Pulmonary Th17 Antifungal Immunity Is Regulated by the Gut Microbiome

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Commensal microbiota are critical for the development of local immune responses. In this article, we show that gut microbiota can regulate CD4+ T cell polarization during pulmonary fungal infections. Vancomycin drinking water significantly decreased lung Th17 cell numbers during acute infection, demonstrating that Gram-positive commensals contribute to systemic inflammation. We next tested a role for RegIIIγ, an IL-22-inducible antimicrobial protein with specificity for Gram-positive bacteria. Following infection, increased accumulation of Th17 cells in the lungs of RegIIIγ−/− and IL22−/− mice was associated with intestinal segmented filamentous bacteria (SFB) colonization. Although gastrointestinal delivery of rRegIIIγ decreased lung inflammatory gene expression and protected IL22−/− mice from weight loss during infection, it had no direct effect on SFB colonization, fungal clearance, or lung Th17 immunity. We further show that vancomycin only decreased lung IL-17 production in mice colonized with SFB. To determine the link between gut microbiota and lung immunity, serum-transfer experiments revealed that IL-1R ligands increase the accumulation of lung Th17 cells. These data suggest that intestinal microbiota, including SFB, can regulate pulmonary adaptive immune responses. The Journal of Immunology, 2016, 197: 97–107.
T cell cytokine production toward *A. fumigatus* has substantial therapeutic value in invasive disease, as well as fungal allergy. Commensal bacteria residing in the intestinal lumen have considerable effects on host defense. Studies in germ-free (GF) mice revealed that commensals protect against pathogenic infections through competitive growth inhibition or stimulation of innate immune receptors (7). Colonization of the gut is critical for the maturation of lymphoid tissues, such as Peyer’s patches, facilitating interactions between B cells and T cells (8). Although helpful for controlling infections, activated T cells in the gut also have the potential to drive colitis (9, 10). These studies demonstrated that microbial products, as well as T cell–intrinsic properties, influence intestinal inflammation. Microbiota also affect immune responses in peripheral tissues (11) and contribute to systemic antimicrobial activity (12–14). Loss of microbial diversity was also implicated in allergic inflammation (15). Altogether, this suggests that host factors that modulate microbial composition in the gut may also regulate peripheral T cell activation during infectious challenges.

The intestinal mucosa provides a physical barrier against microbes, maintained by the generation of a mucus layer, anti-microbial proteins, and IgA (16). Paneth cells shape the composition of intestinal microbiota by producing antimicrobial peptides. For instance, α-defensin 5 increases the ratio of Bacteroidetes/Firmicutes in the distal small intestine (17). RegIIIγ is produced by Paneth cells in response to IL-22 and has bactericidal activity against Gram-positive organisms (18, 19). RegIIIγ contributes to maintaining the spatial separation between microbiota and the intestinal cell wall (20). In the absence of RegIIIγ, the microbiota is enriched for segmented filamentous bacteria (SFB), which are sufficient to induce Th17 cells (21, 22). Although RegIIIγ protects against Gram-positive pathogens in the gut (19, 23), its impact on pulmonary immunity to fungal pathogens is unknown.

In this study, we investigated a role for commensal bacteria in lung immunity to *A. fumigatus*. We found that vancomycin-sensitive bacteria amplify lung IL-17 production early following fungal infection. Endogenous expression of RegIIIγ or Il22 was linked to decreased intestinal SFB colonization, leading to decreased IL-17 production in the lungs and small intestine. The phenotype of expanded CD4+ IL-17+ cells in IL-1RA blunted the IL-17 response, demonstrating that commensals induce IL-1R ligands that contribute to lung IL-17 production. Finally, reconstituting Il22−/− mice with local RegIIIγ protected them from weight loss during *A. fumigatus* infection and reduced inflammatory cytokines in lung tissue. Thus, factors that shape the balance of intestinal commensal species regulate lung inflammatory responses, including Th17 cell priming.

### Materials and Methods

**Mice**

C57BL/6 mice (B6) from The Jackson Laboratory (Bar Harbor, ME; Figs. 2, 3, 5D) or Taconic Farms (Germantown, NY or Cambridge City, IN; Figs. 1, 4, 5A–C, 5E, 6) were used. OT-II mice were purchased from The Jackson Laboratory. Il22−/− mice (24) were bred at Taconic Farms (Cambridge City, IN). Mice with a targeted deletion for all six exons of RegIIIγ were generated as described below and backcrossed to B6 mice for 10 generations. Mice were housed at the Louisiana State University Health Sciences Center and the University of Pittsburgh under specific pathogen–free conditions and were handled in accordance with National Institutes of Health federal guidelines.

**Generation of RegIIIγ−/− mice**

Regions from the mouse RegIIIγ locus were amplified from genomic DNA by PCR and cloned into the pDelboy 3X targeting vector. The fragments included a 3.5-kb 5′ flanking region ending at the RegIIIγ transcription start site, a 2.5-kb fragment cloned between two loxP sites and containing all six exons of the RegIIIγ gene, and a 3.4-kb 3′ flanking region. The targeting vector also contains a neomycin resistance gene, a thymidine kinase gene, and a ß-gal gene. Cre-mediated recombination between the loxP sites results in deletion of all coding portions of the RegIIIγ gene and brings the β-gal gene immediately downstream of the RegIIIγ 5′ promoter.

The vector was linearized and electroporated into embryonic stem cells, and clones underwent positive and negative selection with G418 and ganciclovir. Successfully targeted clones were analyzed by PCR and Southern blot, and three successfully targeted clones were identified. One clone was microinjected into blastocysts using standard techniques, and the resulting chimeric mice were bred to generate heterozygous offspring. Male RegIIIγ−/− heterozygous were crossed with Tie2 Cre females to generate RegIIIγ−/− offspring. These were interbred to generate RegIIIγ−/− offspring and were also backcrossed onto the B6 background for 10 generations.

By quantitative PCR, RegIIIγ−/− mice showed a mean 240-fold lower expression of RegIIIγ mRNA in the trachea compared with control mice. Because these mice have no detectable RegIIIγ coding region, we believe that the residual RegIIIγ signal is from transcripts of other highly related Reg family genes, including RegIIIβ.

**Infections and treatments**

Mice were infected with *A. fumigatus* via oropharyngeal (o.p.) aspiration (107 organisms). For some experiments (Figs. 1, 4B–E, 5A–D, 7), neutrophils were depleted by i.p. injection of Ab IA8 (0.6 mg; Bio X Cell, West Lebanon, NH) 1 d prior to infection. Serum was given i.v. (0.25 ml) in the presence of anakinra (Kineta; Swedish Orphan Biovitrum; Stockholm, Sweden) or vehicle 7 h following infection. Vancomycin was administered in drinking water at 0.5 g/l for ≥ 4 wk and then mice were placed on normal water 1 d prior to infection. Il22−/− mice were gavaged with rRegIIIγ protein (40 µg) (25) or vehicle every other day, beginning on day −9 prior to infection, for a total of six treatments. For fecal-transplant experiments, stool pellets were homogenized in water and poured over a 100-µm cell strainer, and recipient mice were gavaged with 0.4 ml fecal matter.

To examine pulmonary Th17 cell priming, TCR-transgenic OT-II T cells were negatively selected using a CD4 T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), and 0.5 million cells were adoptively transferred into mice via retro-orbital injection. The next day, mice were inoculated via o.p. aspiration with OVA (100 µg; Sigma-Aldrich, St. Louis, MO) plus cholera toxin (1 µg; List Biological Laboratories, Campbell, CA). Lungs were collected on day 7. To examine intestinal Th17 cell priming, mice were infected i.p. with OVA (1 mg) at time 0 and *Escherichia coli*-derived LPS (50–60 µg; Sigma-Aldrich) 18 h later. Small intestinal lamina propria (silP) lymphocytes were isolated 3 wk later.

**Tissue processing**

To analyze gene expression, lungs and distal small intestine were placed in TRIzol reagent (Life Technologies, Carlsbad, CA), homogenized, and processed according to the manufacturer’s protocol. One microgram of RNA was used to synthesize cDNA (Script; Bio-Rad, Hercules, CA). Real-time PCR primers were purchased from Applied Biosystems (Foster City, CA) and used with TaqMan Universal PCR Master Mix (Applied Biosystems) or iSoFast Probes Supermix (Bio-Rad). PCR reactions were run on a Bio-Rad CFX96 Real-Time System.

For ex vivo cytokine production, tissues were placed in cell lysis buffer (Cell Signaling Technology, Danvers, MA) with protease inhibitor mixture tablets (Roche Applied Science, Penzberg, Germany), homogenized, and centrifuged at 10,000 × g. Cytokine levels in supernatants were measured by ELISA (eBioscience, San Diego, CA; BioLegend, San Diego, CA) or MilliPlex Mouse Cytokine/Chemokine Immunoassay (EMD Millipore, Billerica, MA) and normalized to total protein levels (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific, Waltham, MA).

For flow cytometry, lungs were digested with collagenase D (3 mg/ml; Roche Applied Science) in DMEM for 1 h at 37°C, crushed through 100-µm cell strainers (BioExpress, Kayville, UT), and treated with ammonium chloride to lyse RBCs. silP lymphocytes were isolated as previously described (26), with some modifications. Briefly, small intestinal tissue was flushed, sliced open longitudinally, cut into ~1-cm pieces, and washed with Ca/Mg-free balanced salt solution (BSS). Tissue was stirred at 37°C in Ca/Mg-free BSS containing 5 mM EDTA and 0.15 mg/ml dithioerythritol, with the supernatant removed. Then, tissue was incubated at 37°C in BSS containing 1 mM CaCl2, 1 mM MgCl2, and 1 mg/ml collagenase (Roche Applied Science). Cells were fractionated on a 44% and 67% Percoll gradient (Amersham Biosciences, Piscataway, NJ), with lymphocytes partitioning at the interface. Single-cell suspensions were incubated in IMDM...
with 10% FBS, PMA (50 ng/ml; Sigma-Aldrich), ionomycin (750 ng/ml; Sigma-Aldrich), and GolgiPlug (Becton Dickinson Pharmingen, Franklin Lakes, NJ) for 4 h at 37°C. Cells were stained with Abs directed against CD3e, CD4, CD8, TCR Vα2, TCR Vγ, IL-17A, IL-22, or IFN-γ (eBioscience and BD Pharmingen).

For in vitro cytokine production, splenic dendritic cells (DCs) were purified using CD11c MicroBeads (Miltenyi Biotec). Cells were stimulated overnight with heat-killed swollen A. fumigatus (10:1 organisms/DC), LPS (50 μg/ml; Sigma-Aldrich), or zymosan (100 μg/ml; InvivoGen). Cytokine levels were determined by an Milliplex Mouse Cytokine/Chemokine Immunoassay (EMD Millipore).

Microbiota analysis

Real-time PCR. DNA from small intestinal stool or cecum was isolated using a QIAamp DNA Stool Mini Kit (QIAGEN, Venlo, the Netherlands), and 90–180 ng was used as a template for PCR. In some experiments, cDNA from distal small intestine was used as a template. Primers for Eubacteria, SFB (27), Bacteroides, Clostridium leptum (28), and Rumino-coccaceae were purchased from Integrated DNA Technologies (Coralville, IA). Samples were diluted with RNase/DNase-free water and mixed with iQ SYBR Green Supermix (Bio-Rad). PCRs were performed on a CFX96 Real-Time System (Bio-Rad). Relative gene expression for bacteria species (SFB, Bacteroides, C. leptum) was normalized to Eubacteria.

16S next-generation sequencing. DNA from cecal contents was prepared for sequencing as previously described (29). Briefly, DNA was amplified by PCR using 515F and 806R bacterial 16S PCR primers containing sample-specific barcodes and sequence adaptors. PCR products were pooled in equimolar amounts and sequenced on an Illumina MiSeq using a 300 cycle V2 sequencing kit. Sequences were processed in the QIIME pipeline (30). Operational taxonomic unit (OTU) threshold of 97%, and analyzed for taxonomic and α diversity. Data are available on the Metagenomics RAST server under accession number 4541676.3 (http://metagenomics.anl.gov/metageneomics.cgi?page=MetageneOverview&metagene=4541676.3).

Histopathology

B6-Taconic mice on normal water or vancomycin water and IL22−/− mice were injected i.p. with a neutrophil-depleting Ab (1A8; 0.6 mg) 1 d prior to infection. A. fumigatus suspensions were stimulated with PMA plus ionomycin and analyzed by flow cytometry. Approximatively 1% of total CD4+ T cells in WT mice produced IL-17A (Fig. 2B). In RegIIIγ−/− mice, ~2% of total T cells produced IL-17, indicating that SFB colonization in RegIIIγ−/− mice was linked to increased pulmonary Th17 cell priming and/or maintenance (Fig. 2B). This trend was consistent when analyzing the individual CD4+, CD8+, and TCR γδ+ T cell subsets. Thus, lung-resident T cells from infected RegIIIγ−/− mice had a 2-fold increase in IL-17 potential compared with T cells isolated from WT mice.

Microbiota in RegIIIγ-deficient mice regulate pulmonary Th17 cell priming

To test whether intestinal Gram-positive bacteria drive pulmonary Th17 cell priming in RegIIIγ−/− mice, they were placed on vancomycin drinking water for ≥4 wk. In one of two experiments, vancomycin was combined with ampicillin, metronidazole, and neomycin, whereas control mice were maintained on normal water. Following antibiotic treatment, mice were adoptively transferred with TCR-transgenic OT-II CD4+ T cells. One day later, mice were immunized with OVA plus the mucosal adjuvant cholera toxin (33) via o.p. aspiration. On day 7, lung single-cell suspensions were stimulated with PMA plus ionomycin and analyzed for IL-17 production. The total number of OT-II CD4+ T cells recovered from the lungs of RegIIIγ−/− mice on normal water was 1.8-fold higher compared with WT Jackson mice (Fig. 2C). Vancomycin drinking water significantly reduced lung T cell accumulation in RegIIIγ−/− mice to similar numbers as WT

Endogenous RegIIIγ suppresses lung IL-17 production during fungal infection

The bactericidal activity of RegIIIγ is attributed to its binding of cell wall peptidoglycan on Gram-positive bacteria (18). Because oral vancomycin treatment potently decreased IL-17 and IL-22 production in lung tissue (Fig. 1A), and RegIIIγ−/− mice were reported to have increased colonization with SFB (20), we hypothesized that the endogenous antimicrobial factor RegIIIγ may similarly regulate pulmonary Th17 cytokines. Of note, our line of RegIIIγ−/− mice was colonized with SFB, as previously reported for an independent line (20). To test our hypothesis, SFB+ RegIIIγ−/− and B6 Jackson mice that lacked SFB were infected with A. fumigatus via o.p. aspiration. Lungs were harvested 2 d later and analyzed for gene expression by real-time PCR. Pulmonary fungal burden was similar between WT and RegIIIγ−/− mice, suggesting that RegIIIγ was dispensable for antifungal host defense (Fig. 2, A and B). However, expression of the Th17-related genes Il17a, Il17f, and Il22 was elevated in the lungs of RegIIIγ−/− mice, indicating more activated T cells. To determine which T cell subsets contribute to IL-17 production, lymphocytes were isolated from lungs 1 mo following infection, restimulated in vitro with PMA plus ionomycin, and analyzed by flow cytometry. Approximately 1% of total CD3+ T cells in WT mice produced IL-17A (Fig. 2B). In RegIIIγ−/− mice, ~2% of total T cells produced IL-17, indicating that SFB colonization in RegIIIγ−/− mice was linked to increased pulmonary Th17 cell priming and/or maintenance (Fig. 2B). This trend was consistent when analyzing the individual CD4+, CD8+, and TCR γδ+ T cell subsets. Thus, lung-resident T cells from infected RegIIIγ−/− mice had a 2-fold increase in IL-17 potential compared with T cells isolated from WT mice.

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mice, demonstrating that the gut microbiota in RegIII\textsuperscript{g}ε/ε mice contributes to pulmonary Th17 cell accumulation. In contrast, vancomycin did not significantly affect Th17 cells in WT mice from The Jackson Laboratory that were not colonized with SFB, suggesting that their microbiota did not influence Th17 cell accumulation in the lungs (Fig. 2D). These data demonstrate that gut microbiota in RegIII\textsuperscript{g}ε/ε mice inhibits pulmonary Th17 cell priming in a T cell–extrinsic manner.

To further test the link between SFB colonization and pulmonary cytokines, we purchased SFB\textsuperscript{−/−} RegIII\textsuperscript{g}ε/ε mice from The Jackson Laboratory. Following neutrophil depletion and infection, lung IL-17A, IL-22, IFN-γ, and IL-10 were comparable to B6 Jackson mice (Supplemental Fig. 2, top panel). Fungal burdens in RegIII\textsuperscript{g}ε/ε Jackson mice were elevated slightly compared with B6 Jackson mice, but the difference was not statistically different (Supplemental Fig. 2, middle panel). Thus, elevated IL-17 production in RegIII\textsuperscript{g}ε/ε mice (Fig. 2) was linked to SFB colonization.

RegIII\textsuperscript{g} suppresses intestinal Th17 cell priming

Because RegIII\textsuperscript{g} is highly expressed by intestinal epithelium (18), we next determined its effect on intestinal Th cell priming to the model Ag OV\textsubscript{A}. Mice were immunized i.p. with OV\textsubscript{A} plus LPS. Three weeks later, lymphocytes from siLP were restimulated in vitro with PMA plus ionomycin. The total numbers of CD4\textsuperscript{+} T cells isolated from siLP were 8-fold higher in RegIII\textsuperscript{g}ε/ε mice compared with WT Jackson mice (Fig. 3B), suggesting that endogenous RegIII\textsuperscript{g} suppresses T cell priming in the intestinal mucosa. This was accompanied by increased total numbers of CD4\textsuperscript{+} T cells producing the effector cytokine IL-17, IL-22, or IFN-γ. Notably, immunized RegIII\textsuperscript{g}ε/ε mice had 32-fold more Th17 cells than WT mice. As noted above, RegIII\textsuperscript{g} limits colonization with SFB by...
maintaining the spatial separation between commensal microbes and mucosal epithelia (20). Our RegIIIγ−/− mice were also colonized with SFB prior to (data not shown) and after (Fig. 3C) immunization, correlating with increased percentages of CD4+ T cells producing IL-17 (Fig. 3A). Unimmunized RegIIIγ−/− mice contained 2.5- and 3-fold more Th17 cells in the siLP and spleen, respectively, compared with unimmunized WT mice (data not shown). Altogether, these data demonstrate an association between SFB colonization in RegIIIγ−/− mice and preferential induction of the Th17 pathway in mucosal tissues.

Endogenous IL-22 expression suppresses lung IL-17 levels during fungal infection

RegIIIβ and RegIIIγ expression in intestinal epithelium is regulated by IL-22 during gastrointestinal infections (19). In the steady-state, we observed that some naive Il22−/− mice expressed lower levels of RegIII family members in the distal small intestine compared with WT Taconic controls, although this was not true for all mice examined. However, on a consistent basis, we found that Il22−/− mice had increased levels of cecal SFB colonization compared with WT Taconic mice (Fig. 4A). If SFB influences pulmonary IL-17 responses, we reasoned that SFB-colonized Il22−/− mice should have a similar phenotype as the RegIIIγ−/− strain (Figs. 2, 3). To test this, WT-Taconic and Il22−/− mice were infected with A. fumigatus via o.p. aspiration. One day prior to infection, neutrophils were depleted by i.p. injection of Ab 1A8. To determine whether any potential differences between the mouse strains were influenced by gut microbiota, some mice were placed on vancomycin-drinking water for 1 mo prior to the experiment. On day 2, Il22−/− mice had a 1.5-fold increase in lung IL-17 compared with WT Taconic mice (Fig. 4B), consistent with the results observed in RegIIIγ−/− mice colonized with SFB (Fig. 2). Vancomycin drinking water decreased IL-17 levels by 25% in Il22−/− mice, suggesting that IL-22-mediated alterations in intestinal flora regulate pulmonary IL-17 production. In support, vancomycin water significantly decreased SFB colonization in Il22−/− mice (Fig. 4E). In B6 control mice, vancomycin decreased lung IL-17 levels in only one of two experiments (Fig. 4B). Further analysis revealed that B6 Taconic mice were SFB− in one of the experiments. Thus, vancomycin only decreased lung IL-17 levels in SFB-colonized mice. Levels of C. leptum and Ruminococcaceae were similar between WT and Il22−/− mice and were decreased by vancomycin. This is consistent with a study demonstrating that several Clostridium spp., including Ruminococcus, are sensitive to vancomycin (34). Therefore, lung IL-17 levels correlated with intestinal SFB colonization. When analyzing cytokines in the small intestine, Il22−/− mice on normal water had a dramatic 3000-fold increase in Il17a expression compared with WT controls, which was reduced by 99.8% upon pretreatment with vancomycin drinking water (Fig. 4C). There was also a 3.7-fold increase in Il17a expression in Il22−/− mice, which was decreased by vancomycin. Expression of Il4 and Foxp3 was similar between WT Taconic and Il22−/− mice in the intestine and lung (Fig. 4C, 4D), suggesting that endogenous IL-22 did not affect Th2 or Treg differentiation. Lung Il17a expression correlated with Il10a in the lungs and small intestine of Il22−/− mice (Fig. 4C, 4D). Therefore, the absence of IL-22 creates a permissive environment for SFB growth, augmenting mucosal IL-17 production in the gut and lung.

To examine the pulmonary T cell subsets affected by microbiota, B6 Taconic mice on vancomycin water and normal water were infected with A. fumigatus via o.p. aspiration. Two days later, lung single-cell suspensions were stimulated with PMA plus ionomycin and stained for intracellular IL-17. Neutrophils were depleted by IFN-γ staining, gated on CD4 T cells. Vancomycin significantly decreased the numbers of γδ T cells producing IL-17 in only one of two experiments (Fig. 4B). Further analysis revealed that B6 Taconic mice were SFB− in one of the experiments. Thus, vancomycin only decreased lung IL-17 levels in SFB-colonized mice. Levels of C. leptum and Ruminococcaceae were similar between WT and Il22−/− mice and were decreased by vancomycin. This is consistent with a study demonstrating that several Clostridium spp., including Ruminococcus, are sensitive to vancomycin (34). Therefore, lung IL-17 levels correlated with intestinal SFB colonization. When analyzing cytokines in the small intestine, Il22−/− mice on normal water had a dramatic 3000-fold increase in Il17a expression compared with WT controls, which was reduced by 99.8% upon pretreatment with vancomycin drinking water (Fig. 4C). There was also a 3.7-fold increase in Il17a expression in Il22−/− mice, which was decreased by vancomycin. Expression of Il4 and Foxp3 was similar between WT Taconic and Il22−/− mice in the intestine and lung (Fig. 4C, 4D), suggesting that endogenous IL-22 did not affect Th2 or Treg differentiation. Lung Il17a expression correlated with Il10a in the lungs and small intestine of Il22−/− mice (Fig. 4C, 4D). Therefore, the absence of IL-22 creates a permissive environment for SFB growth, augmenting mucosal IL-17 production in the gut and lung.
Notably, the number of lung CD4+ IL-17+ cells directly correlated with SFB colonization in the small intestine (Fig. 5C) and the cecum (data not shown). A previous study (35) found that microbiota-induced IL-1β increases intestinal Th17 cell numbers. We found that SFB-high microbiota from Il22−/− donors increased Il1a/b gene expression in the lungs following fungal infection (Fig. 5E). Our data demonstrate that vancomycin-sensitive bacteria in the gut augment IL-17 production by CD4+ T cells during pulmonary fungal infection and that increased Th17 responses observed in Il22−/− mice are transferrable with SFB-containing microbiota.

**IL-22 suppresses pulmonary Th17 cell accumulation through serum IL-1R ligands**

We next addressed a potential role for SFB-containing microbiota in the regulation of lung DC function. In vitro, lung CD11c+ cells from B6 Taconic mice on normal water or vancomycin were nearly equivalent at inducing IL-17 from polyclonal naive CD4+ T cells in the presence of heat-killed *A. fumigatus* (Supplemental Fig. 1B). This suggested that lung DCs from vancomycin-treated mice can mount a normal response to *A. fumigatus* in vitro. In support, overnight stimulation of lung CD11c+ cells from B6 Taconic mice on normal water or vancomycin resulted in similar secretion of IL-1α, IL-1β, and IL-6 (Fig. 6). In vivo, we found that infection increased the number of lung CD11c+ CD11b+ cells similarly in B6 Taconic, B6 plus vancomycin, and Il22−/− mice (Supplemental Fig. 1E). Further, SFB colonization did not impact IL-1α or IL-1β production by lung APCs, as measured by intracellular cytokine staining. If SFB
colony activates lung DCs, then \( \text{II22}^{--} \) DCs would be more potent than B6 at inducing Th17 differentiation. Coculture of lung CD11c\(^+\) cells with \( \text{A. fumigatus} \)-specific naive T cells revealed that IL-17 secretion in vitro did not correlate with SFB colonization in DC donor mice (Supplemental Fig. 1C). Overall, these data suggest that lung DCs were not directly influenced by gastrointestinal microbiota, and additional factors contribute to IL-17 production following pulmonary fungal infection.

To determine the role of serum factors in pulmonary Th17 cell accumulation, SFB\(^-\) B6 Jackson mice were infected with \( \text{A. fumigatus} \), and adoptively transferred with serum from naive B6 Taconic or II22\(^-\) mice 7 h later. Some groups of mice received serum premixed with IL-1RA (anakinra), because \( \text{II1a} \) expression was upregulated in the gut of II22\(^--\) mice infected with \( \text{A. fumigatus} \) (Fig. 4C). Two days following infection, lung single-cell suspensions were restimulated with PMA plus ionomycin and stained for intracellular IL-17A. Mice receiving sera from SFB-colonized II22\(^--\) mice had increased numbers of CD4\(^+\) IL-17\(^+\) T cells (Fig. 5D). This was dependent on IL-1R signaling, because preincubation of II22\(^--\) serum with IL-1RA significantly decreased Th17 cell numbers, as well as TCR \( \gamma \delta \) IL-17\(^+\) cells. Notably, donor serum from II22\(^--\) mice contained elevated levels of IL-1\( \alpha \) protein (II22\(^--\) serum: 958 ± 198 pg/ml; B6 Taconic serum: 501 ± 35 pg/ml), whereas IL-1\( \beta \) protein levels were similar to B6 Taconic (II22\(^--\) serum: 99 ± 4 pg/ml; B6 serum: 107 ± 3 pg/ml). In contrast to the role of IL-1R signaling, systemic IL-23 administration did not significantly affect IL-17 levels in the lung on day 2 (data not shown). Overall, these data demonstrate that IL-22 modulates serum IL-1R ligands that augment lung Th17 cell accumulation during fungal infection.

**Intestinal RegIII\( \gamma \) delivery is anti-inflammatory during pulmonary fungal infection**

Next, we tested whether rRegIII\( \gamma \) can rescue the phenotype in II22\(^--\) mice, because RegIII\( \gamma \) is an IL-22-inducible gene. To directly deliver RegIII\( \gamma \) to the gut, mice were gavaged with the recombinant protein at 48-h intervals from 1 to 9 d prior to infection. A total of five treatments were given, and tissues were collected 2 d following infection. RegIII\( \gamma \) gavage protected mice from weight loss, losing only 4% of body mass on day 1, whereas the group receiving PBS vehicle lost 9% (Fig. 7A). \( \text{A. fumigatus} \) burden was not significantly different between the two groups (Fig. 7B, A.f.), suggesting that, rather than having a direct antifungal effect, RegIII\( \gamma \) was anti-inflammatory in this model. Supporting this, expression of II1\( \alpha \), II1\( \beta \), Ilb, and Ilny was significantly reduced in lungs of mice receiving rRegIII\( \gamma \) (Fig. 7B), and the cytokines IL-1\( \alpha \), IL-1\( \beta \), and IL-6 were also decreased (Fig. 7C). Surprisingly, IL-17 protein levels were comparable between RegIII\( \gamma \)-treated and control mice. Analysis of the small intestine and cecum revealed that RegIII\( \gamma \) had no effect on SFB colonization (data not shown). FACS was used to sort RegIII\( \gamma \) and RegIII\( \gamma \) fractions. This was followed by PCR, which revealed that...
nous RegIII
g16S rDNA sequencing was performed on cecal contents. Exoge-
species that may contribute to weight loss during fungal infection,
tion correlates with lung Th17 responses. To characterize bacteria
findings complement our data demonstrating that SFB coloniza-
tonary Th17 cell priming. To address this, we compared B6 mice
SFB colonization augments Th17 immunity during pulmonary
by transferring SFB-low microbiota from Jackson donors into
them use this drinking water significantly decreased lung Th17 cell
hemocyte, including SFB, because the phenotype ob-
ning pulmonary challenges (Fig. 2). This was attributed to
endogenous RegIII
does not preferentially bind to SFB-enriched microbiota (P. Kumar,
complements our data demonstrating that SFB colonization cor-
erves SFB-enriched microbiota from Jackson re-
with previous experiments using this vendor. This cohort was
gavaged with SFB-high fecal suspensions from other Taconic
dons in-house and rested for 2 wk on normal or vancomycin
lephants significantly increased total numbers of IL-17+ and CD4+
but not in SFB-low Jackson mice (Supplemental Fig. 3). Accord-
vancomycin significantly decreased SFB colonization in Taconic mice.
Although C. leptum was also decreased by vancomycin, there were no differences in C. leptum
colonization between Taconic and Jackson mice (Supplemental
This provides further evidence that intestinal SFB colo-
nition promotes lung Th17 immunity.
To confirm that SFB-enriched microbiota augment lung Th17
immunity, vancomycin-treated mice were gavaged with fecal suspensions from Taconic donors (SFB-high) or Jackson donors
(SFB-low). Three weeks following microbiota transfer, mice were
ed by metronidazole (28, 37). In contrast, vancomycin de-
been shown to induce the development of colonic Tregs and are
ndolates (8, 9). We demonstrate that another consequence of RegIII
gy (40). We demonstrate that another consequence of RegIII
deployment may lead to increased accumulation of Th17 cells fol-
owing pulmonary challenges (Fig. 2). This was attributed to
ommensal bacteria, including SFB, because the phenotype ob-
served in RegIII
g/− mice was restored with vancomycin drinking
thus, our data show that modulating the balance of intes-
tinal bacteria species significantly affects T cell polarization at
onlocal sites during infections.
Beneficial effects of commensal bacteria include stimulating the
development of intestinal lymphoid tissue, antimicrobial protein
secretion, and lymphocyte activation. It is not surprising that GF or
ommensal-depleted animals are susceptible to intestinal infec-
tions; however, they also succumb to systemic, skin, and respiratory
pathogens (12–14, 41). Two mechanisms were proposed to explain
the role of commensals in systemic inflammation (42). First, in-
nate immune agonists, such as bacterial cell wall products, may
directly provide an adjuvant effect. Second, chronic low-level

FIGURE 6. Microbiota condition splenic DCs for antifungal cytokine
responses. Splenic CD11c+ cells were purified and stimulated overnight
with heat-killed swollen A. fumigatus (A.f.), LPS, or zymosan, and cytoto-
cline levels were measured in supernatants. Concentrations of IL-1β (A),
IL-6 (B), and TNF (C) in culture supernatants are shown. Data (mean ±
SEM) are from one representative experiment of two performed (four or
five wells). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

SFB colonization augments Th17 immunity during pulmonary fungal infection

Our data suggest that colonization with SFB, rather than host
genotype for RegIII or Il22, determines the strength of pulmo-
nary Th17 cell priming. To address this, we compared B6 mice
that were SFB-high versus SFB-low. Routine screening revealed a
cohort of Taconic mice that was SFB− upon arrival, in contrast

Discussion

Commensal microbiota support the development of immune re-
ponses by stimulating pattern-recognition receptors. By targeting
growth pathways that are shared among classes of bacteria, anti-
biotics decrease intestinal species diversity, which modulates the
immune tone of the gut (36). For instance, Clostridium spp. have
shown to induce the development of colonic Tregs and are
depleted by metronidazole (28, 37). In contrast, vancomycin de-
letes Gram-positive bacteria and decreases the population of
small intestinal Th17 cells (38). In this article, we show that oral
vancomycin has a significant effect on lung IL-17 production
during an acute fungal infection (Fig. 1). Antibiotics also were
shown to inhibit Th17-mediated asthma (39). These data dem-
strate that antibiotics, although required for treating intestinal
infections, have systemic anti-inflammatory effects that can
hibit responses to secondary infections. In support, oral vanco-
mycin renders mice susceptible to vancomycin-resistant enterococci
through the downregulation of antimicrobial proteins, including
RegIII (40). We demonstrate that another consequence of RegIII
deployment may lead to increased accumulation of Th17 cells fol-
owing pulmonary challenges (Fig. 2). This was attributed to
ommensal bacteria, including SFB, because the phenotype ob-
served in RegIII−/− mice was restored with vancomycin drinking
water. Thus, our data show that modulating the balance of intes-
tinal bacteria species significantly affects T cell polarization at
onlocal sites during infections.

Beneficial effects of commensal bacteria include stimulating the
development of intestinal lymphoid tissue, antimicrobial protein
secretion, and lymphocyte activation. It is not surprising that GF or
ommensal-depleted animals are susceptible to intestinal infec-
tions; however, they also succumb to systemic, skin, and respiratory
pathogens (12–14, 41). Two mechanisms were proposed to explain
the role of commensals in systemic inflammation (42). First, in-
nate immune agonists, such as bacterial cell wall products, may
directly provide an adjuvant effect. Second, chronic low-level
stimulation of APCs by commensal products, termed “tonic signaling,” epigenetically programs them to respond robustly to a future infection (12, 13). We observed that vancomycin-sensitive commensals condition splenic DCs to produce increased levels of inflammatory cytokines in response to fungal products (Fig. 6), providing evidence for epigenetic programming. Further, Quintin et al. (43) demonstrated that a primary fungal infection trains monocytes for increased cytokine production during a secondary infection. In our model, lung DCs from vancomycin-treated mice were not hyporesponsive to *A. fumigatus* (Supplemental Fig. 1D), and it remains possible that commensals provide an acute adjuvant effect. Future studies will be aimed at deciphering the mechanism of how Gram-positive commensals amplify lung IL-17 production.

Secretion of RegIIIg into the small intestine promotes the physical separation of luminal bacteria from epithelium (20). Therefore, RegIIIg may block DC stimulation with commensal products or microbial translocation across the gastrointestinal tract. SFB adhere directly to intestinal villi and provide an adjuvant effect that restores small intestinal Th17 cells in GF mice (21, 22, 44). Because RegIIIg was found to inhibit mucosa-associated SFB colonization (20), this may explain why small intestinal CD4+ T cells, including Th17 and Th1 cells, are significantly increased in *RegIIIg−/−* mice following i.p. immunization (Fig. 3). It is not known whether RegIIIg has direct bactericidal activity against SFB or inhibits colonization indirectly. Nevertheless, several inflammatory diseases are associated with a compromised intestinal epithelial barrier. Microbial translocation is increased during inflammatory bowel disease, chronic HIV infection, hepatitis B or C infection, and pancreatitis, contributing to systemic immune activation (45). IL-22 is critical for epithelial repair following injury (19, 46), and production of IL-22 by innate lymphoid cells prevents microbial translocation in the steady-state (47). This suggests that intestinal IL-22 activity may have systemic anti-inflammatory effects. We found that *Il22−/−* mice have increased IL-17 in the lungs.

**FIGURE 7.** Intestinal RegIIIg delivery is anti-inflammatory during pulmonary fungal infection. (A) *Il22−/−* mice were gavaged with recombinant mouse RegIIIg (40 µg; ○) or vehicle control (■), as described in Materials and Methods. Mice were depleted of neutrophils and infected. Percentage body weight relative to day 0 (mean ± SEM). (B) Lung gene expression on day 2, normalized to *Hprt*. (C) Lung cytokines measured by ELISA on day 2, normalized to total protein. Data are combined from two experiments with *n* = 8–10. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**FIGURE 8.** Selective depletion of Clostridiales Ruminococcaceae by RegIIIg treatment. DNA from cecal contents was isolated from *Il22−/−* mice used in Fig. 7 and used as a template for bacterial 16S rDNA PCR analysis, as described previously (29). Amplicons were sequenced using Illumina MiSeq and analyzed for OTUs at the phylum, class, order, and family levels. (A) Total number of OTUs defined as species in PBS versus RegIIIg treatment groups (upper panel) and the distribution of major phyla (lower panel). (B) Total number of individual species that were changed by ≥2-fold in response to RegIIIg treatment, as categorized by phyla or order (upper panel). Total number of individual species within Clostridiales that were changed by RegIIIg treatment, categorized by family (lower panel). Data are combined from two experiments with *n* = 8–10.
and small intestine during acute fungal infection compared with WT mice (Fig. 4). This phenotype was vancomycin sensitive, correlated with SFB colonization, and was transferable with intestinal microbiota (Fig. 5B), demonstrating that IL-22 was modulating microbial composition in the gut. Overall, cumulative evidence points to a model suggesting that IL-22 helps to prevent systemic immune activation by inhibiting SFB colonization, blocking microbial translocation, and decreasing pulmonary Th17 cell polarization toward fungal Ags.

The cellular sources of IL-17 during infections may influence its physiological effects. Deletion of integrin αβ8 on CD11c+ cells inhibits Ag-specific Th17 responses and protects against airway hyperresponsiveness independently of airway inflammation (48). IL-17 produced by CD4 T cells, not γδ T cells, enhances airway smooth muscle contraction. In our study, SFB colonization led to preferential accumulation of Th17 cells in lung tissue and had no effect on the number of γδ T cells making IL-17 (Fig. 5A, 5B). Further studies are necessary to define the role of SFB colonization on airway hyperresponsiveness.

The regulation and function of RegIIIγ in vivo are compartmentalized. In Paneth cells, IL-22 upregulates RegIIIγ mRNA (19, 47, 49), whereas stimulation of the MyD88 pathway induces RegIIIγ release from secretory granules in an inactive proform (50, 51). Following its secretion into the intestine, trypsin cleaves 11 aa from the N terminus of RegIIIγ, resulting in a 15-kDa isoform with potent bactericidal activity (51). In lung epithelium, IL-6 upregulates RegIIIγ expression during Staphylococcus aureus infection (52). We showed that RegIIIγ neutralization delays S. aureus clearance, whereas rRegIIIγ delivery to the lungs accelerates clearance. Although the cleaved 15-kDa isoform is detectable in bronchoalveolar lavage fluid, the enzyme responsible for RegIIIγ cleavage in lungs is not known. Thus, IL-22 and IL-6 control RegIIIγ expression in the gut and lungs, respectively. Because both of these cytokines activate the STAT3 transcription factor, the compartmentalized regulation of RegIIIγ expression may be determined by the local release of STAT3-activating cytokines and/or epithelial receptor expression. The regulation of RegIIIγ at multiple checkpoints is one way that the innate immune system controls bacteria colonization on mucosal surfaces.

The role of RegIIIγ in host defense is pathogenic specific. Although RegIIIγ binds to the surface of A. fumigatus, it does not significantly affect fungal viability at pH 7 (data not shown). This is similar to what was reported for other fungi (18). Following infection, RegIIIγ did not significantly affect pulmonary fungal clearance (Figs. 2A, 7B, Supp. Fig. 2). Our finding that vancomycin drinking water decreases lung Th17 cell numbers in RegIIIγ−/− mice demonstrates that RegIIIγ expression is not sufficient to regulate Th17 immunity (Fig. 2D). The increased Th17 cell numbers in RegIIIγ−/− mice compared with B6 controls was due to microbiota differences (Fig. 2). Thus, when using SFB-negative strains, fungal clearance and IL-17 levels were comparable in B6 and RegIIIγ−/− mice (Supplemental Fig. 2). Our data suggest that RegIIIγ does not play a direct role in pulmonary antifungal immunity.

In this article we demonstrated that differences in microbiota between strains of mice can impact pulmonary immunity. Certain bacteria, including SFB, may stimulate the systemic release of IL-1R ligands, augmenting lung Th17 cell accumulation during fungal infections. The source of IL-1R ligands remains an interesting question. We observed that SFB-enriched microbiota increased the expression of ILβ in lung tissue (Fig. 5E) but did not affect the number of lung CD11c+ CD11b+ cells producing IL-1α or IL-1β (Supplemental Fig. 1E). This suggests that non-DCs may provide a source of IL-1R ligands during infection. It also remains possible that T cell responsiveness to IL-1R ligands might be regulated by microbiota. Our work complements a study showing that increased activity of the NLRP3 inflammasome in response to a high-fat diet leads to IL-17–dependent airway hyperresponsiveness (53). The investigators found that IL-1β produced by macrophages drives the accumulation of group 3 innate lymphoid cells that mediate disease through IL-17. Our findings suggest that microbiota can influence susceptibility to autoimmune disease. Animal studies identified commensals that provoke or prevent autoimmunity by modulating the balance of Th17 cells and Tregs (54, 55). Intestinal Tregs recognize microbial Ags (56, 57) and increase the ratio of Firmicutes to Bacteroidetes species (58), suggesting that T cells can, in turn, influence microbial composition. Thus, commensal organisms have integral roles in the development of local and systemic immune responses.

Intestinal SFB colonization may regulate pulmonary host defense through various mechanisms, including antimicrobial protein secretion, immune cell activation, and recruitment. A recent study found that SFB protects against acute S. aureus pneumonia (59). SFB-colonized mice had significantly increased levels of IL-6 and IL-22, as well as neutrophil cell numbers, in bronchoalveolar lavage fluid. In that study, IL-22 action in the lung was critical for host defense against S. aureus. Our data show that IL-22 action in the gut may also regulate lung immunity by modulating the microbiota. This is consistent with other studies measuring the impact of IL-22 on gut microbiota (60, 61). Although we provide evidence that systemic IL-1R ligands contribute to pulmonary Th17 immunity (Fig. 5D), more studies are necessary to fully define the link between SFB colonization and host defense.

Disclosures
W.O. is an employee of Genentech; the other authors have no financial conflicts of interest.

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