 d postimmunization, and IFN-γ production from CD4 T cells and CD8 T cells was measured by ELISA. Serum was collected at 14 d postimmunization and OVA-specific antibodies titers were measured by ELISA.

Bacterial Recovery and Identification. Stool specimens harvested directly from the mouse large intestine were weighed and homogenized in PBS to a concentration of 50 mg/mL, and 10 μL was aliquoted to nonselective 5% sheep's blood, chocolate, and CDC anaerobic agar plates (Remel). Aliquots were also spread to agar plates selective for both Gram-positive (colistin, nalidixic acid) and Gram-negative (MacConkey) bacteria. After incubation at 35 °C for 24–48 h, the bacterial colonies were counted and separated on the basis of morphology for additional analysis. Reported bacterial counts are from the blood agar plate. Growth on selective media did not identify bacteria that failed to grow on the nonselective blood and chocolate agar plates. The nasal specimens were harvested as described (6). Briefly, nasal washes

were collected from decapitated animals by back-flushing 200 µL of sterile PBS from the posterior nasopharyngeal opening and plated without dilution as above, except nasal washes were not plated to CDC anaerobic plates.

Bacteria were identified in the clinical microbiology laboratory by standard techniques. Briefly, bacteria from single colonies were classified by colony morphology, hemolysis on the blood plate, Gram stain, and phenotypic assays: Gram-positive cocci were identified as *Staphyloccus sp*, *Streptococcus sp*, or *Enterococcus sp* on the basis of catalase activity, a Staphaurex latex agglutination test (Remel), and pyrolidonyl arylamidase (PYR) activity (Remel). Gram-negative organisms were tested for oxidase and indole activity and the identification confirmed by the Vitek 2 (BioMeriux) clinical instrument using the Gram-negative identification card. Yeast were identified on the Vitek with the yeast identification card. All Vitek generated results were classified as excellent with a probability of correct identification at >99%. Gram-positive rods were examined for morphology, the presence of bacterial spores, catalase, and PYR activity. Any organisms that could not be definitively identified by phenotypic assays were identified by 16sRNA DNA sequence analysis as previously described (7). A 500-bp portion of the 16sRNA was amplified by PCR, purified (Qiagen), sequenced, and the sequence compared by Blast analysis to known bacterial sequences (8). This was necessary for two organisms that were identified as *Enterococcus sp*, and *Bacillus sp*. A *Lactobaccilus* isolate was also sequenced to confirm the validity of the phenotypic analyses.

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Isolation of Mediastinal Lymph Node DCs and Restimulation of Naïve p14 CD8 T Cells. Water-fed and antibiotic-treated mice were infected intranasally with 1,000 pfu of PR8-GP33 influenza virus. Three days later, $CD11c^+$ DCs were isolated from the mediastinal LN of PR8-GP33 influenza virus-infected mice using anti-CD11c microbeads (Miltenyi Biotec). Naïve p14 CD8 T cells were isolated from single-cell suspension of spleen from the p14 TCR transgenic mice using a CD8 T-cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. A total of 2×10^5 naïve p14 CD8 T cells were restimulated with 1×10^4, 5×10^4, or 1×10^5 DCs for 72 h at 37 °C. A total of 1×10^5 splenic $CD11c^+$ DCs isolated from mice infected with PR8-GP33 influenza virus were cocultured with 2×10^5 naïve p14 CD8 T cells as a negative control. IFN-γ production in supernatants was measured by ELISA in triplicates.

In Vivo Depletion of Treg Cells. Water-fed and antibiotic-treated mice were injected i.p. with 0.2 mg of anti-CD25 (clone PC61) or corresponding isotype control antibody (purified rat IgG1) 1 and 3 d before influenza virus infection. Twenty-four hours later, spleen, lung, mediastinal LN, and Peyer's patch were isolated and the numbers of $CD4^+CD25^+Foxp3^+$ cells were determined by flow cytometry.
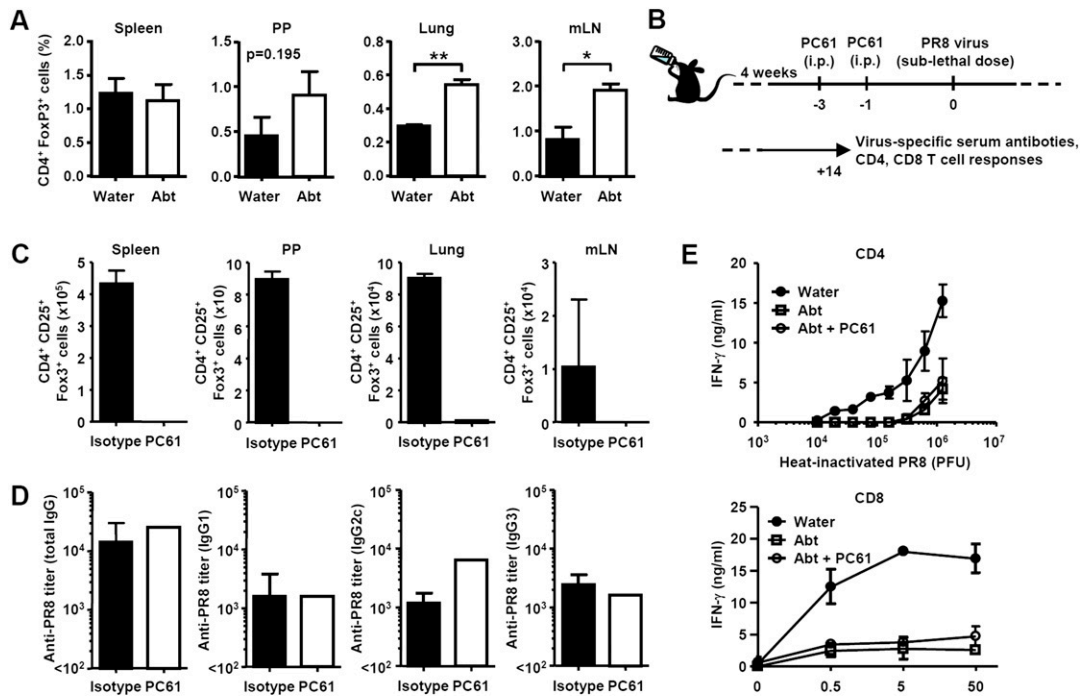
In Vivo Staining of Respiratory DCs with CFSE. CFSE was dissolved at 25 mM in DMSO and subsequently diluted to 8 mM in PBS. CFSE (50 µL) was administered intranasally to each mouse after anesthesia as previously described (9).

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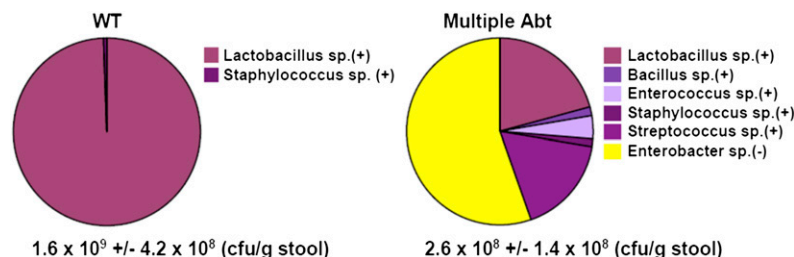
Fig. S1. Effect of four antibiotic treatments on bacterial colonization. C57BL/6 mice were given four combinatorial antibiotics in drinking water for 4 wk. Bacterial load and compositions in the stool from water-fed and antibiotic-treated mice are depicted. Purple and yellow tones denote Gram-positive and Gram-negative bacteria, respectively.

Fig. S2. Antibiotic-treatment does not impair adaptive immune responses against immunization or respiratory HSV-2 or *L. pneumophila* infections. Mice were given antibiotics in drinking water for 4 wk before immunization with OVA in CFA (*A* and *B*) or intranasal inoculation with 10^6 pfu of TK-HSV-2 (*C* and *D*) or 10^6 cfu of *L. pneumophila* JR32 (*C* and *D*). Two wk later, serum was collected and OVA-specific antibody titers were measured (*A*), T-cells were isolated from popliteal LN and restimulated with OVA peptide for 72 h, and IFN-γ production from CD4 T cells and CD8 T cells (*B*) was measured. CD4 and CD8 T cells were isolated from spleen at 14 d postinfection and cocultured with APCs with indicated amount of heat-inactivated HSV-2 Ag (*C*), gB peptide (*D*), or *L. pneumophila* (*E* and *F*) for 72 h. IFN-γ levels were measured by ELISA.

Fig. S3. Schematic representation of possible mechanisms by which commensal bacteria support antiviral responses.

Fig. S4. Treg depletion fails to restore immune responses to influenza virus in antibiotic-treated mice. C57BL/6 mice were given antibiotics in drinking water or regular water for 4 wk. (*A*) Percentages of CD4+Foxp3+cells from spleen, Peyer's patch, lung, and mediastinal LN (mLN) are shown. (*B*) Antibiotic-treated mice were injected with 200 μg of either PC61 monoclonal Ab against CD25 or purified rat IgG1 Ab twice at days -3 and -1 and infected intranasally with 10 pfu of PR8 influenza viruses. Two weeks later, immune responses were assessed. (*C*) On day 0, numbers of CD4+CD25+Foxp3+cells in organs were determined. Serum antibody titers against influenza virus (*D*) and T-cell responses to flu virion or NP peptides (*E*) were measured. Data represent the mean ± SD and are representative of at least three independent experiments. *P < 0.05; **P < 0.01.
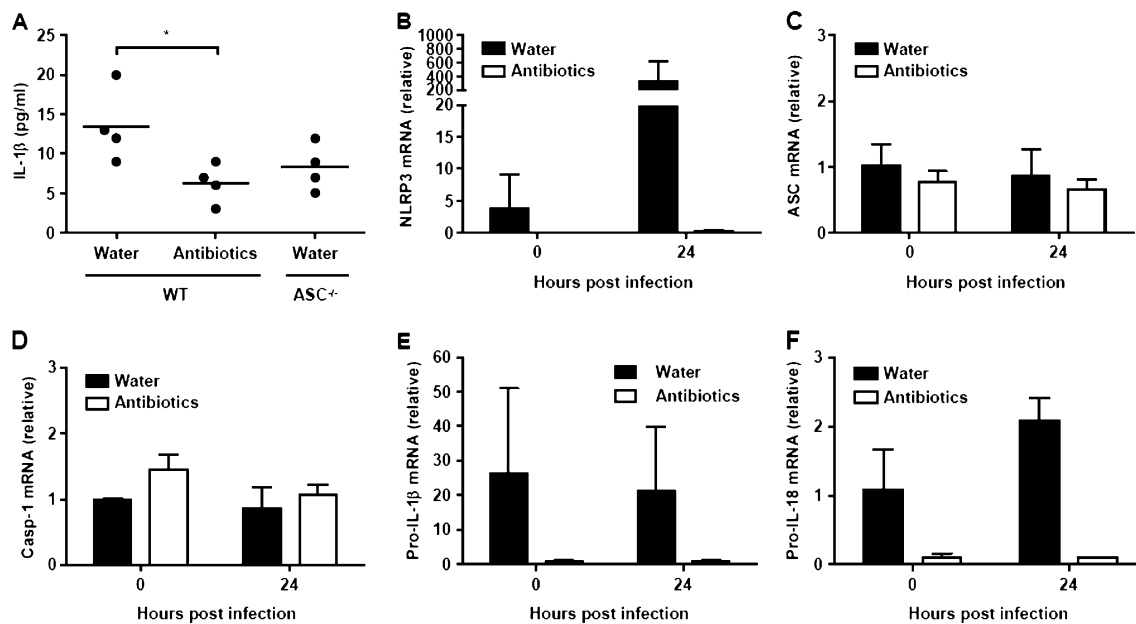
Fig. S5. Commensal microbiota provides signals leading to constitutive expression of pro–IL-1β, pro–IL-18 and NLRP3. C57BL/6 mice were given antibiotics in drinking water for 4 wk before intranasal infection with 1,000 pfu of PR8 influenza viruses. (*A*) The BAL was collected from water-fed, antibiotic-treated and ASC-deficient mice at 2 d postinfection. IL-1β levels in BAL were determined by ELISA. (*B–F*) Total RNAs were extracted from the lung of water-fed and antibiotic-treated mice at 0 and 24 h postinfection. mRNA levels of NLRP3 (*B*), ASC (*C*), caspase-1 (*D*), pro–IL-1β (*E*), and pro–IL-18 (*F*) were assessed by quantitative RT-PCR. GAPDH was used as an internal control. *P < 0.05. These data are representative of three repeated experiments.
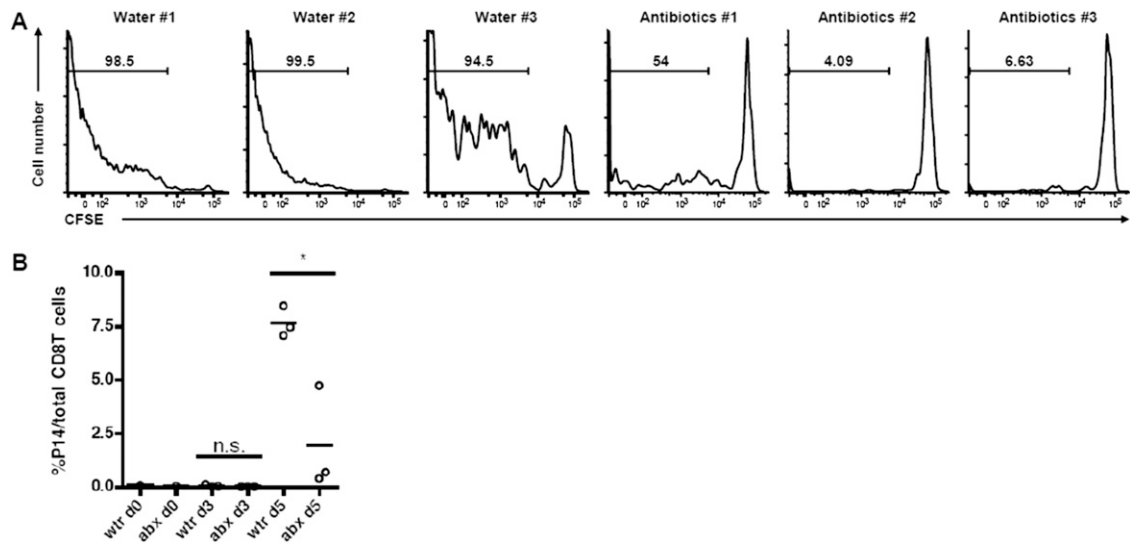
Fig. S6. Naïve CD8 T cells fail to proliferate in mLNs of antibiotic-treated mice after viral challenge. C57BL/6 mice were given antibiotics in drinking water for 4 wk before intranasal infection with 10 (*A*) or 100 (*B*) pfu of PR8-GP33 viruses. Water-fed or antibiotic-treated mice were injected with CFSE-labeled P14 tg CD8 T cells 1 d before the intranasal infection of PR8-GP33 virus. Five days (*A*) or indicated times (*B*) after influenza infection, mLNs were taken and T-cell proliferation was assessed by CFSE dilution.