

# Perinatal antibiotic-induced shifts in gut microbiota have differential effects on inflammatory lung diseases

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**Background:** Resident gut microbiota are now recognized as potent modifiers of host immune responses in various scenarios. Recently, we demonstrated that perinatal exposure to vancomycin, but not streptomycin, profoundly alters gut microbiota and enhances susceptibility to a T<sub>H</sub>2 model of allergic asthma.

**Objective:** Here we sought to further clarify the etiology of these changes by determining whether perinatal antibiotic treatment has a similar effect on the T<sub>H</sub>1/T<sub>H</sub>17-mediated lung disease, hypersensitivity pneumonitis.

**Methods:** Hypersensitivity pneumonitis was induced in C57BL/6 wild-type or recombination-activating gene 1-deficient mice treated perinatally with vancomycin or streptomycin by repeated intranasal administration of *Saccharopolyspora rectivirgula* antigen. Disease severity was assessed by measuring lung inflammation, pathology, cytokine responses, and serum antibodies. Microbial community analyses were performed on stool samples via 16S ribosomal RNA pyrosequencing and correlations between disease severity and specific bacterial taxa were identified.

**Results:** Surprisingly, in contrast to our findings in an allergic asthma model, we found that the severity of hypersensitivity pneumonitis was unaffected by vancomycin, but increased dramatically after streptomycin treatment. This likely reflects an effect on the adaptive, rather than innate, immune response because the effects of streptomycin were not observed during the early phases of disease and were abrogated in

recombination-activating gene 1-deficient mice. Interestingly, *Bacteroidetes* dominated the intestinal microbiota of streptomycin-treated animals, while vancomycin promoted the expansion of the *Firmicutes*.

**Conclusions:** Perinatal antibiotics exert highly selective effects on resident gut flora, which, in turn, lead to very specific alterations in susceptibility to T<sub>H</sub>2- or T<sub>H</sub>1/T<sub>H</sub>17-driven lung inflammatory disease. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

**Key words:** Antibiotics, *Bacteroidetes*, *Firmicutes*, gut microbiota, hypersensitivity pneumonitis, asthma, perinatal

Hypersensitivity pneumonitis (HP), also known as extrinsic allergic alveolitis, is an exaggerated inflammatory response that develops after repeated exposure to a wide variety of inhaled environmental antigens. It is largely driven by inhalation of organic dust found in a range of occupational and residential settings. A number of environmental antigens are causative agents, including those derived from bacteria, fungi, parasites, animal and plant proteins, and inorganic chemical compounds (reviewed in Girard et al<sup>1</sup>). Because of the diversity of environmental triggers and the lack of a consistent definition of HP, current epidemiological data are thought to severely underestimate the prevalence of the disease.<sup>2</sup>

Unlike asthma, which is classically defined as a T<sub>H</sub>2-mediated peribronchial airway inflammatory disease, HP is characterized by T<sub>H</sub>1/T<sub>H</sub>17-mediated inflammation affecting the parenchyma and small airways (alveoli) of the lungs (reviewed in Schuyler et al<sup>3</sup> and McSharry et al<sup>4</sup>). In HP, initial antigen exposure induces an influx of neutrophils that, after repeated exposures, is replaced by the infiltration of lymphocytes secreting IFN- $\gamma$ <sup>5</sup> and IL-17A.<sup>6-8</sup> Acute lymphocytosis involves mainly CD4<sup>+</sup> T cells, whereas chronic forms require CD8<sup>+</sup> T cells that can cause irreversible lung damage in the form of fibrosis or emphysema, resulting in significant morbidity and mortality.<sup>4</sup> Treatment options are limited to corticosteroids and contact avoidance.<sup>2</sup>

The most widely used murine model of HP involves repeated intranasal exposure to antigens isolated from the gram-positive thermophile, *Saccharopolyspora rectivirgula* (SR), a bacterium isolated from moldy hay, which, in humans, causes the disease “farmer’s lung.” Repeated inhalation of SR induces a T<sub>H</sub>1/T<sub>H</sub>17-type inflammation in the lung characterized by high levels of IFN- $\gamma$ <sup>9</sup> and IL-17A,<sup>7,8</sup> antigen-specific IgG<sub>2a</sub> antibodies,<sup>1</sup> and formation of parenchymal granulomas.<sup>10</sup> In animal models, T-cell activation is involved in the development of HP<sup>11</sup> and IL-10 is an important modulator of disease severity.<sup>12</sup> Dendritic cells (DCs) are also critical for the initiation of antigen-specific T-cell responses; high DC maturation status<sup>13</sup>

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**Abbreviations used**

BAL: Bronchoalveolar lavage  
 DC: Dendritic cell  
 HP: Hypersensitivity pneumonitis  
 OTU: Operational taxonomic unit  
 RAG 1: Recombination-activating gene 1  
 SR: *Saccharopolyspora rectivirgula*  
 Treg: Regulatory T

and CD34-dependent DC trafficking<sup>14</sup> are required for robust inflammatory responses to HP antigens. A number of innate immune cells have also been identified as early sources of IL-17A in experimental HP, including neutrophils, monocytes/macrophages, and  $\gamma\delta$  T cells.<sup>6,15</sup>

Mounting evidence in human and animal models suggests that gut microbes have a profound effect on the development of allergic asthma.<sup>16–18</sup> By using germ-free mice<sup>19,20</sup> and antibiotics,<sup>21–23</sup> our laboratory and others have shown that the intestinal microbiota profoundly influence disease susceptibility in murine models of allergic asthma. In our studies, we found that mice receiving clinically relevant doses of vancomycin early in life develop more severe asthma than do untreated controls,<sup>21,22</sup> which is likely mediated by a mechanism involving elevated IgE levels and reduced regulatory T (Treg) cells.<sup>21,22</sup> Interestingly, this effect was not observed in mice given streptomycin, nor when either antibiotic was administered to adult mice.<sup>21</sup> Thus, our data point to a perinatal “window” when alterations in specific members of gut microbiota can influence asthma susceptibility. Whether this enhanced sensitivity to T<sub>H</sub>2-driven disease leads to protection from T<sub>H</sub>1/T<sub>H</sub>17-driven disease is unknown.

Unlike allergic asthma,<sup>24,25</sup> there is currently no epidemiological evidence linking antibiotic use to an increased risk of HP, perhaps because of the diversity in causative agents or the complex nature of diagnosis and reporting strategies. Likewise, no studies have addressed the role of the microbiota in murine models of HP. Therefore, in this study, we have used a murine model to test the hypothesis that antibiotic perturbation of the intestinal microbiota alters susceptibility to experimental HP.

**METHODS****Mice**

C57BL/6J and B6.129S7-*Rag1<sup>tm1Mom</sup>/J* (Rag1KO) mice (Jackson Laboratories, Bar Harbor, Me) were bred and maintained in a specific pathogen-free facility at The Biomedical Research Centre. All experiments were conducted in accordance with the UBC Animal Care Committee guidelines.

**Antibiotic treatment**

C57BL/6J and Rag1KO breeding pairs were given vancomycin or streptomycin (Sigma-Aldrich, St Louis, Mo) at 200 mg/L in drinking water. Pups born from respective breeding pairs were reared on antibiotic-treated water for the duration of the experiment. These antibiotics were chosen because they are poorly absorbed when given orally, minimizing the risk of systemic effects on the host. The rationale behind the clinically relevant concentrations used has been described in detail previously.<sup>39</sup>

**Induction of experimental HP**

HP was induced in 7-week-old mice as previously described.<sup>11,14</sup> Mice were sensitized intranasally with 40  $\mu$ L of 4 mg/mL SR antigen in

PBS 3 d/wk for 3 weeks. Mice were sacrificed at the time points indicated. Mice were anesthetized with 500 mg/kg Avertin and blood was collected by cardiac puncture. After sacrifice, bronchoalveolar lavage (BAL) was performed by 3  $\times$  1 mL washes with PBS. Total BAL cells were counted by a hemocytometer and immune cells were differentially quantified from cytopsin preparations (Thermo Shandon, Pittsburgh, Pa) stained with HemaStain (Fisher Scientific, Ottawa, Canada) on the basis of standard morphological criteria. Cell types were confirmed by flow cytometry (see below).

**Histology**

Lungs were collected and fixed in 10% formalin, embedded in paraffin, cut longitudinally into 5- $\mu$ m sections, and stained with hematoxylin and eosin. Inflammation was blindly assessed from 5 fields per section, each graded on a scale of 0 to 5 (0 = no signs of disease, 5 = severe disease) for each of the following parameters: (1) peribronchial infiltration, (2) perivascular infiltration, (3) parenchymal infiltration, and (4) granulomas (number and size) for a maximum score of 20.

**Determination of SR-specific antibody responses**

SR-specific IgG<sub>1</sub> and IgG<sub>2a</sub> in serum was measured by ELISA as previously described.<sup>14</sup> Plates were coated with 10  $\mu$ g/mL SR antigen in 0.1 M sodium carbonate buffer overnight at 4°C. Serum was serially diluted from 1:2000 to 1:32000. SR-specific IgGs were detected using biotin-conjugated rat anti-mouse IgG<sub>1</sub> and IgG<sub>2a</sub> followed by streptavidin-horseradish peroxidase and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (both BD Biosciences, Franklin Lakes, NJ). Absorbance was measured at 450 nm using a Tecan Infinite 200 plate reader (Männedorf, Switzerland).

**RNA isolation and quantitative PCR**

RNA samples were prepared using an RNeasy Mini Kit, and cDNA was synthesized using the QuantiTect RT Kit (both Qiagen, Valencia, Calif). Real-time PCR was performed using a QuantiTect SYBR Green Master Mix (Qiagen) and a 7500 Fast Real-Time System (Applied Biosystems, Carlsbad, Calif). Values were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase for each sample. Primers for quantitative PCR: *Ifng*: 5'-TCAAGTGGCATAGATGTGGAAGAA-3' (F) and 5'-TGGC TCTGCAGGATTTTCATG-3' (R); *Il17a*: 5'-CTCTCCACCGCAATGAA GAC-3' (F) and 5'-AGCTTCCCTCCGCATTGA-3' (R); *Gapdh*: 5'-ATT GTCAGCAATGCATCCTG-3' (F) and 5'-ATGGACTGTGGTCATGAGCC-3' (R).

**Cytokine analysis of lung homogenates and cell supernatants**

Lung lobes were collected and homogenized in PBS plus protease inhibitors (Roche, Branford, Conn). Supernatants were analyzed for cytokines using cytokine bead array flex sets for IL-17A and IFN- $\gamma$  (BD Biosciences). Analysis was performed using an LSR II as described above. Cytokine concentrations were normalized to total protein, assessed by Bradford assay reagent (Pierce, Rockford, Ill).

**Isolation of immune cells and flow cytometry**

BAL, lung, and lung-draining lymph node (mediastinal lymph node) cells were isolated and stained with fluorochrome-conjugated antibodies against 7/4 (Abcam, Cambridge, Mass), B220, CD25, CD19, CD3, CD4, CD8, Gr-1, CD11b, CD11c, NK1.1, and CD45 (eBioscience, San Diego, Calif) and Siglec-F (BD Biosciences). Innate lymphoid cells were defined as CD25<sup>+</sup>, lineage<sup>−</sup> (CD19<sup>−</sup>, CD3<sup>−</sup>, Gr-1<sup>−</sup>, CD11c<sup>−</sup>, CD11b<sup>−</sup>, NK1.1<sup>−</sup>), alveolar macrophages as CD11c<sup>Hi</sup> SiglecF<sup>+</sup>, lymphocytes as CD3<sup>+</sup>B220<sup>+</sup>, and neutrophils as CD11c<sup>−</sup> SiglecF<sup>−</sup>CD11b<sup>+</sup>Gr-1<sup>+</sup>. Intracellular cytokine production was assessed by incubating 2  $\times$  10<sup>6</sup> lung or lymph node cells with 0.5  $\mu$ g/mL phorbol 12-myristate 13-acetate and 1  $\mu$ g/mL ionomycin for 4 hours with Brefeldin A (10  $\mu$ g/mL). Stimulated cells were stained intracellularly

(BD Cytofix/Cytoperm) with fluorochrome-conjugated antibodies to IFN- $\gamma$  or IL-17A (eBioscience). Flow cytometry was performed using an LSR II (BD Biosciences), and data were analyzed with FlowJo 8.7 software (TreeStar, Ashland, Ore). Lung cells were isolated and analyzed by using flow cytometry as previously described.<sup>14</sup>

## Microbial analysis

For composition analyses, stool pellets from untreated or antibiotic-treated SR-challenged mice were homogenized using a 60s bead-beating method (FastPrep instrument, MP Biomedicals, Solon, Ohio) and total DNA was extracted (QIAamp DNA Stool Mini Kit, Qiagen). 16S ribosomal RNA gene fragments were amplified using 33 nucleotide-bar-coded primer pairs (27F; 5'-AGAGTTTGATCMTGGCTCAG-3'), (519R; 5'-GWATTACCGC GGCKGCTG-3'). PCR products were gel-purified (QIAquick gel extraction kit, Qiagen). Each amplicon (100 ng) was pooled and pyrosequenced using a 454 Titanium platform (Roche). Further information is listed in the [Methods](#) section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

## Bacterial taxa associated with disease severity

We used Mann-Whitney *U* tests to compare the relative abundances of all OTUs identified in streptomycin-treated mice relative to untreated control mice after 3 weeks of SR exposure. Significantly different OTUs after false-discovery rate correction using the Benjamini-Hochberg method ( $P < .05$ ) were used to generate a heat map, displaying log-transformed relative abundances (relative abundances were multiplied by 100 before log-transforming, so that a scale of 2 [most abundant, RED] to zero [below the limit of detection, BLUE] is created). This transformation produced the best visual separation among samples. Correlations between OTUs that differed significantly between control and streptomycin treatment were generated by comparing BAL counts from individual control and streptomycin-treated animals with relative abundances of these OTUs. OTUs that positively correlated with disease severity are shown in green, whereas OTUs that negatively correlated with disease severity are shown in brown. (*R* values shown in boxes). A Spearman correlation coefficient (*R*) was generated for each OTU in the relative abundance heat map. Coefficients are shown to the right of the heat map in green/brown boxes. Stronger correlations are indicated by the color and value of *R*.

## Statistics

Differences between control and experimental groups were compared using 1-way ANOVA to calculate statistical significance (GraphPad Prism software, version 6.0, San Diego, Calif).

## RESULTS

### Antibiotic treatment increases the severity of HP

HP was induced in mice exposed pre- and perinatally to vancomycin or streptomycin by intranasal instillation of SR antigen 3 times a week for 3 weeks. In contrast to what we observed in a  $T_H2$  model of allergic asthma,<sup>21,22</sup> mice treated with streptomycin, but not vancomycin, exhibited exacerbated disease after repeated exposure to SR as assessed by total leukocytes in the BAL ([Fig 1, A](#)). Differential analyses of infiltrating airway leukocytes from SR-challenged animals revealed that streptomycin-treated mice had increased numbers of lymphocytes and macrophages ([Fig 1, B](#)), consistent with these cell types playing important roles in HP pathogenesis. Interestingly, relative frequencies of these cell types in the BAL of streptomycin-treated mice were not different from those of control SR-challenged mice (see [Fig E1, A](#), in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), arguing for increased severity but not a major alteration in disease course. As expected, no differences

in total cell numbers or cell types were observed in the BAL of PBS-challenged control or antibiotic-treated animals, with alveolar macrophages making up the vast majority of leukocytes in the airways ([Fig 1, A](#); see [Fig E1, B](#)).

The effects of vancomycin and streptomycin on the severity of HP were also examined histologically. Unlike allergic asthma in which lung inflammation is predominantly localized to the bronchi and neighboring arteries, HP is characterized by the formation of granulomatous cell aggregates deep in the alveolar spaces.<sup>26</sup> Pathology scores were consistently higher in SR-challenged, streptomycin-treated animals ([Fig 1, C](#)) than in control mice across all parameters evaluated: perivascular infiltration, peribronchial infiltration, parenchymal infiltration, and granuloma formation. Interestingly, vancomycin-treated, SR-challenged mice scored higher than SR-challenged control animals in a few categories (perivascular infiltration and granuloma formation). No pathology was observed in PBS-treated control mice and PBS-treated vancomycin and streptomycin-treated animals.<sup>21</sup> In summary, on the basis of an evaluation of BAL infiltrates and histopathology, we find that streptomycin treatment enhances the severity of experimental HP.

### Streptomycin-treated SR-challenged mice have elevated $T_H1$ and $T_H17$ responses

Next, we characterized the immune response generated in control and antibiotic-treated mice after SR exposure. We detected equivalent levels of SR antigen-specific IgG<sub>2a</sub> and IgG<sub>1</sub> in the serum of untreated and antibiotic-treated mice ([Fig 2, A](#)), indicating that antibiotics did not dramatically alter the humoral immune responses in HP. This is consistent with human data that suggest that antigen-specific antibody titers are not good indicators of disease severity and that individuals with high serum antibody titers to HP-inducing antigens can remain asymptomatic.<sup>27</sup>

It is well established that  $T_H1$  and  $T_H17$  immune responses are important contributors to disease pathogenesis in HP.<sup>7,28,29</sup> Correspondingly, we found increased mRNA transcripts for *Ifng* and *Il17a* in lungs of streptomycin-treated mice relative to vancomycin-treated and control mice after SR challenge ([Fig 2, B](#)), and elevated IFN- $\gamma$  and IL-17A cytokines in lung homogenates from the same mice ([Fig 2, C](#)). Lung cells from SR-challenged mice restimulated with SR antigen trended toward increased IL-17A production in the streptomycin-treated mice; however, this did not reach significance (data not shown). There were no differences in the frequency of lung or mediastinal lymph node CD4<sup>+</sup> T cells producing IL-17A or IFN- $\gamma$  in streptomycin-treated mice compared with control animals after SR exposure.

Several members of the intestinal microbiota have been implicated in the development of  $T_H1$ ,<sup>30</sup>  $T_H17$ ,<sup>31</sup> and Treg-cell responses.<sup>32,33</sup> To determine whether there was an intrinsic alteration in a  $T_H1$  or  $T_H17$  pathway, we investigated whether the DCs or T cells from antibiotic-treated mice were skewed toward  $T_H1$  or  $T_H17$  responses in the steady state. There were no differences in cytokine secretion by OT-II T cells conditioned by ovalbumin-pulsed DCs from control, vancomycin-treated, or streptomycin-treated mice (see [Fig E2, A](#), in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Similarly, splenic T cells isolated from control or antibiotic-treated mice, cultured in neutral,  $T_H1$ -inducing, or  $T_H17$ -inducing conditions, did not differ in their intracellular IFN- $\gamma$  or IL-17A production,

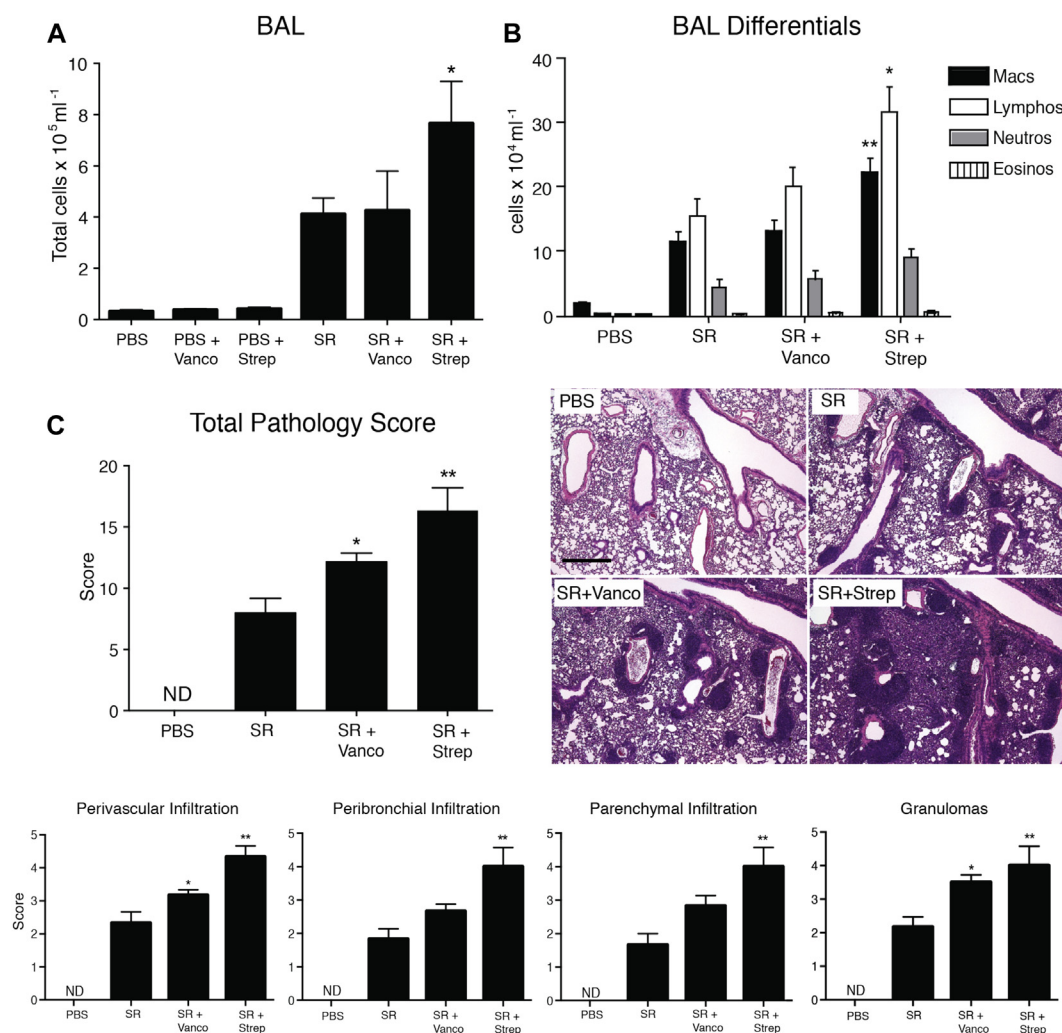


**FIG 1.** Early life streptomycin treatment exacerbates HP. **A** and **B**, BAL and differential leukocyte counts of control or antibiotic-treated mice challenged for 3 weeks with SR antigen or PBS and sacrificed on day 20 after initial SR exposure. **C**, Pathological scores and representative H&E-stained lung sections. Scale bar, 300  $\mu\text{m}$ . Error bars are means  $\pm$  SEM, representative of at least 3 independent experiments ( $n = 3-6$ ). Statistics shown are based on comparisons to SR-challenged controls. H&E, Hematoxylin and eosin; ND, none detected; Strep, streptomycin; Vanco, vancomycin. \* $P < .05$  and \*\* $P < .01$ .

suggesting that there is no systemic skewing of T-cell responsiveness after streptomycin treatment (see Fig E2, B).

### Adaptive immunity likely contributes to streptomycin-exacerbated HP

To determine whether the enhanced severity of HP in mice treated with streptomycin was due to altered innate or adaptive immune responses, we challenged mice with SR antigen for 1 or 3 consecutive days and measured lung inflammation 24 hours later to highlight contributions from innate cells before robust adaptive responses could be generated. After 1 or 3 SR exposures, there were no significant differences in total inflammatory cells in the BAL between SR-challenged, streptomycin-treated animals and untreated controls (Fig 3, A). Early SR exposure is characterized by an influx of neutrophils, which we observed on differential analysis of the BAL. However, there were no significant differences in the frequency of neutrophils, macrophages, or lymphocytes in the BAL of mice treated with streptomycin

relative to controls (Fig 3, B). Similarly, there were no differences in *Ifng* or *Il17a* expression (Fig 3, C) or IL-17A<sup>+</sup>/IFN- $\gamma$ <sup>+</sup>-producing innate lymphoid cells (Fig 3, D) in the lung after short-term SR exposure.

Because IL-17A-producing  $\gamma\delta$  T cells have been implicated in HP pathogenesis,<sup>6</sup> we investigated the prevalence of IL-17A-producing, ROR $\gamma$ t<sup>+</sup>  $\gamma\delta$  T cells in the lung after early SR exposure in antibiotic-treated mice. We observed no differences in ROR $\gamma$ t<sup>+</sup>  $\gamma\delta$  T cells in streptomycin- or vancomycin-treated mice compared with controls (data not shown).

In a further attempt to determine whether adaptive immunity plays a role in streptomycin-exacerbated HP, we treated recombination-activating gene 1 (RAG 1)-deficient mice (lacking mature T and B cells) with vancomycin or streptomycin perinatally, and then exposed them to SR for 3 weeks. Interestingly, although RAG 1-deficient mice showed increased BAL cell counts after SR exposure, no differences were detected in BAL total cells (Fig 4, A) or differential cell counts (Fig 4, B and C) between streptomycin-treated and control

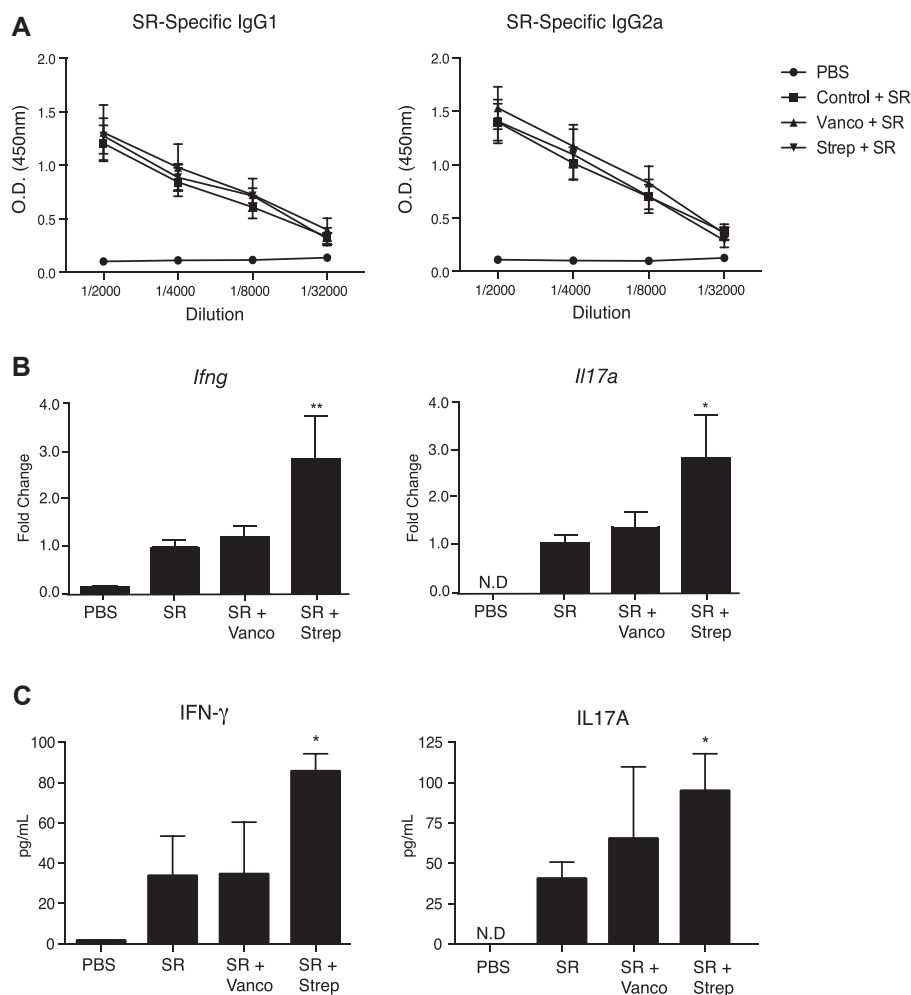


**FIG 2.** IFN- $\gamma$  and IL-17A are upregulated in streptomycin-exacerbated HP. **A**, Serum SR-specific IgG<sub>1</sub> and IgG<sub>2a</sub> responses after 3 weeks of SR exposure. **B** and **C**, *Ifng* and *Il17a* mRNA expression and IFN- $\gamma$  and IL-17A cytokine production in lung homogenates from control and antibiotic-treated mice (means of 3-6 mice per group  $\pm$  SEM, 1 of at least 2 independent experiments). Data in Fig 2 (C) are pooled from 2 experiments. Statistics shown are based on comparisons to SR-challenged controls. *Eosinos*, Eosinophils; *Lymphos*, lymphocytes; *Macs*, macrophages; *ND*, none detected; *Neutros*, neutrophils; *Strep*, streptomycin; *Vanco*, vancomycin. \* $P < .05$  and \*\* $P < .01$ .

SR-challenged RAG 1-deficient mice, suggesting that an adaptive immune cell lineage is likely mediating this streptomycin-induced HP phenotype in wild-type mice.

### Streptomycin treatment alters the composition of the intestinal microbiota

Previously, we found that perinatal vancomycin treatment profoundly altered the gut microbiota of mice pre- and post-induction of experimental asthma, whereas streptomycin treatment resulted in more subtle shifts in microbial composition.<sup>21</sup> We therefore sought to determine whether these same trends existed after SR-induced HP.

Changes in gut composition after antibiotic treatment and HP induction were assessed by pyrosequencing the V1 to V2 regions of bacterial 16S ribosomal RNA genes (64,659 quality reads obtained from 33 samples). As expected, there were distinct differences among antibiotic-treated groups and untreated controls after HP induction (Fig 5, A). As previously observed,<sup>21</sup> vancomycin induced profound shifts in microbial community

composition at the family level relative to control and streptomycin-treated animals (Fig 5, A). Also, at the operational taxonomic unit (OTU) level, the effect of vancomycin was much greater than that of streptomycin with respect to community similarity, demonstrated by principle coordinate analyses (Fig 5, B), Bray-Curtis similarity index (control vs control, 64.7; control vs streptomycin, 29.1; control vs vancomycin, 1.81) and diversity (Inverse Simpson's diversity index: control,  $12.3 \pm 1.6$ ; streptomycin,  $14.0 \pm 1.0$ ; vancomycin,  $2.7 \pm 0.3$ ). Despite their similar diversity, there were notable changes at the family level between control and streptomycin-treated mice. Some of the bacterial families were similarly depleted in both vancomycin- and streptomycin-treated mice. For example, *Clostridiaceae 1* had a relative abundance of  $1.8\% \pm 0.7\%$  in control mice but were undetectable in both antibiotic-treated groups (Fig 5, A). Conversely, many bacterial families were differently affected by the 2 antibiotic treatments. Vancomycin-treated mice exhibited a loss/reduction in a number of bacterial families (predominantly *Bacteroidetes*), which were largely replaced by

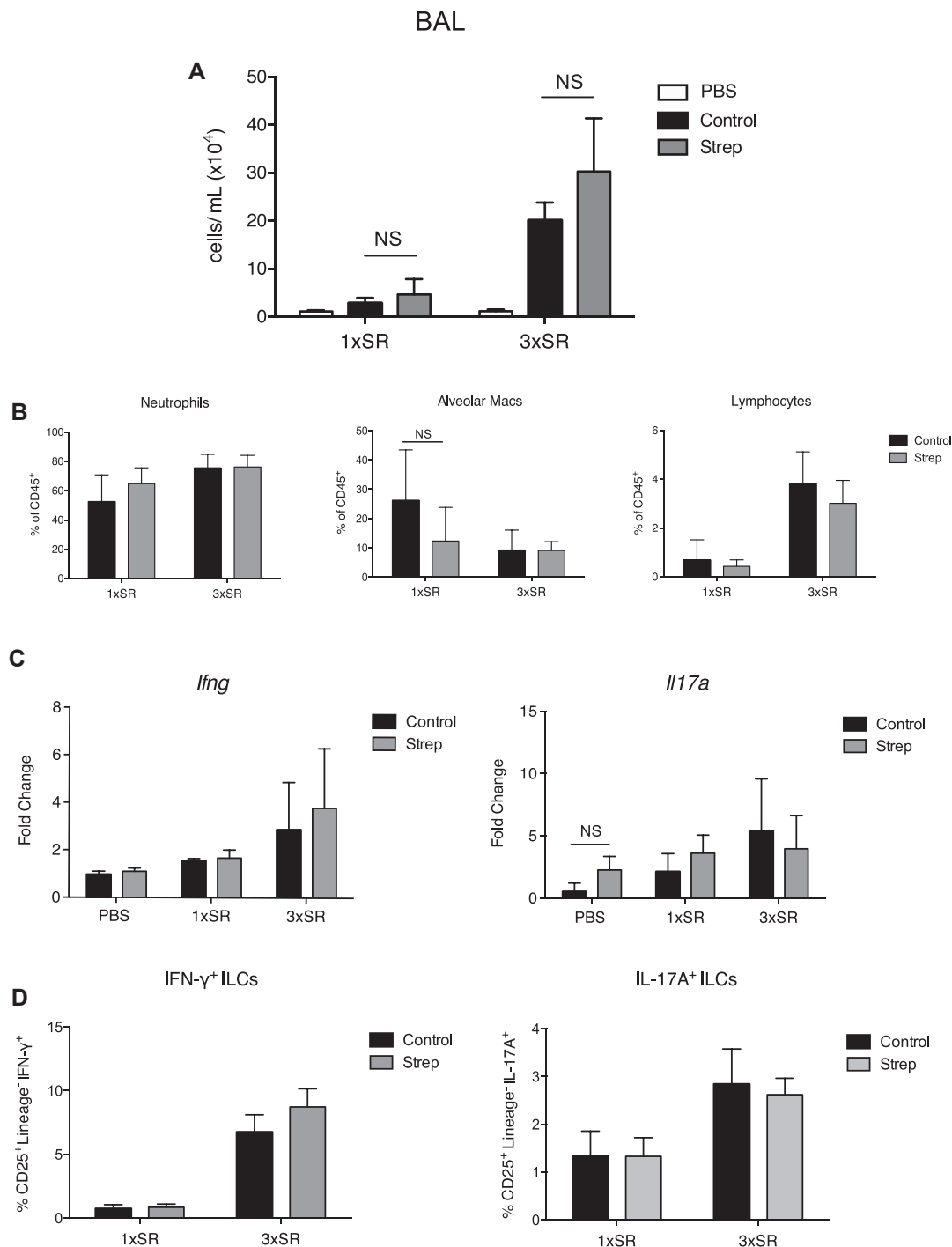


**FIG 3.** Streptomycin does not alter early innate immune responses in HP. **A**, Total BAL counts of control or streptomycin-treated mice challenged with SR for 1 (1 × SR) or 3 (3 × SR) consecutive days. **B**, Frequency of leukocyte subsets in BAL of SR-challenged mice. **C**, *Ifng* and *Il17a* mRNA expression in lung tissue from SR-challenged control and streptomycin-treated mice. **D**, Intracellular IFN-γ or IL-17A production by innate immune cells (CD45<sup>+</sup>CD25<sup>+</sup>lineage<sup>-</sup>) isolated from lung tissue of SR-challenged mice. Means of 3 to 6 mice per group ± SEM, representative of 2 independent experiments. NS, Not significant; Strep, streptomycin.

*Firmicutes* (*Lactobacillus* sp), *Paenibacillaceae* (*Paenibacillus*), *Verrucomicrobia* (specifically *Akkermansia*), and *Enterobacteriaceae*. In contrast, streptomycin-treated mice exhibited an expansion of *Bacteroidetes* from 52.2% ± 6.4% to 76.5% ± 18.0% relative abundance, involving only 2 families: *Porphyromonadaceae* and *Bacteroidaceae*.

### Specific members of the microbiota correlate with disease severity in HP

There were 26 OTUs significantly affected by streptomycin treatment post-HP induction (Fig 6, B). All these OTUs were undetected in vancomycin-treated mice. All OTUs identified as *Clostridiales* were depleted in mice treated with streptomycin,

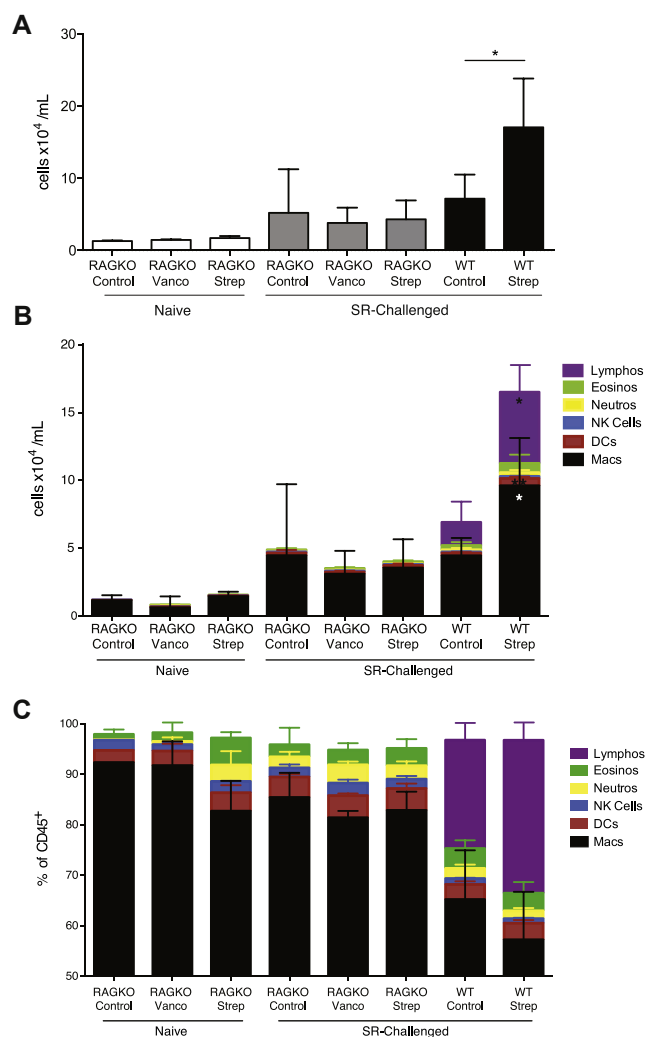


**FIG 4.** Adaptive immunity plays a role in streptomycin-exacerbated HP. **A**, BAL counts of control or antibiotic-treated, SR-challenged (3 weeks) RAG 1-deficient or wild-type (WT) mice. Total number (**B**) or frequency (**C**) of leukocyte subsets in BAL of SR-challenged mice. Data shown are means of 3 to 6 mice per group  $\pm$  SEM. Statistics shown are based on comparisons to WT controls. *Eosinos*, Eosinophils; *Lymphos*, lymphocytes; *Macs*, macrophages; *Neutros*, neutrophils; *NK*, natural killer; *Strep*, streptomycin; *Vanco*, vancomycin. \* $P < .05$  and \*\* $P < .01$ .

whereas 12 of 14 OTUs classified within the *Bacteroidetes* phylum that differed between streptomycin and control were increased after streptomycin treatment. These data coincide with the increase in *Bacteroidetes* at the family and phylum levels after streptomycin treatment (Fig 5, A). Microbiota profiles of individual mice from SR-challenged control and streptomycin-treated groups were highly variable while following the above trends (see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

There was also substantial variation within the streptomycin-treated mice with respect to HP severity. Thus, to determine whether this was due to the presence or absence of particular bacterial groups, the abundance of select taxa was compared with disease severity (measured by total cells in BAL from streptomycin-treated, SR-challenged mice compared with control-challenged mice; Fig 6, A and B; see Fig E4 in this

article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). First, given the apparent shift toward OTUs belonging to *Bacteroidetes* after streptomycin treatment, and *Firmicutes* after vancomycin treatment, we sought to determine whether there was a correlation between disease severity and all OTUs belonging to *Bacteroidetes* and *Firmicutes*. Intriguingly, members of the microbiota belonging to *Bacteroidetes* (all OTUs) correlated positively with disease severity, while those belonging to *Firmicutes* correlated negatively with disease severity (Fig 6, A).

Individual OTUs (Fig 6, B, green/brown boxes) and select taxa (Fig E4) also correlated with disease severity. Interestingly, the *Bacteroidetes* (all OTUs,  $R = 0.87$ , and specific OTUs that corresponded to unclassified members of *Bacteroidetes* phylum,  $R = 0.89$ , and *Bacteroidales* order,  $R = 0.78$ ) positively correlated with disease severity ( $P < .05$ ), while the *Firmicutes* (all OTUs,  $R = -0.75$ , and *Lachnospiraceae*,  $R = -0.80$ ) negatively correlated with disease severity. The abundance of other related taxa, such as *Bacteroidaceae*, *Clostridiaceae*, and *Ruminococcaceae*, showed no correlation with disease severity (Fig E4).

## Discussion

On the basis of our previous findings that perinatal vancomycin, but not streptomycin, treatment had profound effects on disease severity in a  $T_H2$  model of allergic asthma,<sup>21,22</sup> we set out to investigate how these animals responded in a  $T_H1/T_H17$  lung disease model of HP in order to gain further insights into how 2 different antibiotics promote distinct immunological outcomes during disease development. Vancomycin and streptomycin shift the composition of the intestinal microbiota in very distinct/contrasting ways, suggesting that alterations in resident gut microbiota may be responsible for their differential effects on asthma severity. Several potential immune mechanisms could account for the increased susceptibility observed in vancomycin-treated mice: (1) a general, microbiota-driven immune skewing away from  $T_H1/17$  responses and toward  $T_H2$  responses, as has been suggested by others,<sup>23</sup> (2) a general, microbiota-driven loss of a key immunosuppressive population (eg, Treg cells), or (3) a much more selective set of microbiota-driven changes that lead to distinct alterations in a specific immune response pathway. To explore these possibilities, we assessed the influence of vancomycin and streptomycin on susceptibility to the  $T_H1/T_H17$ -mediated disease, HP. Surprisingly, the severity of HP was relatively unaffected by vancomycin, but was exacerbated by streptomycin as evidenced by increased airway inflammation, enhanced recruitment of lymphocytes and macrophages, increased IFN- $\gamma$  and IL-17A production, and more severe lung pathology. Analyses of the intestinal microbiota in these mice revealed a subtle shift in community membership by streptomycin, similar to what we described previously.<sup>21</sup> Thus, the data suggest that these antibiotics do not induce global immune skewing toward a specific subset of  $T_H$  cells, but instead select for microbial communities that directly or indirectly alter specific immunological pathways involved in pulmonary responses to environmental antigens.

A number of innate and adaptive immune cells play important roles in HP, so we attempted to investigate mechanistically whether streptomycin treatment enhanced innate or adaptive arms of the immune response. We observed no striking

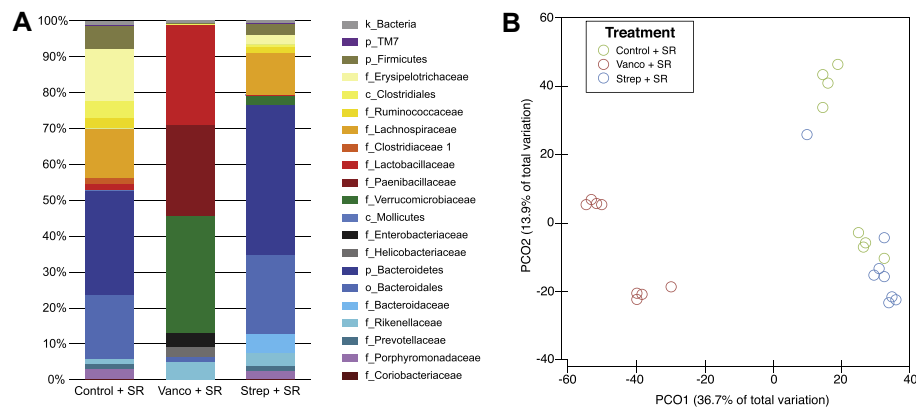


**FIG 5.** *Bacteroidetes* are enriched in mice treated with streptomycin. Pyrosequencing analysis of 16S rRNA gene amplicons was performed on fecal DNA samples from the indicated mice. **A**, Relative abundance (%) of OTUs grouped at the family level from the indicated SR-challenged mice. Classification scheme: *k*, kingdom; *p*, phylum; *c*, class; *o*, order; *f*, family. Each bar represents an average of 8 mice per group, from 2 independent experiments. Rare taxa were removed from the legend, but still included in the graph. **B**, Principal coordinate analysis (PCO) of bacterial communities. Each colored circle represents 1 mouse. rRNA, Ribosomal RNA; Strep, streptomycin; Vanco, vancomycin.

differences in total BAL counts, neutrophils,  $\gamma\delta$  T cells, innate lymphoid cells, or IFN- $\gamma$  and IL-17A cytokines in mice treated with streptomycin compared with untreated controls after exposing mice to SR for just 1 or 3 consecutive days. Because this time frame would be too early to elicit a specific adaptive immune response, the data suggest that early innate immune responses are not responsible for the exacerbated HP phenotype we observed in streptomycin-treated mice. In addition, we observed no differences in disease severity between streptomycin-treated and control SR-challenged RAG 1-deficient mice. Although this experiment needs to be interpreted with caution because inactivation of the RAG 1 gene could itself shift the microbiota in ways that obscure the effect of streptomycin, at face value, it further supports a role for the adaptive immune response in the streptomycin-induced exacerbation of disease. Although we were unable to identify the specific cell type involved in mediating streptomycin-exacerbated HP, we were able to rule out a number of possibilities: polarized DCs and T cells, systemic SR-specific IgG antibodies, and a number of innate immune cell lineages. Thus, our data would argue for heightened adaptive, T-cell-mediated responses in streptomycin-treated mice exposed repeatedly to SR.

Previously, we provided evidence that vancomycin treatment increases asthma severity by mechanisms involving elevated production of IgE and a decreased frequency of Treg cells.<sup>21,22</sup> The lack of an overt exacerbation of HP pathology or cytokine production in vancomycin-treated mice would argue against a systemwide defect in the immunosuppressive capacity of Treg cells. In addition, it was previously shown that overexpression of the T<sub>H</sub>2-polarizing transcription factor GATA3 provides protection against HP<sup>34</sup> rather than exacerbation. Together, these observations suggest that global T<sub>H</sub>2 skewing does not mediate vancomycin-exacerbated asthma because these animals were not protected from experimental HP. Thus, different antibiotics appear to exert more subtle effects on responses in specific disease states.

Examination of the gut bacterial communities in mice exposed to vancomycin or streptomycin perinatally, compared with untreated controls, reveals intriguing changes that may

account for the differences in susceptibility to asthma<sup>21</sup> and HP. Remarkably, despite the drastic shift in microbiota composition induced by vancomycin (with diversity measures dropping more than 50% compared with controls), and the minimal shifts in composition induced by streptomycin, only streptomycin had profound effects on HP. Regardless of the degree of perturbation, it is clear that distinct phyla dominate microbiota treated with vancomycin (*Firmicutes*) versus streptomycin (*Bacteroidetes*). *Bacteroidetes* demonstrated the strongest positive association with HP disease severity in streptomycin-treated animals, while loss of select members of the *Firmicutes* (specifically those belonging to *Lachnospiraceae* and *Clostridiaceae 1*) also correlated with disease severity, suggesting that the proportions of 2 dominant bacterial phyla in the gut may play a defining role in HP pathogenesis. In addition, community composition, rather than diversity, appears to be the major driver of dysregulated immune responses in the HP disease model.

*Bacteroidetes* (the most over-represented bacterial group after streptomycin treatment) is a diverse phylum, with limited characterization of specific clades. The most well-studied organism, *Bacteroides fragilis*, has been shown to play a role in the maturation of T<sub>H</sub>1-driven immune responses early in life by increasing IFN- $\gamma$  production by CD4<sup>+</sup> T cells,<sup>30</sup> a result that could be important in the context of HP. Intriguingly, there is an enterotoxigenic strain of *B fragilis* that promotes colon tumorigenesis in a Stat3 and T<sub>H</sub>17-dependent manner,<sup>35</sup> which further supports a role for this group in influencing T<sub>H</sub>17 disease.

Although the precise immunological mechanisms underlying streptomycin-exacerbated HP remain undefined, there is compelling evidence to suggest that members of the microbiota elicit distinct immune cell responses (eg, Treg cell,<sup>32,33,36</sup> T<sub>H</sub>17<sup>31</sup>) and alter pathogenesis in several disease models including colitis, allergy, and arthritis. Microbiome studies reveal great interindividual variation in the bacterial species that reside in the intestinal microbiota of humans<sup>37</sup> and mice.<sup>38</sup> This has fueled the notion that different commensal species provide similar functional roles in different microbial environments. Thus, instead of focusing on the presence or absence of individual species in different

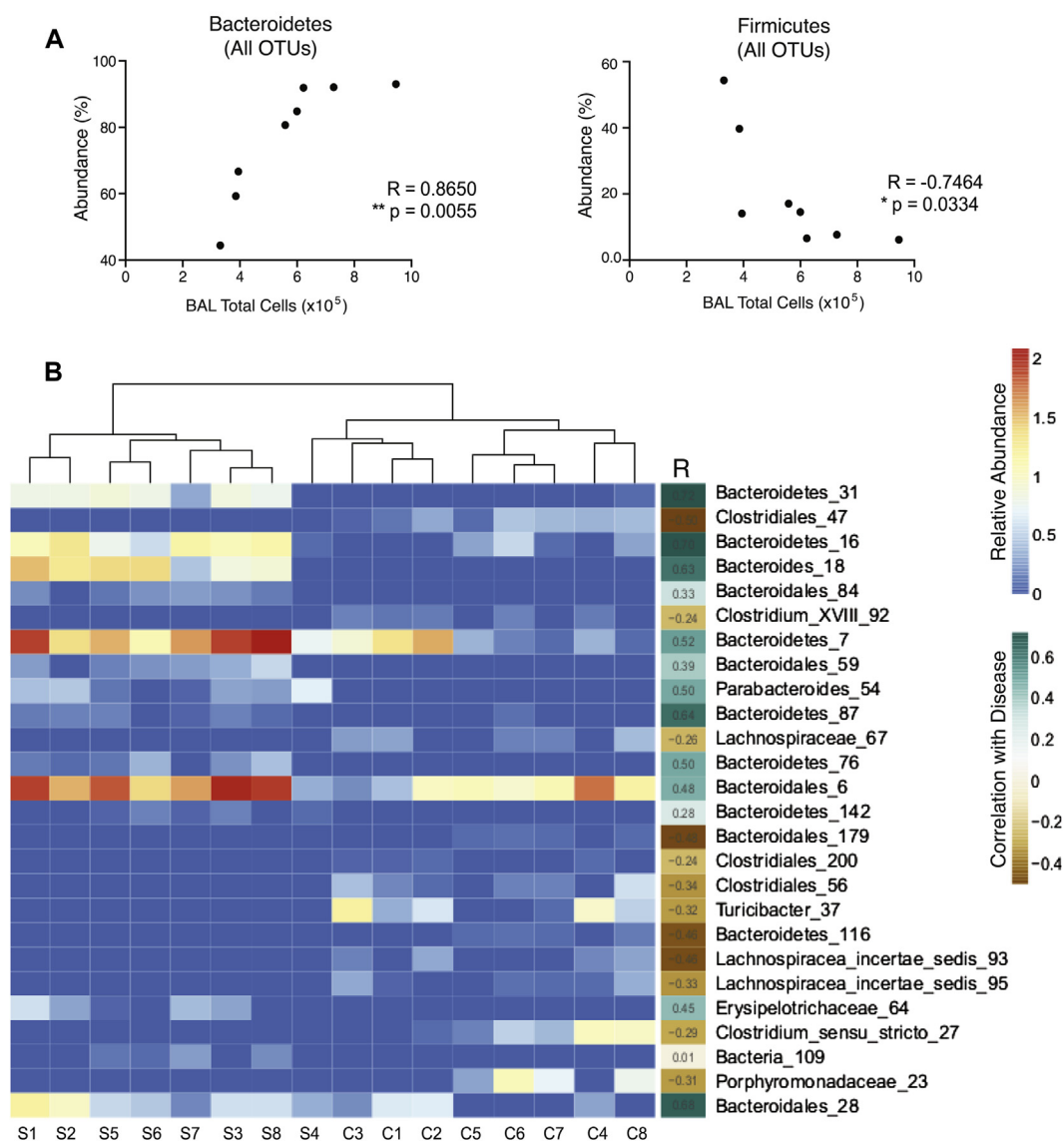


**FIG 6.** Specific bacterial taxa correlate with disease severity in HP. **A**, Relative abundance (%) of all OTUs classified as *Bacteroidetes* or *Firmicutes* was plotted against BAL infiltrates for individual streptomycin-treated mice. **B**, Heatmap displaying OTUs enriched in either control (C) or streptomycin (S)-treated mice. Samples were hierarchically clustered using the complete linkage method. Colored “cells” represent log-transformed % relative abundances and vary between blue and red. The numbered vertical bar represents Spearman correlations between differentially abundant OTUs and BAL counts in the lungs of control and streptomycin-treated, SR-challenged mice. Correlation coefficient values are represented by colors ranging from brown (negative correlations) to green (positive correlation). Each column represents 1 mouse.

disease settings, the microbiome can be viewed as a complex network of interactions that impinge on key functions of the host by the community as a whole. In the context of HP, perhaps streptomycin promotes the overgrowth of bacteria that promote an “inflammatory” milieu that, directly or indirectly, exaggerates  $T_H1/T_H17$  adaptive immune responses. Further studies will be needed to delineate these mechanisms, but it will be essential to compare and contrast the microbes, their nutrient environments, and host immune cell homeostasis in streptomycin- versus vancomycin-treated backgrounds.

In summary, we have described a new role for the commensal microbiota in a  $T_H1/T_H17$ -driven inflammatory lung disease model. Our data suggest that the previously documented shifts

in gut microbiota that exacerbate allergic asthma have no effect on HP and that instead, HP is exacerbated by a more subtle shift in microbiota induced by streptomycin. Together, these studies argue for a more nuanced role for the microbiota in altering disease development and demonstrate how divergent shifts in microbiota have very different consequences in 2 immunologically distinct disease models. These novel findings have important implications for clinical interventions with both antibiotics and probiotics.

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## Key messages

- Perinatal antibiotic-induced shifts in gut microbiota enhance susceptibility to a murine model of hypersensitivity pneumonitis.
- Microbiotas dominated by *Firmicutes* or *Bacteroidetes* have differential effects on T<sub>H</sub>2 and T<sub>H</sub>1/T<sub>H</sub>17-driven disease models.

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## METHODS

### DC-T-cell polarization assay

DCs (CD11c<sup>+</sup>, CD64<sup>+</sup>) were sorted using fluorescence-activated cell sorting from the mesenteric lymph nodes of untreated or antibiotic-treated mice, and CD4<sup>+</sup> T cells were magnetically sorted (EasySep Mouse CD4<sup>+</sup> T cell Isolation Kit; StemCell Technologies, Vancouver, British Columbia, Canada) from the peripheral lymph nodes of untreated OT-II transgenic mice. DCs and OT-II CD4<sup>+</sup> T cells were cultured together for 3 days in the presence of ovalbumin peptide (Invivogen, San Diego, Calif). Culture supernatants were then taken and analyzed for the presence of cytokines using cytokine bead array assays (BD Biosciences).

### Splenic T-cell polarization assay

CD4<sup>+</sup> T cells were isolated from the spleens and lymph nodes of control, vancomycin-, and streptomycin-treated mice by negative selection with magnetic beads (StemCell Technologies). Purified CD4<sup>+</sup> cells ( $2.5 \times 10^5$ ) were cultured for 6 days in culture media (Iscove modified Dulbecco medium supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 150 µM monothioglycerol) with 1 µg/mL each of plate-bound anti-CD3 (145-2C11) and anti-CD28 (37.51) in the presence of neutral (10 ng/mL IL-2), T<sub>H</sub>1 (10 ng/mL IL-2, 10 ng/mL IL-12, and 10 µg/mL anti-IL-4 [1B11]), or T<sub>H</sub>17 (10 ng/mL IL-1b, 20 ng/mL IL-6, 10 ng/mL IL-23, 10 ng/mL TNF-α, 1 ng/mL TGF-β, 10 µg/mL anti-IL-4 [1B11], and 10 µg/mL anti-IFN-γ [XMG1.2]) conditions. For intracellular staining, polarized cells were stimulated for 4 hours with 50 ng/mL phorbol 12-myristate 13-acetate and 750 ng/mL ionomycin in the presence of brefeldin A (GolgiPlug, BD). Cells were stained with fixable viability dye (eBioscience), stained for CD4 (GK1.5, eBioscience), fixed and permeabilized with intracellular staining buffers (eBioscience), and then stained with antibodies against IL-17A (17B7) and IFN-γ (XMG1.2, eBioscience).

### Bioinformatics for microbial analyses

High-quality sequence reads were determined using MOTHUR according to standard operating procedures described by Schloss et al.<sup>E1</sup> Quality sequences were obtained by removing sequences with ambiguous bases, read lengths less than 200 bases, and chimeras (identified using chimera.uchime). Quality sequences were aligned to the Silva bacterial reference alignment and OTUs were generated using a dissimilarity cutoff of 0.03. Sequences were classified using the classify.seqs command within the Ribosomal Database Project as reference. For Fig 5, A, sequences were grouped on the basis of terminal classification, family level and above. If sequences could not be classified at the family level, they were grouped into the taxonomic group (ie, order, class, phylum, etc) in which they could be confidently classified.

### Multivariate analysis of community structures and diversity

Data were standardized by dividing the number of reads in each taxonomic unit by the total number of reads in each sample. A resemblance matrix was calculated using Bray-Curtis similarities on the basis of standardized data. Principal Coordinate Analysis implemented in PRIMER6+ was used to display similarities in microbial community structures among all samples. Principal Coordinate Analysis ordination was plotted with STATISTICA 8.0 (StatSoft, Tulsa, Okla). Inverse Simpson's diversity index was used to calculate community diversity between treatment groups.

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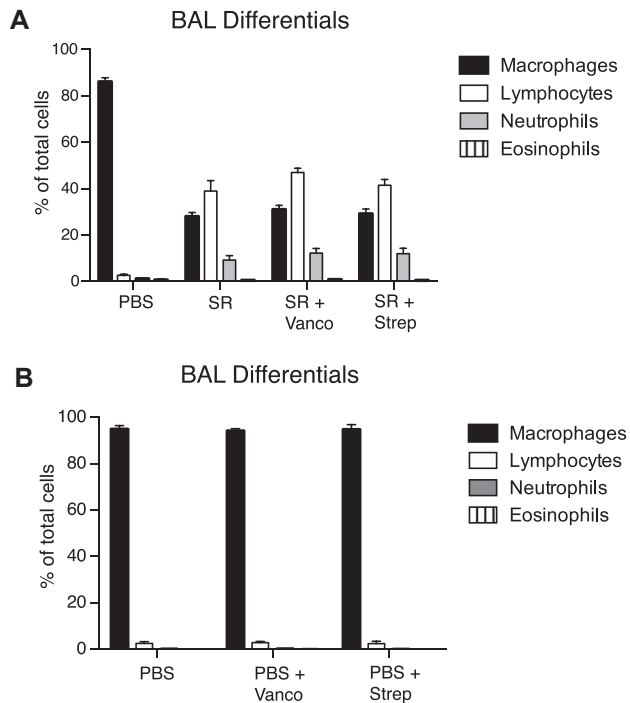


**FIG E1.** Abundance of immune cell subsets in BAL of SR-challenged antibiotic-treated mice. Frequency (%) of leukocyte subsets relative to total BAL cells from **(A)** 3-week SR-challenged or **(B)** 3-week PBS-challenged antibiotic-treated mice, quantified by cytopsin or flow cytometry. Means of 3 to 6 mice per group  $\pm$  SEM, representative of at least 2 independent experiments. *Strep*, Streptomycin; *Vanco*, vancomycin.

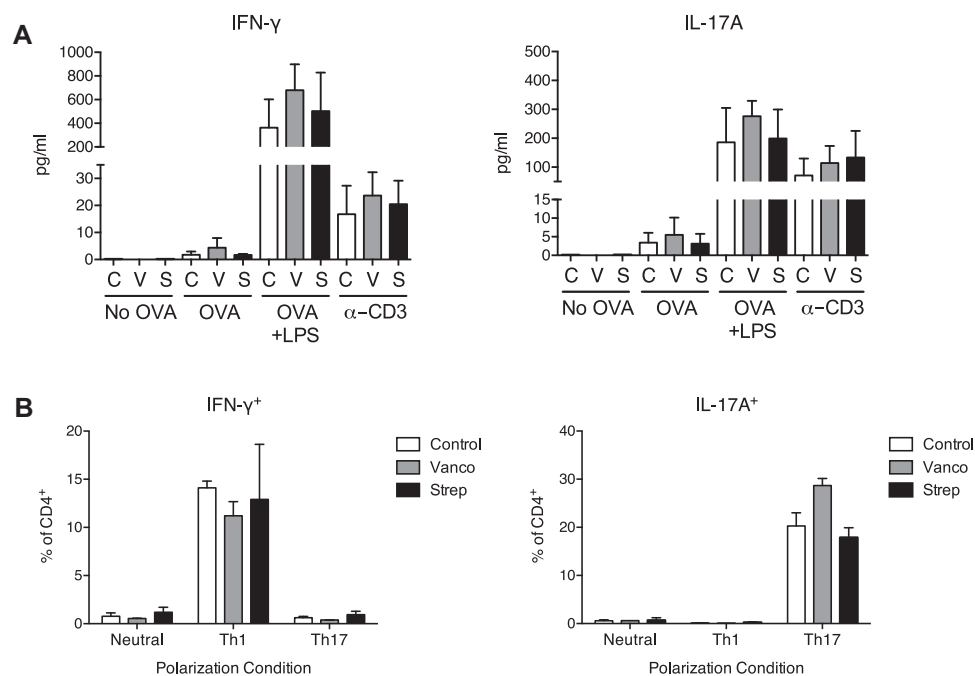


**FIG E2.** Streptomycin treatment does not polarize systemic DC or T-cell responses toward  $T_H1/T_H17$  effector phenotypes. **A**, IFN- $\gamma$  and IL-17A secretion by OT-II cells cultured for 3 days with DCs isolated from mesenteric lymph nodes of control or antibiotic-treated naive animals with or without OVA in the presence of LPS or anti-CD3. Supernatant cytokines from cocultures were quantified by cytokine bead array. **B**, Intracellular IFN- $\gamma$  and IL-17A cytokine production by splenic CD4<sup>+</sup> T cells cultured for 7 days in neutral,  $T_H1$ -, or  $T_H17$ -inducing conditions. Means of 3 mice per group  $\pm$  SEM and representative data from 1 (A) or 2 (B) independent experiments. OVA, Ovalbumin; Strep, streptomycin; Vanco, vancomycin.

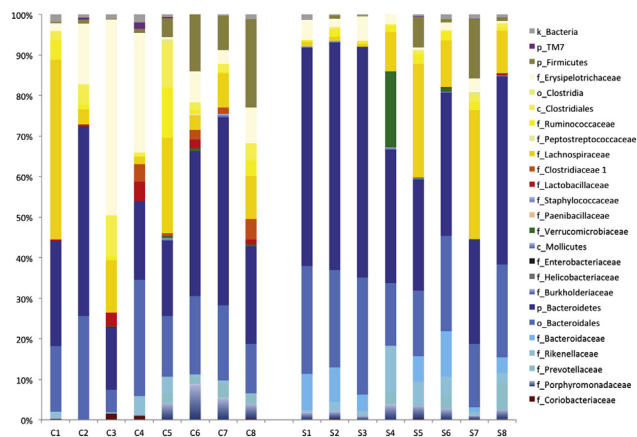


**FIG E3.** Interindividual differences exist between streptomycin-treated and control microbial communities. Family-level phylogenetic classification of 16S rRNA gene frequencies in fecal DNA from SR-challenged control (C) and streptomycin-treated (S) animals. Those indicated with a classification level other than family level (f) could be identified confidently only to the level indicated: k, kingdom; p, phylum; c, class; o, order; f, family. Each bar represents 1 mouse. Rare taxa were removed from the legend, but still included in the graph. *rRNA*, Ribosomal RNA.

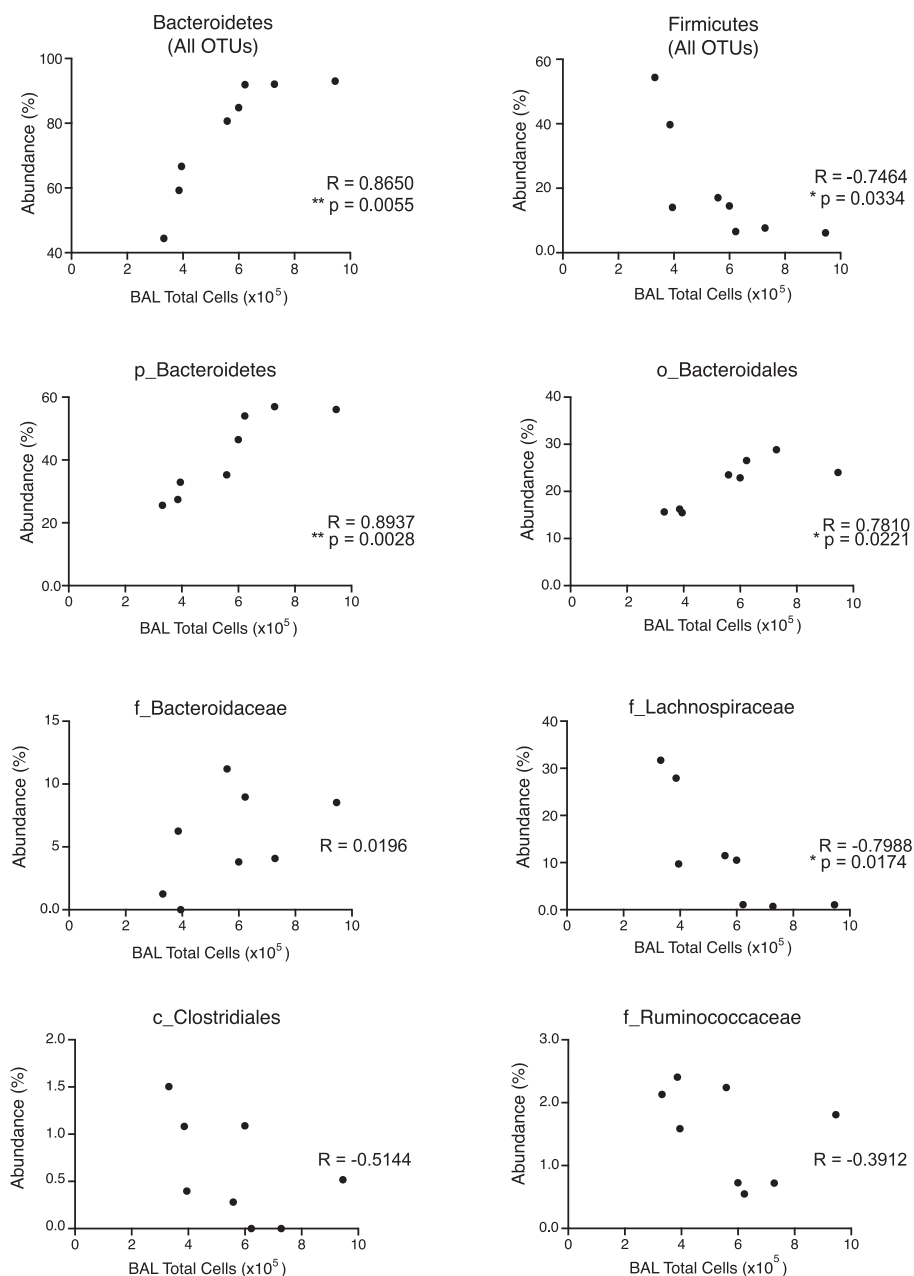


**FIG E4.** Specific bacterial taxa correlate with disease severity in streptomycin-treated mice. Relative abundance (%) of select bacterial taxa grouped at family level (unless otherwise specified) was plotted against total BAL infiltrates of individual mice treated with streptomycin and challenged for 3 weeks with SR antigen. Significant positive or negative correlation coefficients are indicated beside each corresponding graph. Those with a classification level other than family (*f*) could be identified confidently only to the level indicated: *p*, phylum; *c*, class; *o*, order; *f*, family. Each dot on the graph represents abundance/BAL from 1 mouse.