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This information is current as of November 19, 2019.

J Immunol published online 8 November 2019

<http://www.jimmunol.org/content/early/2019/11/07/jimmunol.1900711>

Supplementary Material

<http://www.jimmunol.org/content/suppl/2019/11/07/jimmunol.1900711.DCSupplemental>

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



The *Pglyrp1*-Regulated Microbiome Enhances Experimental Allergic Asthma

Sunil Banskar,* Ashley A. Detzner,* Maria D. Juarez-Rodriguez,*¹ Iztok Hozo,[†] Dipika Gupta,* and Roman Dziarski*

Changes in intestinal or respiratory microbiomes in infants correlate with increased incidence of asthma, but the causative role of microbiome in the susceptibility to asthma and the host genes that regulate these changes in microbiome are mostly unknown. In this study, we show that decreased responsiveness to allergic asthma in *Pglyrp1*^{-/-} mice (lacking bactericidal peptidoglycan recognition protein 1) could be transferred to germ-free wild-type mice by colonization of mothers and newborns with microbiota from *Pglyrp1*^{-/-} mice. These colonized mice had decreased airway resistance and fewer inflammatory cells, less severe histopathology, and lower levels of IgE and proallergic cytokines and chemokines in the lungs. This microbiome-dependent decreased responsiveness to asthma was most pronounced in colonized germ-free BALB/c mice (genetically predisposed to asthma), only partially evident in outbred germ-free Swiss Webster mice, and marginal in conventional BALB/c mice following depletion of microbiome with antibiotics. Mice with a low asthmatic response colonized with microbiota from *Pglyrp1*^{-/-} mice had increased abundance of *Bacteroidetes* and decreased abundance of *Firmicutes*, *Tenericutes*, *Deferribacteres*, and *Spirochaetes* in the feces and increased abundance of *Pasteurella* in the oropharynx. These changes in bacterial abundance in the feces and oropharynx correlated with lower asthmatic responses in the lungs. Thus, our results show that *Pglyrp1* enhances allergic asthmatic responses primarily through its effect on the host intestinal microbiome and identify several bacteria that may increase or decrease sensitivity to asthma. This effect of microbiome is strong in asthma-prone BALB/c mice and weak in asthma-resistant outbred mice and requires germ-free conditions before colonization with microbiota from *Pglyrp1*^{-/-} mice. *The Journal of Immunology*, 2019, 203: 000–000.

Asthma is an inflammatory disease of the airways manifested by airway hyperresponsiveness, increased mucus secretion, chronic inflammation, and airway remodeling. The prevalence of asthma has been steadily increasing over the last several decades and is now >7% in the United States, with the annual healthcare expenditures exceeding \$50 billion. Both multigenic predisposition to an aberrant immune response and environmental factors determine sensitivity to asthma. In about half of asthma cases, the primary trigger is allergy to environmental allergens (atopic or allergic asthma), and the remaining cases have no known allergic trigger (nonatopic or nonallergic asthma) but similar pathology (1). One of the frequent allergens involved in

asthma is house dust mite (HDM) allergen, with 10% of individuals with asthma allergic to HDM (2). HDM induces experimental asthma in mice that resembles human asthma (3–5). Genome-wide profiling of the lung transcriptome of patients following exposure to environmental allergens (6) and of mice with HDM-induced asthma (4) identified *Pglyrp1* as one of the genes involved in lung inflammation.

Pglyrp1 codes for peptidoglycan recognition protein (Pglyrp) 1, which is one of the four mammalian antibacterial innate immunity proteins (7–9). Pglyrp1, Pglyrp3, and Pglyrp4 are directly bactericidal (10–13) and kill bacteria by inducing exaggerated oxidative, thiol, and metal stress in bacteria (14–16), whereas Pglyrp2 is an *N*-acetylmuramoyl-L-alanine amidase that hydrolyzes bacterial peptidoglycan and is also bactericidal (17, 18). Pglyrps are expressed in cells and tissues that usually come in contact with bacteria: Pglyrp1 is highly expressed in neutrophil and eosinophil granules and also in epithelial and other cells (7, 10–12, 19–21), whereas Pglyrp2, Pglyrp3, and Pglyrp4 are expressed in epithelial cells in the skin and mucous membranes, and Pglyrp2 is also expressed in the liver (7, 11, 22–25).

In vivo *Pglyrps* modulate inflammatory responses in the intestine, skin, lungs, and joints. All *Pglyrps* are anti-inflammatory in the intestine and protect from colitis (22, 26, 27). *Pglyrp3* and *Pglyrp4* also protect from atopic dermatitis (23), and *Pglyrp2* protects from psoriasis-like skin inflammation (24). However, some *Pglyrps* have opposite effects: *Pglyrp1* has a proinflammatory effect in experimental asthma (21, 28) and in three mouse models of inflammatory skin diseases (atopic dermatitis, contact dermatitis, and psoriasis) (23, 24), whereas *Pglyrp2* promotes the development of experimental arthritis (25) and inflammatory response that protects against bacterial lung infection (29). Thus, *Pglyrp1* is often proinflammatory, whereas other *Pglyrps* are often anti-inflammatory.

*Indiana University School of Medicine–Northwest, Gary, IN 46408; and
[†]Department of Mathematics, Indiana University–Northwest, Gary, IN 46408

¹Current address: Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, KS.

ORCID: 0000-0002-2638-1935 (S.B.); 0000-0003-2475-0984 (A.A.D.); 0000-0003-2349-5707 (I.H.); 0000-0002-1911-2104 (R.D.).

Received for publication June 24, 2019. Accepted for publication October 8, 2019.

This work was supported by U.S. Department of Defense Discovery Award PR150219.

The sequencing data presented in this article have been submitted to the National Center for Biotechnology Information Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/bioproject/565191>) under accession number PRJNA565191.

Address correspondence and reprint requests to Dr. Roman Dziarski, Indiana University School of Medicine–Northwest, 3400 Broadway, Gary, IN 46408. E-mail address: rdziars@iun.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: BAL, bronchoalveolar lavage; FDR, false discovery rate; HDM, house dust mite; OTU, operational taxonomic unit; PAS, periodic acid–Schiff; PCoA, principal coordinate analysis; Pglyrp, peptidoglycan recognition protein; QIIME, Quantitative Insights Into Microbial Ecology; WT, wild-type.

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These effects are nonredundant because each of the single *Pglyrp* knockout mice (*Pglyrp1*^{-/-}, *Pglyrp2*^{-/-}, *Pglyrp3*^{-/-}, or *Pglyrp4*^{-/-}) exhibits its characteristic phenotype not compensated by other *Pglyrps*.

In vivo *Pglyrps* also maintain a beneficial healthy intestinal microbiome, and this microbiome is responsible for the protective effects of *Pglyrp3* and *Pglyrp2* in experimentally induced ulcerative colitis (22, 26, 27). Because each *Pglyrp* has a unique effect on the intestinal microbiome, these unique effects of *Pglyrps* on microbiome may explain the nonredundant in vivo effects of *Pglyrps*. It is not known, however, whether in vivo effects of *Pglyrp1* or other in vivo effects of *Pglyrp2*, *Pglyrp3*, and *Pglyrp4* are due to their effects on microbiome. Thus, the objective of this study was to test the hypothesis that the decreased responsiveness of *Pglyrp1*^{-/-} mice to allergic asthma is due to the effect of *Pglyrp1* on the host microbiome.

The rationale for this hypothesis was as follows: 1) compelling epidemiologic evidence showing association between the incidence and severity of asthma and other allergic and inflammatory lung diseases and the composition of intestinal or respiratory tract microbiomes or exposure to environments rich in microorganisms (30–44); 2) the ability of *Pglyrp1* to control the composition of the intestinal microbiome (22, 27); and 3) the microbiome-mediated effect of *Pglyrp2* and *Pglyrp3* on the sensitivity to colitis (22, 26, 27).

Because clinical correlation studies cannot show the causative effect of microbiome, we used the same mouse model of HDM-induced allergic asthma that in two previous independent studies revealed a greater resistance of *Pglyrp1*^{-/-} mice to HDM-induced asthma (21, 28). To prove the effect of *Pglyrp1*-controlled microbiome, we used germ-free mice colonized with microbiomes from conventionally raised *Pglyrp1*^{-/-} mice or wild-type (WT) mice (*Pglyrp1*^{+/+}). We colonized pregnant mothers and their pups because clinical correlation studies and some experimental data suggested that the exposure to diverse microbiome in the first few months of life is especially critical for subsequent development of resistance to asthma and other allergic diseases (34, 36–39, 41–44). Our results show that the decreased responsiveness to HDM-induced asthma seen in *Pglyrp1*^{-/-} mice can be transferred to WT germ-free mice by colonization of pregnant mothers and their pups with microbiome from *Pglyrp1*^{-/-} mice.

Materials and Methods

Mice

Pglyrp1^{-/-} and WT mice, both on BALB/c background, were generated and maintained as described previously (20–22). The original founder WT BALB/c mice were obtained from Harlan-Sprague-Dawley. All WT and *Pglyrp1*^{-/-} mice on BALB/c background were bred and kept under conventional pathogen-free conditions in the same room in our facility to minimize the influence of differences in the environment. For each experiment, mice from several different cages and breeder pairs were used. To avoid changes in microbiome that could accumulate over an extended period of time, we backcross our *Pglyrp1*^{-/-} mice to WT females once every 2 y and rederive our homozygous knockout breeding pairs. The latter strategy also minimized genetic drift in the population. Some investigators use knockout and WT littermates from heterozygous breeding pairs to minimize parent-to-parent and cage-to-cage variations. We do not use this strategy for two reasons. First, this strategy may skew the results to the particular microbiota present only in this breeding pair. Second, and most importantly, we noticed that the effect of innate immunity genes on the composition of microbiota is not instantaneous, but takes time, and stabilization of microbiota characteristic of a given knockout takes more than one generation and thus cannot be accomplished in one generation (25–27). The BALB/c background of knockout mice and their negative status for all common viral and bacterial pathogens and parasites (including negative PCR stool tests for mouse norovirus) were confirmed as previously described (22, 27).

Germ-free BALB/c mice, female and male, 4–5 wk old, were obtained from the University of North Carolina National Gnotobiotic Rodent Resource Center (Chapel Hill, NC). Germ-free outbred Swiss Webster mice, female and male, 4–5 wk old, were obtained from Taconic Farms (Hudson, NY). Germ-free mice were received in sterile containers and kept and handled under sterile conditions (including feeding with sterile food and sterile drinking water) in sterile isolators in a separate room devoted to keeping these mice for the entire duration of each experiment.

All experiments on mice were performed according to the guidelines of and approved by the Indiana University School of Medicine–Northwest Institutional Animal Care and Use Committee (approval number IUSM-NW-IACUC-41) and U.S. Army Medical Research and Materiel Command Animal Care and Use Review Office. All efforts were made to minimize suffering of animals.

Depletion of microbiota with antibiotics

We used an established non-germ-free model of WT mice depleted of their gastrointestinal tract microbiota by a 3- to 4-wk treatment of breeding pairs of conventional BALB/c mice with antibiotics in drinking water (containing 100 µg/ml ciprofloxacin, 250 µg/ml metronidazole, and 20 mg/ml Kool-Aid mix) (26, 27, 45, 46). Anaerobic stool cultures at the end of antibiotic treatment were negative (<10³ bacteria per gram of feces, freshly collected into a reducing buffer). Microbiota-depleted pregnant mothers 1 wk before delivery were then given sterile drinking water for 2 d and colonized with microbiota from WT or *Pglyrp1*^{-/-} mice.

Preparation of feces and oropharyngeal washings for colonization

Fresh feces (20 freshly defecated pellets = 300 mg/mouse) were collected and pooled from 24 female 8-wk-old mice of each strain (WT or *Pglyrp1*^{-/-}). For each strain, mice originated from six to seven different litters from different parents (three to four mice per litter) and were weaned into separate cages, but all were kept in the same room in our animal facility. Feces were immediately placed in a preserving buffer (Dulbecco's PBS with 0.15% thioglycollate, 0.05% cysteine, 15% glycerol) on ice to maintain the original composition of live bacteria, including anaerobes, homogenized with Polytrone, and filtered through a 100-µm nylon cell strainer (BD Falcon) to remove debris (22, 27). The concentration of the fecal suspension from both strains of mice was equalized based on OD₆₆₀, and aliquots were kept at -80°C and thawed out once. All colonization experiments were done with the same batches of stools pooled from WT or *Pglyrp1*^{-/-} mice.

Oropharyngeal microbiota were collected from isoflurane-anesthetized WT or *Pglyrp1*^{-/-} (48 each) 8-wk-old female mice by vigorously washing the posterior oral cavity and oropharynx five times with 50 µl of preserving buffer (Dulbecco's PBS with 0.15% thioglycollate, 0.05% cysteine, 15% glycerol). The washings were combined from each strain and centrifuged at 10,000 × g at 4°C for 15 min, and the sediment was resuspended in 1/10 original volume of preserving buffer, aliquoted, and kept at -80°C. All colonization experiments were done with the same batches of oropharyngeal washings pooled from WT or *Pglyrp1*^{-/-} mice.

Colonization with microbiota

Several harems of two female and one male germ-free WT BALB/c or outbred Swiss Webster mice or antibiotic-treated conventional BALB/c mice were set up, and from each harem 1 wk before delivery, one pregnant female was randomly assigned for colonization with microbiota from WT mice and the other female for colonization with microbiota from *Pglyrp1*^{-/-} mice. All mice were colonized by three methods: 1) gavaging into the stomach with 10 mg of preserved stools (equivalent of 5 × 10⁹ bacteria in 0.2 ml) (22, 27), 2) instilling 5 µl of preserved oropharyngeal washings into the mouth, and 3) cohousing for 1 wk with three female conventionally raised WT or *Pglyrp1*^{-/-} BALB/c mice. All mice were simultaneously colonized by these three methods to assure as complete as possible transfer of WT or *Pglyrp1*^{-/-} microbiota. Colonized mice were kept in sterile cages with sterile food and water. Colonization by gavaging and oral instillations was repeated weekly on nursing mothers until weaning and on pups starting at weaning at 4 wk of age and was continued throughout the experiment (as shown in the figures) to maintain the microbiota that were transferred (WT or *Pglyrp1*^{-/-}) and to avoid reversal of the *Pglyrp1*^{-/-} microbiota to the WT microbiota during the course of the experiment because the colonized mice were of WT genotype.

HDM asthma model

To induce asthma-like lung inflammation, female mice were sensitized 5 d/wk for 5 wk with 10 µl (25 µg) per application of purified HDM

allergen (from *Dermatophagoides pteronyssinus*, Der p 1, catalog no. XPB70D3A25, endotoxin content of ~400 endotoxin units per milligram of protein; Stallergenes Greer, Lenoir, NC) in PBS instilled into the nose under isoflurane anesthesia (3–5, 21). Three days after the last sensitization, mice were evaluated for the extent of asthma and inflammatory response in the lungs and microbiome composition. Unsensitized similarly colonized mice were used as controls.

Lung airway resistance

Lung airway resistance was measured in response to aerosol of 0.25 to 8 mg/ml of methacholine, as previously described (21). Mice were anesthetized by s.c. injection of 0.3 ml of a mixture of 6.7 mg/ml ketamine hydrochloride, 0.67 mg/ml xylazine, and 0.2 mg/ml acepromazine; an incision was made to access the trachea; and intratracheal cannula was inserted. Mice were placed on a warming pad in the Buxco Fine Pointe RC System (Buxco Research Systems, Wilmington, NC), and intratracheal cannula was connected to the respirometer, which maintained respirations at 140 respirations per minute, delivered methacholine aerosol intratracheally, and measured lung airway resistance and compliance in response to methacholine (3–5, 21). The presented results are resistance data, and the compliance data are not shown because they mirror the resistance with similar statistically significant differences.

Bronchoalveolar lavage, lung cells, blood, cytostaining, and histology

To collect bronchoalveolar lavage (BAL) fluid, after measuring airway resistance, lungs were lavaged five times with 0.9 ml PBS. Total numbers of cells in the BAL fluid were then counted, and cells from the BAL fluid were spun onto microscope slides in a cytocentrifuge, fixed with methanol at 4°C for 5 min, dried, and stained with Diff Stain Kit (no. K7128; IMEB Inc, San Marcos, CA), followed by differential counts of inflammatory cells (21). Blood was collected for serum IgE assay, and lungs were excised. For inflammatory cell counts, lungs were minced into ~1-mm pieces in RPMI 1640 with 5% FBS with 3 mg/ml of collagenase D and 0.15 µg/ml DNase I and digested for 3 h at 37°C (21), followed by cytospin and staining with Diff Stain Kit. For histology, lungs were placed in 10% Buffered Protocol Formalin for 48 h, processed for paraffin embedding and periodic acid–Schiff (PAS) or H&E staining, and evaluated microscopically (21). Histological changes in the lungs (cellular infiltration and goblet cell hyperplasia and metaplasia) were evaluated semiquantitatively on a scale of 0–4 (0 = no change compared with unsensitized mice; 1–4 = minimum to maximum change in sensitized mice).

IgE, cytokine, and chemokine ELISAs

The amounts of IgE, IL-4, IL-5, IL-13, CCL11, CCL17, CCL22, and CCL24 in the lungs, BAL fluid, and serum were measured by ELISA, as previously described (21), using detection kits from BioLegend (IgE, IL-4, IL-5) or R&D Systems (IL-13, CCL11, CCL17, CCL22, CCL24). Lungs (100 mg) were homogenized in 400 µl of PBS (without Ca/Mg) with 1 mM EDTA, 1 mM PMSF, 1:100 dilution of protease inhibitors (P1860; Sigma-Aldrich), and 0.5% Triton X-100 by shaking with tungsten carbide beads at 4°C for 20 min, followed by centrifugation at $13,000 \times g$ at 4°C for 15 min. All the cytokines and chemokines were below the level of detection in the BAL fluid.

Sample collection, DNA extraction, and PCR amplification for microbiome analysis

Freshly defecated fecal pellets, lungs, and sediments from $10,000 \times g$ centrifugation of oropharyngeal washings and BAL fluid (collected at the time of asthma evaluation) were homogenized by shaking with silica beads for 15 min at 4°C and used for extraction of DNA with the DNeasy PowerSoil kit (Qiagen). DNA from feces and oropharyngeal washings was used directly for the sequencing of 16S rRNA genes, but DNA from BAL fluid and lungs was amplified by two-step nested PCR with Advantage 2 Taq Polymerase (Takara Bio) because of low DNA yield. The first PCR was with universal eubacterial 8F (5'-AGAGTTT-GATCCTGGCTCAG-3') and 1492R (5'-CGGYTACCTTGTTCAG-CTT-3') primers (94°C for 5 min, 32 cycles at 94°C for 45 s and 55°C for 45 s, 72°C for 1 min, and 72°C for 7 min). In the second PCR, the products from the first amplification were used as DNA template with 16S universal eubacterial primers 515F (5'-GTGCCAGCMGCCGCG-GGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') for V4 V regions (94°C for 5 min, 32 cycles at 94°C for 45 s and 54°C for 35 s, 72°C for 45 s, and 7 min at 72°C). The ~291-bp amplification products were then gel purified using QIAquick Gel Extraction kit (Qiagen) and used for 16S sequencing.

Sequencing of 16S rRNA genes and microbiome analysis

Genetic phylotyping was performed using Illumina sequencing of the V4 V regions of bacterial 16S rRNA genes. We selected the V4 region because the majority of previous studies comparing microbiomes in asthma were based on V4 region sequencing (47). We used 16S universal eubacterial primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTAC-HVGGGTWTCTAAT-3') with barcodes on the forward primer and 30 (for purified DNA) or 5 (for PCR products) PCR cycles and HotStarTaq Plus Master Mix Kit (Qiagen) under the following conditions: 94°C for 3 min; followed by 30–35 cycles of 94°C for 30 s, 53°C for 40 s, and 72°C for 1 min; and final elongation at 72°C for 5 min. After amplification, PCR products were checked in 2% agarose gel to confirm the success of amplification and the relative intensity of bands. Multiple samples were pooled, purified using calibrated Ampure XP beads, and used to prepare Illumina DNA library. Sequencing was performed at MR DNA Molecular Research LP (Shallowater, TX) on a MiSeq following the manufacturer's guidelines. Sequence data were processed using MR DNA analysis pipeline (www.mrdnalab.com) (27, 48). In summary, sequences were joined and depleted of barcodes, and then sequences <150 bp and sequences with ambiguous base calls were removed. Sequences were then denoised, operational taxonomic units (OTUs) were generated, and chimeras were removed. OTUs were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated database derived from the National Center for Biotechnology Information and Ribosomal Database Project II (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu) and assigned to species at >97% identity to the reference sequence (<3% divergence), to genus at 95–97% identity, to family at 90–95% identity, to order at 85–90% identity, to class at 80–85% identity, to phylum at 77–80% identity, and as unclassified at <77% identity. Further detailed phylogenetic diversity and taxonomic analyses were performed using Quantitative Insights Into Microbial Ecology (QIIME; http://qiime2.org/) (49). Briefly, the sequences were demultiplexed and quality filtered using the DADA2 (50) and feature tables were generated. Phylogenetic trees were generated using Fast-Tree for phylogenetic diversity analysis, and Greengenes (51) 16S rRNA database (version gg-13-8) was used for taxonomic assignments. The α -diversity matrices, such as the Shannon diversity index ($H = -\sum p_i \ln p_i$) and Shannon equitability index ($E_H = H/\ln(S)$) (where p_i is the proportion of the i th OTU and S is the total number of OTUs), and Faith phylogenetic distance were also calculated. β -diversity indices (weighted and unweighted Unifrac matrices, Jaccard distance, Bray Curtis) were calculated, and plots and F , p , and q values were generated in QIIME. For differential abundance analysis, QIIME2 plugin Gneiss was also used (https://biocore.github.io/gneiss/). The entire microbiome sequencing data have been deposited in the National Center for Biotechnology Information Sequence Read Archive (accession no. PRJNA565191, http://www.ncbi.nlm.nih.gov/bioproject/565191).

Statistical analysis

All quantitative lung function and immunological test results are presented as means $\pm$ SEM, with statistical significance of the differences between groups determined by the two-sample two-tailed Student t test, with p and n (number of mice per group = independent biological replicates) indicated in the figure legends; $p \leq 0.05$ was considered significant. The differences in microbiome α diversity were determined using Kruskal–Wallis test, and differences in β diversity were determined using two-sample two-tailed Student t test and permutational multivariate ANOVA, followed by calculation of false discovery rate (FDR) q values, using QIIME2 for overall differences (Supplemental Table I) and the Benjamini–Hochberg procedure (52) using the qqvalue package in STATA (53) for differences between the abundance of individual taxonomic units, with a significance threshold of $q \leq 0.05$. Spearman rank test R program (https://www.r-project.org/) was used to determine correlations between various parameters of asthmatic response and abundance of bacterial genera, with the r values presented as correlation heatmaps. The microbiome abundance heatmaps were generated using Java TreeView and represent mean fold changes in *Pglyrp1*^{−/−} mice relative to WT mice, after converting <1 ratios to negative fold difference using the formula $(-1)/\text{ratio}$.

Results

Germ-free BALB/c mice colonized with microbiota from *Pglyrp1*^{−/−} mice have reduced response to HDM-induced asthma

We mated germ-free WT BALB/c male and female mice, and then we colonized pregnant females with microbiota from either WT or

from *Pglyrp1*^{-/-} mice by gavaging with preserved fresh stools, instilling oropharyngeal washings, and cohousing with conventionally raised mice. We continued recolonizing nursing mothers until weaning, and then we continued recolonizing pups after weaning throughout the entire experiment. At 6 wk of age, we began intranasal sensitization of the pups with HDM allergen, which we continued for 5 wk to induce chronic asthma-like lung inflammation. We then measured the severity of asthma and lung and airways inflammation using lung function and histopathologic and immunologic tests (Fig. 1A).

HDM-sensitized germ-free WT BALB/c mice colonized with microbiota from *Pglyrp1*^{-/-} mice, compared with germ-free mice colonized with microbiota from WT mice, had significantly reduced lung airway resistance (~25% difference), which is a measure of hyperresponsiveness of airway smooth muscle cells to methacholine

(Fig. 1B). Methacholine is a muscarinic receptor agonist, which induces bronchoconstriction, characteristically more severe in asthma. These results demonstrate significantly lower lung resistance in germ-free mice colonized with microbiota from *Pglyrp1*^{-/-} than in mice colonized with microbiota from WT mice and indicate that microbiota from *Pglyrp1*^{-/-} mice can diminish lung resistance response in allergic asthma.

We then compared the numbers and types of inflammatory cells in dissociated lungs and in BAL fluid in HDM-sensitized germ-free mice colonized from birth with microbiota from either WT or *Pglyrp1*^{-/-} mice because allergic asthma is characterized by pronounced inflammatory response in the lungs. Inflammatory cells are mostly undetectable in BAL fluid and lungs in unsensitized mice (21). High numbers of inflammatory cells were found in the lungs and BAL fluid of mice colonized with microbiota

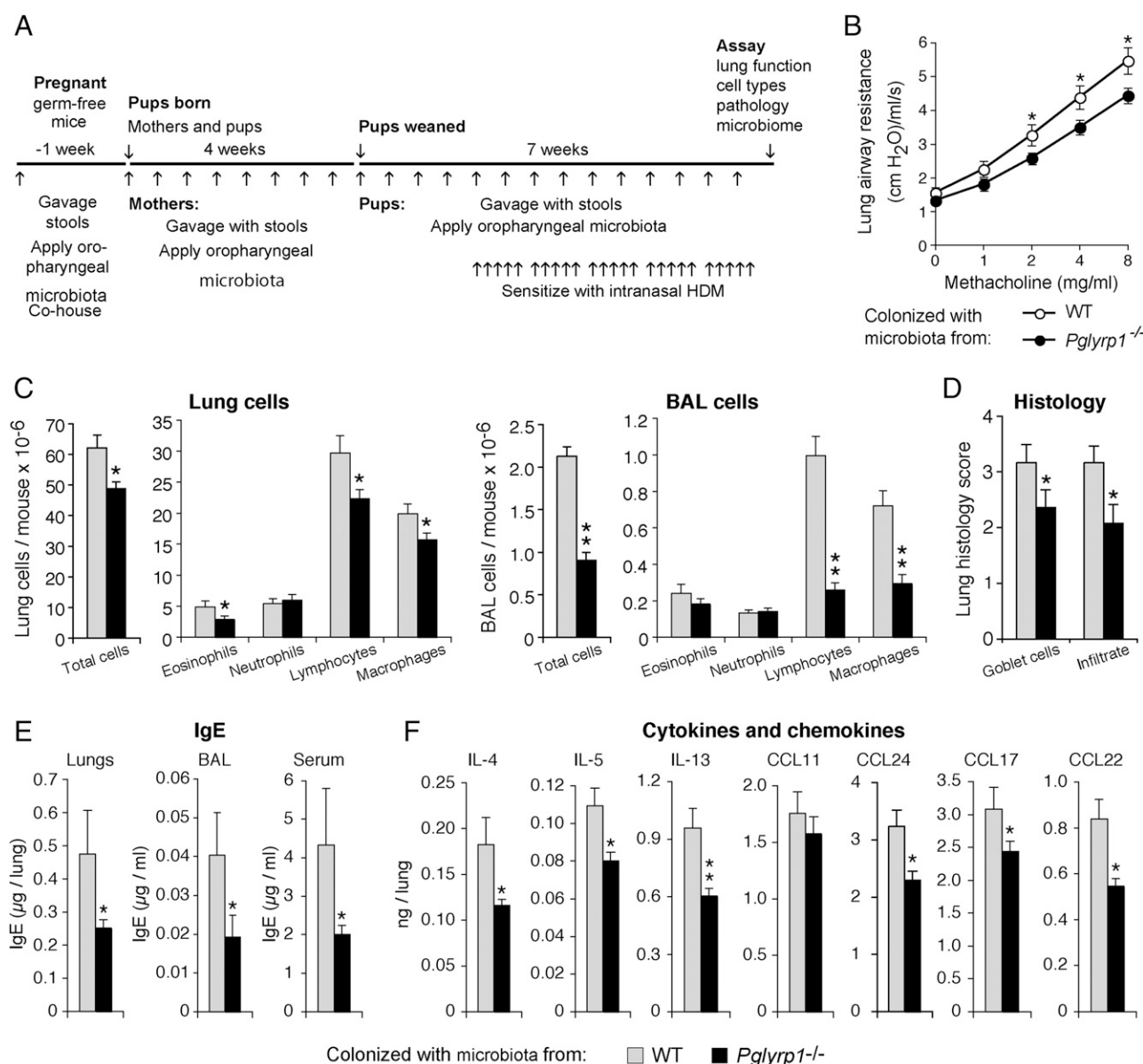


FIGURE 1. Germ-free BALB/c mice colonized with microbiota from *Pglyrp1*^{-/-} mice have low lung airway resistance, few inflammatory cells, and low levels of IgE, cytokines, and chemokines in the lungs following HDM sensitization. (A) Experimental timeline for colonization of germ-free pregnant WT BALB/c mice and pups with fecal and oropharyngeal microbiota from WT or *Pglyrp1*^{-/-} mice, sensitization of pups with HDM, and assays. (B) Lung airway resistance in response to methacholine; (C) inflammatory cells in lungs and BAL fluid; (D) lung histopathology scores; (E) IgE in the lungs, BAL fluid, and serum; and (F) IL-4, IL-5, IL-13, CCL11 (eotaxin 1), CCL24 (eotaxin 2), CCL17, and CCL22 in the lungs in germ-free WT BALB/c mice colonized with microbiota from WT or *Pglyrp1*^{-/-} mice and sensitized with HDM. The results are means ± SEM of 12–14 mice per group. **p* < 0.05, ***p* < 0.001 for WT versus *Pglyrp1*^{-/-} groups (*t* test).

from WT mice, including lymphocytes, macrophages, eosinophils, and neutrophils, whereas germ-free mice colonized with microbiota from *Pglyrp1*^{-/-} mice had a significantly lower total number of inflammatory cells, lymphocytes, and macrophages in both lungs and BAL fluid and eosinophils in lungs than mice colonized with microbiota from WT mice (~30–70% difference in lung cells and ~140–280% difference in BAL cells) (Fig. 1C). These results demonstrate significantly lower cellular infiltration of HDM-sensitized lungs in germ-free mice colonized with microbiota from *Pglyrp1*^{-/-} mice than with microbiota from WT mice and indicate that microbiota from *Pglyrp1*^{-/-} mice can diminish the severity of lung inflammation in allergic asthma.

We then compared lung histology of unsensitized and HDM-sensitized colonized germ-free mice to determine the pathologic manifestations of this lower response to HDM sensitization in mice colonized with microbiota from *Pglyrp1*^{-/-} mice. Both groups of unsensitized mice showed normal histology and no PAS⁺ cells. HDM-sensitized germ-free mice colonized with microbiota from WT mice showed massive inflammatory cell infiltrates mainly around bronchi and bronchioles and also around pulmonary arteries and veins along the bronchial tree (Fig. 2A). The inflammatory infiltrates contained numerous mononuclear cells, eosinophils, and neutrophils. These mice also showed numerous large PAS⁺ mucin-producing goblet cells lining the bronchial air spaces, showing pronounced goblet cell hyperplasia and metaplasia, which indicate primarily bronchial involvement and extensive remodeling. Additionally, there was smooth muscle hypertrophy and fibrosis, also indicative of remodeling. All of these histopathologic changes are highly characteristic of allergic asthma (1–5). By contrast, HDM-sensitized germ-free mice colonized with microbiota from *Pglyrp1*^{-/-} mice had significantly smaller cellular infiltrates, consistent with lower inflammatory cell counts (in Fig. 1C), and significantly less hyperplasia and metaplasia of PAS⁺ mucin-producing goblet cells (Figs. 1D, 2). These results demonstrate less severe histopathologic changes characteristic of asthma in HDM-sensitized lungs in mice colonized with microbiota from *Pglyrp1*^{-/-} mice, compared with mice colonized with microbiota from WT mice, and further indicate that microbiota from *Pglyrp1*^{-/-} mice can diminish inflammatory lung infiltrates in allergic asthma.

We then compared the levels of IgE in the lungs and serum in HDM-sensitized colonized germ-free mice because atopic allergies, including allergic asthma and atopic dermatitis, are characterized by increased local and systemic production of IgE. HDM-sensitized germ-free mice colonized with microbiota from WT mice had high levels of IgE in lung homogenates, BAL fluid, and serum, whereas similarly sensitized germ-free mice colonized with microbiota from *Pglyrp1*^{-/-} mice had significantly lower levels of IgE in the lungs, BAL fluid, and serum (90–215% difference) (Fig. 1E). These results are consistent with the lower asthmatic responses of these mice and indicate that colonization of WT mice with microbiota from *Pglyrp1* mice is sufficient to reduce IgE production following HDM sensitization. These results also suggest that mice colonized with microbiota from *Pglyrp1*^{-/-} mice have lower Th2 response to HDM because high IgE production typically reflects Th2 polarization of T cell responses and Th2 polarization promotes type I hypersensitivity, including our HDM asthma model (21).

We also compared the levels of IL-4, IL-5, IL-13, CCL11 (eotaxin 1), CCL24 (eotaxin 2), CCL17, and CCL22 in the lungs of HDM-sensitized mice colonized with microbiota from WT or *Pglyrp1*^{-/-} mice because IL-4, IL-5, and IL-13 are the main Th2 cytokines; CCL11 and CCL24 are the main eosinophil-attracting chemokines; and CCL17 and CCL22 are CCR4 ligands involved

in Th2 cell recruitment. HDM-sensitized germ-free mice colonized with microbiota from *Pglyrp1*^{-/-} mice had significantly lower levels of IL-4, IL-5, IL-13, CCL24, CCL17, and CCL22 (but not CCL11) in the lungs than similarly sensitized germ-free mice colonized with microbiota from WT mice (25–60% difference) (Fig. 1F). In the serum, concentrations of the cytokines were below the level of detection and concentrations of the chemokines were not significantly different (data not shown). These results are consistent with the cell counts and histopathological findings (Figs. 1, 2) and suggest that colonization of WT mice with microbiota from *Pglyrp1*^{-/-} mice reduces local (in the lung) Th2-promoting and eosinophil-attracting proinflammatory responses.

Several characteristics of the increased resistance to asthmatic inflammation in germ-free mice colonized with microbiota from *Pglyrp1*^{-/-} mice showed high positive correlation ($r \geq 0.6$, Fig. 2B). Lower airway resistance correlated with lower numbers of total inflammatory cells, macrophages, and PAS⁺ goblet cells; low overall cell infiltration; and lower levels of IL-13, CCL11, CCL24, CCL17, and CCL22 in the lungs. Lower total numbers of inflammatory cells, eosinophils, macrophages, and PAS⁺ goblet cells and low overall cell infiltration correlated with lower levels of CCL24. Lower numbers of eosinophils and PAS⁺ goblet cells also correlated with lower levels of CCL11, and lower numbers of macrophages, in addition, correlated with lower levels of IL-13, CCL17, and CCL22 in the lungs. Lower numbers of eosinophils in the BAL fluid correlated with lower levels of IgE in the lungs.

Altogether, our results demonstrate that the low responsiveness to allergic asthma in *Pglyrp1*^{-/-} mice is due to the effect of *Pglyrp1* deficiency on the microbiome because this effect can be fully reproduced by colonization of germ-free BALB/c mice with microbiome from *Pglyrp1*^{-/-} mice. These results thus indicate that *Pglyrp1* in WT mice promotes proinflammatory (“proasthmatic”) microbiota and also suggest that these microbiota are lost or diminished in *Pglyrp1*^{-/-} mice.

*Germ-free Swiss Webster mice colonized with microbiota from *Pglyrp1*^{-/-} mice have reduced response to HDM-induced asthma*

The effect of microbiome on the sensitivity to asthma is likely highly dependent on the genetic background and genetic predisposition of the host, as shown by several clinical correlation and epidemiologic studies (41, 43, 44, 54). BALB/c mice are genetically predisposed to Th2 responses and development of IgE-mediated allergic and asthmatic responses (21, 28), but development of asthma is not always dependent on Th2 bias, as asthma can be classified into “Th2-high” and “Th2-low” subgroups (55). Therefore, to determine the role of genetic predisposition in the effect of *Pglyrp1*-controlled microbiome on the development of asthma, we performed similar colonization and asthma-induction experiments using germ-free outbred (WT) Swiss Webster mice, which are genetically more resistant to allergic asthma than BALB/c mice and have no Th2 bias. We used the same protocol for mating, colonization, and induction of asthma in colonized pups of germ-free outbred Swiss Webster mice as we used in germ-free BALB/c mice (Fig. 1A), and we then similarly measured the severity of asthma and lung and airways inflammation.

HDM-sensitized germ-free outbred Swiss Webster mice colonized with microbiota from *Pglyrp1*^{-/-} mice had significantly reduced lung airway resistance in response to methacholine (29% difference) (Fig. 3A), significantly lower numbers of all inflammatory cell types in the lungs and BAL fluid (~45–240% difference in lung cells and ~60–170% difference in BAL cells) (Fig. 3B), and less-pronounced infiltrates in the lungs (Fig. 3E), compared with germ-free mice colonized with microbiota from WT mice.

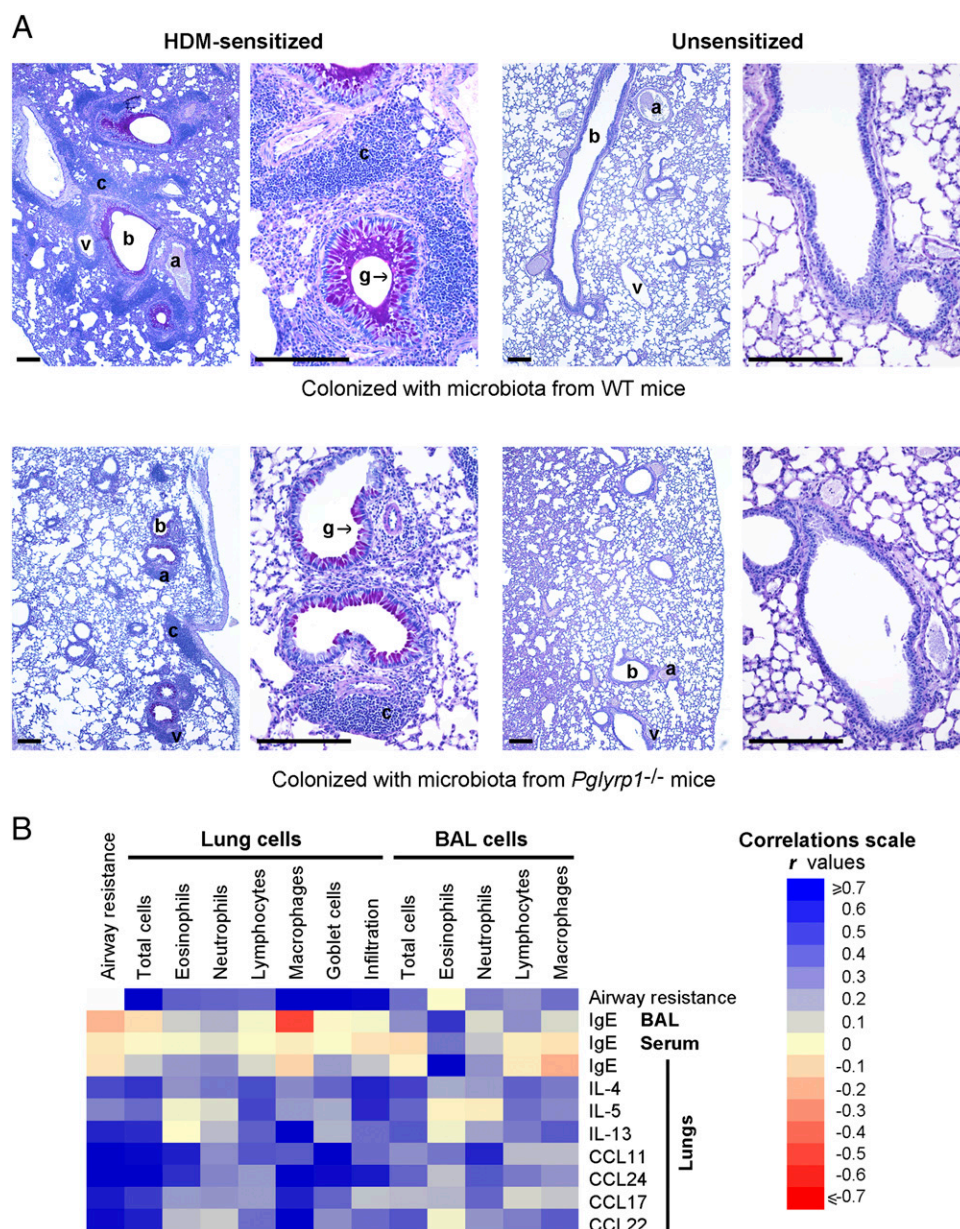


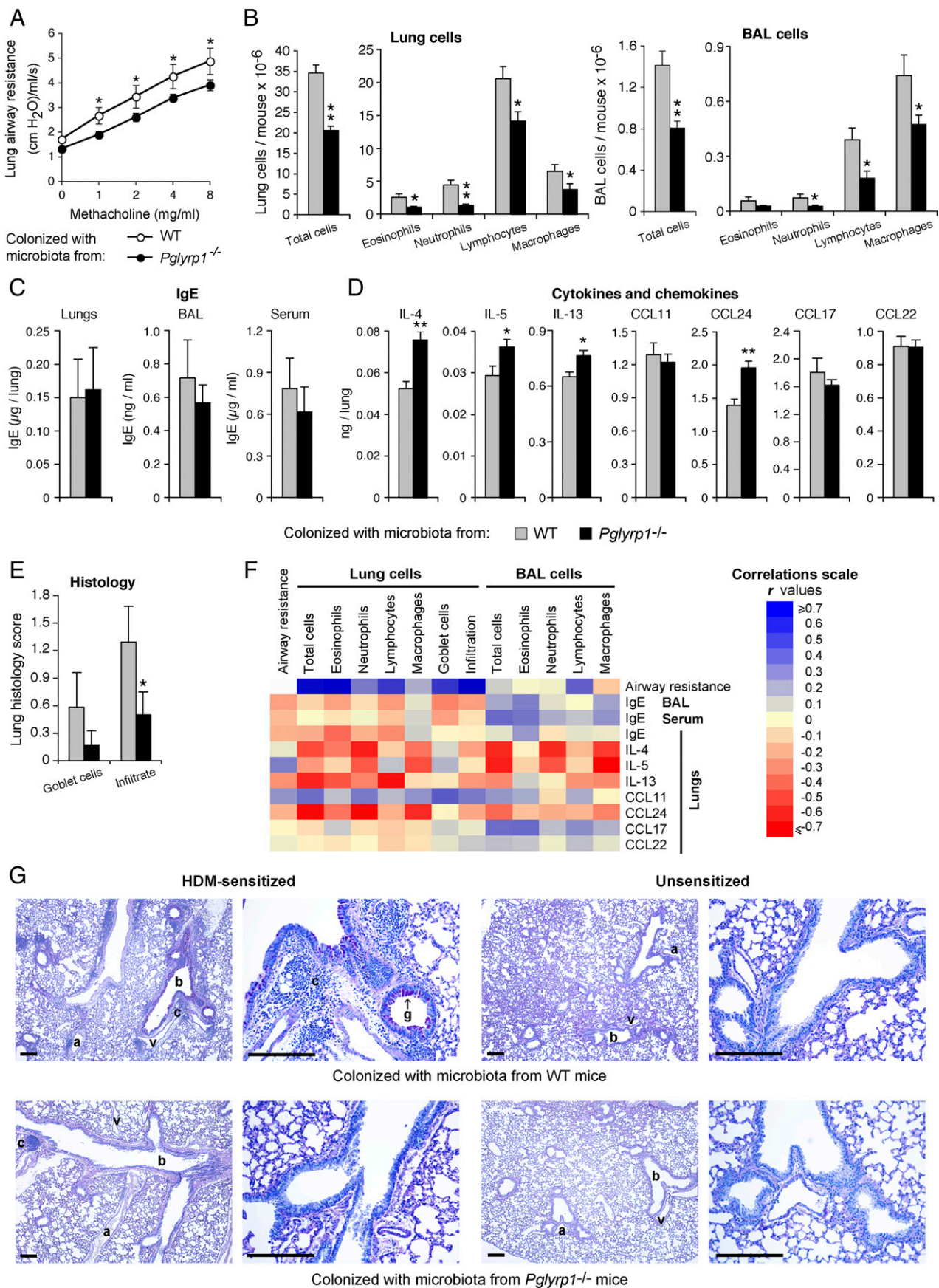
FIGURE 2. Germ-free BALB/c mice colonized with microbiota from *Pglyrp1*^{-/-} mice have low cell infiltration and goblet cell hyperplasia and metaplasia in the lungs following HDM sensitization, and their decreased air resistance and cell numbers in the lungs and BAL correlate with decreased IgE, cytokine, and chemokine levels in the lungs. **(A)** PAS staining of lung sections with massive inflammatory cell infiltrates (c) around bronchi and bronchioles (b), pulmonary arteries (a), and veins (v) along the bronchial tree and numerous large PAS⁺ mucin-producing goblet cells (g) lining the bronchial air spaces in HDM-sensitized mice colonized with microbiota from WT mice (top left) and much lower infiltrations and fewer and smaller PAS⁺ goblet cells in mice colonized with microbiota from *Pglyrp1*^{-/-} mice (bottom left). Normal histology in similarly colonized unsensitized mice is shown on the right. Low- and high-magnification views are on the left and right (respectively) for each panel; scale bars, 100 μ m. **(B)** Correlation between the airway resistance and cell numbers in lungs and BAL and IgE, cytokine, and chemokine levels in germ-free WT BALB/c mice with HDM-induced asthma colonized with microbiota from WT or *Pglyrp1*^{-/-} mice. Mice were colonized as shown in Fig. 1A, and samples were collected at the time of lung function assays. The *r* values were calculated by Spearman rank test; *n* = 12–14 mice per group.

However, the absolute numbers of cells (especially eosinophils) were lower than in BALB/c mice (Fig. 1C).

HDM-sensitized germ-free Swiss Webster mice colonized with microbiota from WT mice had inflammatory cell infiltrates around bronchi, bronchioles, and pulmonary arteries and veins and some PAS⁺ goblet cells lining the bronchial air spaces (Fig. 3G), but both were much less prominent than in similarly colonized and sensitized BALB/c mice (Fig. 2A). Germ-free Swiss Webster mice colonized with microbiota from *Pglyrp1*^{-/-} mice and sensitized with HDM showed very few cell infiltrates and almost no PAS⁺ goblet cells, compared with mice colonized with microbiota from

WT mice (Fig. 3E, 3G). Unsensitized germ-free Swiss Webster mice colonized with microbiota from either WT or *Pglyrp1*^{-/-} mice showed normal histology and no PAS⁺ cells.

There was no difference in the levels of IgE in the lungs, BAL fluid, and serum in HDM-sensitized germ-free Swiss Webster mice colonized with microbiota from WT and *Pglyrp1*^{-/-} mice (Fig. 3C), and these levels of IgE were much lower than in similarly colonized and sensitized BALB/c mice (Fig. 1E). Moreover, the levels of CCL11, CCL17, and CCL22 in the lungs were similar in both groups of Swiss Webster mice, but unexpectedly, the levels of IL-4, IL-5, IL-13, and CCL24 were actually higher in



the lungs of Swiss Webster mice colonized with microbiota from *Pglyrp1*^{-/-} mice than from WT mice (Fig. 3D). In the serum, concentrations of the cytokines were below the level of detection and concentrations of the chemokines were not significantly different (data not shown). Lower airway resistance showed positive correlation with lower numbers of total inflammatory cells, eosinophils, lymphocytes, goblet cells, and low overall cell infiltration (as expected), but again, unexpectedly low numbers of several inflammatory cells showed negative correlation with increased levels of IL-4, IL-5, IL-13, and CCL24 (Fig. 3F).

Altogether, these results are consistent with general lower sensitivity of Swiss Webster mice to asthma than BALB/c mice and demonstrate that the full effect of microbiome on asthma requires allergy-prone genetic background of the host because in outbred asthma-resistant Swiss Webster mice, colonization with microbiota from *Pglyrp1*^{-/-} mice significantly diminishes only some, not all, aspects of lung inflammation. However, unexpectedly increased levels of IL-4, IL-5, IL-13, and CCL24 in Swiss Webster mice colonized with microbiota from *Pglyrp1*^{-/-} mice (in which several other manifestations of asthma were less severe) suggest that unbalanced microbiome may have unpredictable effects on some aspects of immune response in some genetic backgrounds.

*Antibiotic-treated BALB/c mice colonized with microbiota from *Pglyrp1*^{-/-} mice have reduced IgE, CCL11, and CCL17 response to HDM sensitization but no changes in other manifestations of asthma*

Transfer of changes in sensitivity to asthma in germ-free mice is the ultimate proof for the role of microbiome in the sensitivity to asthma. However, germ-free condition is unnatural and unrealistic in humans, and if modifications of microbiome were to be applied in humans to increase resistance to asthma, the procedure would have to involve modification of the existing microbiome. Therefore, next we tested whether depletion of microbiome with antibiotics in conventionally raised WT BALB/c mice, followed by colonization with microbiota from *Pglyrp1*^{-/-} mice, decreases their responsiveness to HDM-induced asthma.

We depleted microbiota in conventional WT BALB/c male and female mice using 3-wk-long antibiotic treatment. We mated these mice and colonized pregnant females, nursing mothers, and their pups with microbiota from WT or from *Pglyrp1*^{-/-} mice and then sensitized 6-wk-old pups with HDM, the same way as we previously colonized and sensitized germ-free mice (Supplemental Fig. 1A). Both groups of mice (colonized with microbiota from WT or from *Pglyrp1*^{-/-} mice) had similar severity of asthma and lung inflammation (as measured by lung resistance test), extent of infiltration with inflammatory cells, and levels of IL-4, IL-5, IL-13, CCL22, and CCL24 in the lungs (Supplemental Fig. 1B–E). However, IgE levels in the lungs, BAL fluid, and serum and levels of CCL11 and CCL17 in the lungs in mice colonized with microbiota from *Pglyrp1*^{-/-} mice were significantly lower than in mice colonized with microbiota from WT mice (~100–225% difference in IgE and 25–44% difference in CCL11 and CCL17) (Supplemental Fig. 1D, 1E). In the serum, these cytokines

were not detectable, and the levels of chemokines were not significantly different (data not shown).

Despite only few significant differences in the overall inflammatory responses of these colonized conventional mice, lower airway resistance showed positive correlation ($r \geq 0.6$) with lower numbers of eosinophils in lungs and BAL and total inflammatory cells in BAL; low IgE levels in BAL, serum, and lungs; and low CCL11 levels in lungs (Supplemental Fig. 1F). Also, lower numbers of eosinophils in lungs and BAL and low total numbers of inflammatory cells in lungs showed a positive correlation with low levels of IgE in BAL and of IL-4, IL-5, CCL11, CCL24, CCL17, and CCL22 in lungs, and low numbers of neutrophils in lungs and BAL showed positive correlation with low levels of CCL11, CCL24, and CCL17 in lungs and of IgE in BAL, which indicates a trend toward low inflammatory responses in these mice.

These results indicate that depletion of microbiota with antibiotics in conventional mice and colonization with microbiota from *Pglyrp1*^{-/-} mice are not sufficient for the complete transfer of low allergic asthmatic responses, and thus, complete transfer of this low responsiveness requires colonization of germ-free recipients. However, colonization of antibiotic-treated conventional mice can still transfer low IgE, CCL11, and CCL17 responsiveness.

Differences in α and β diversity of microbiomes in colonized mice

We next analyzed α and β diversity of fecal, oropharyngeal, and lung microbiomes in germ-free WT BALB/c mice colonized with microbiota from WT or from *Pglyrp1*^{-/-} mice, using Illumina sequencing of bacterial 16S rRNA genes, to reveal correlations between the composition of these microbiomes and the sensitivity to asthma.

α -Diversity analysis revealed no significant differences in the numbers of OTUs per mouse in the feces and oropharynx between both groups of colonized mice, reflecting similar microbiome richness. However, Shannon diversity index (H) and equitability index (E_H , reflecting microbiome evenness) and also Faith phylogenetic distance were significantly lower in fecal microbiomes from germ-free mice colonized with microbiota from *Pglyrp1*^{-/-} mice than from WT mice (Supplemental Fig. 2). By contrast, in the oropharynx, Shannon diversity index and equitability index were significantly higher in germ-free mice colonized with microbiota from *Pglyrp1*^{-/-} mice than from WT mice. Lung microbiomes did not show any significant differences in α diversity (data not shown).

More striking were the differences in microbiome β diversity. Fecal microbiomes from germ-free BALB/c mice colonized with microbiota from WT or *Pglyrp1*^{-/-} mice clearly separated in the principal coordinate analysis (PCoA) and Gneiss analysis (Fig. 4A, 4B, Supplemental Table I) and had many statistically significant differences in the abundance of various bacterial groups at all taxonomic levels (Figs. 4C, 5). For example, in the feces, the abundance of five out of nine detected phyla showed statistically significant differences by both t test and FDR q test (Figs. 4C, 5): germ-free mice colonized with microbiota from *Pglyrp1*^{-/-} mice had significantly higher abundance of *Bacteroidetes*

chemokine levels; and (G) PAS staining of lung sections with moderate inflammatory cell infiltrates (c) around bronchi and bronchioles (b), pulmonary arteries (a), and veins (v) along the bronchial tree and few PAS⁺ mucin-producing goblet cells (g) lining the bronchial air spaces in HDM-sensitized Swiss Webster mice colonized with microbiota from WT mice (top left) and very few infiltrations and no PAS⁺ goblet cells in mice colonized with microbiota from *Pglyrp1*^{-/-} mice (bottom left); normal histology in similarly colonized unsensitized mice is shown on the right; low- and high-magnification views are on the left and right (respectively) for each panel; scale bars, 100 μ m. Germ-free Swiss Webster mice were colonized and sensitized with HDM as in Fig. 1A. (A)–(E) are means $\pm$ SEM. * $p < 0.05$, ** $p < 0.001$ for WT versus *Pglyrp1*^{-/-} groups (t test). (F) The r values were calculated by Spearman rank test; $n = 12$ mice per group.

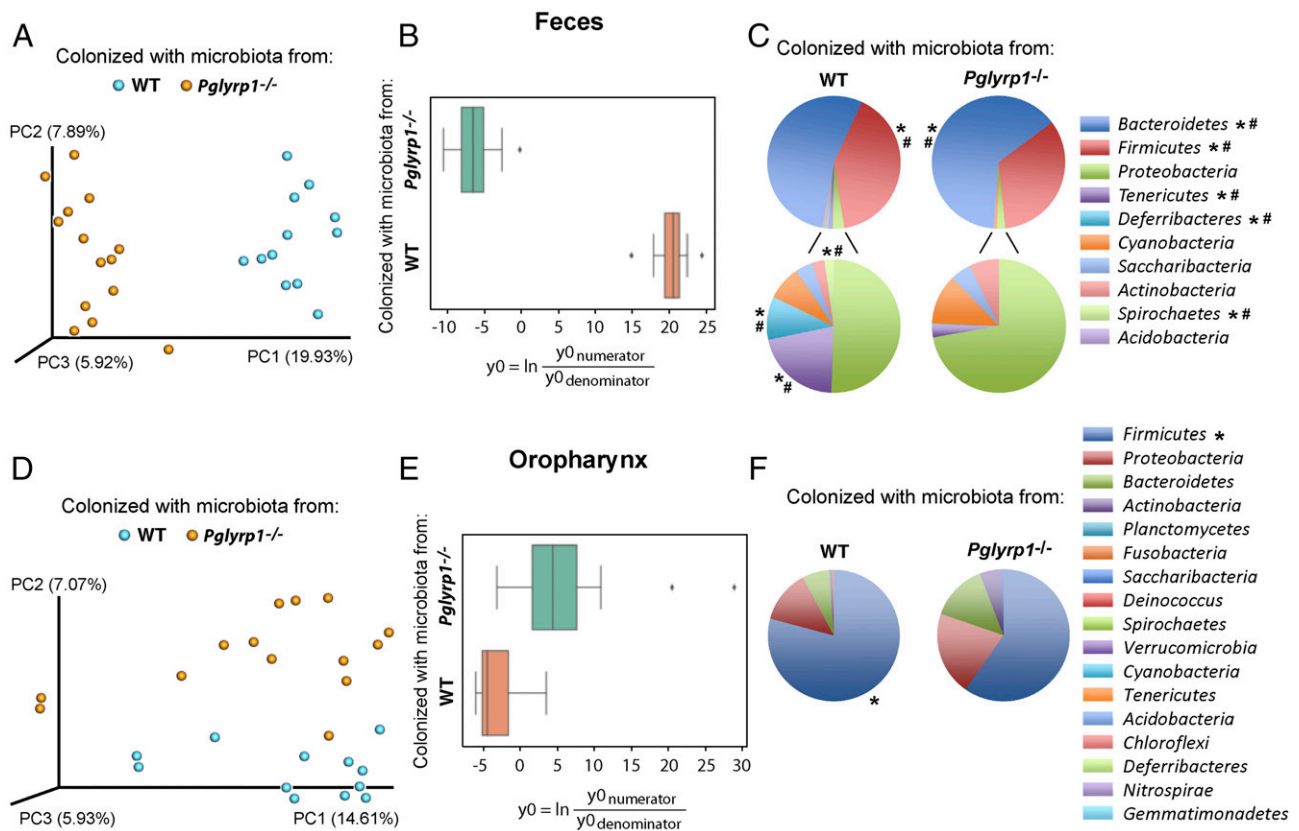


FIGURE 4. β diversity of fecal and oropharyngeal microbiomes in germ-free WT BALB/c mice colonized with microbiota from WT or *Pglyrp1*^{-/-} mice. Separation of microbiomes on unweighted UniFrac PCoA (A and D) and Gneiss analysis (B and E) and percentage of abundance of bacterial phyla (C and F). Mice were colonized as shown in Fig. 1A, and microbiome samples were collected at the time of lung function assays; $n = 12$ –14 mice per group. * t test $p < 0.05$, #FDR $q < 0.05$ for WT versus *Pglyrp1*^{-/-} groups).

and significantly lower abundance of *Firmicutes*, *Tenericutes*, *Deferribacteres*, and *Spirochaetes* than mice colonized with microbiota from WT mice. Also, the abundance in the feces of 8 of 22 bacterial classes, 9 of 34 orders, 13 of 62 families, 29 of 145 genera (Fig. 5), 63 of 321 species, and 370 of 4916 OTUs (data not shown) significantly differed by FDR q test between germ-free mice colonized with microbiota from WT and *Pglyrp1*^{-/-} mice, with the majority of these changes representing significantly lower abundance of bacteria at all taxonomic levels in mice colonized with microbiota from *Pglyrp1*^{-/-} mice (>5-fold difference in most cases) (Fig. 5).

The oropharyngeal microbiomes from germ-free BALB/c mice colonized with microbiota from WT or *Pglyrp1*^{-/-} mice showed almost complete separation on PCoA and Gneiss analysis (Fig. 4D, 4E) and significantly different abundance of one phylum (*Firmicutes*) of 18 bacterial phyla by t test but not FDR q test (Fig. 4F). At other taxonomic levels (38 classes, 79 orders, 151 families, 312 genera, 529 species, and 2822 OTUs), there were no statistically significant differences by FDR q test in β diversity of oropharyngeal microbiomes between the two groups of mice, except for higher abundance of genus *Pasteurella* in mice colonized with microbiota from *Pglyrp1*^{-/-} mice (15.9% versus 0.18%, $q = 0.039$). Microbiomes recovered from BAL fluid and lungs had no significant differences by FDR q test in β diversity between mice colonized with microbiota from WT and *Pglyrp1*^{-/-} mice (data not shown), partial separation on PCoA, and no difference on Gneiss analysis (Supplemental Fig. 3).

These results suggested that the composition of fecal microbiome may have the main influence on the sensitivity to experimental asthma and motivated us to determine which differences in

fecal microbiome correlated with which clinical manifestations of asthmatic response.

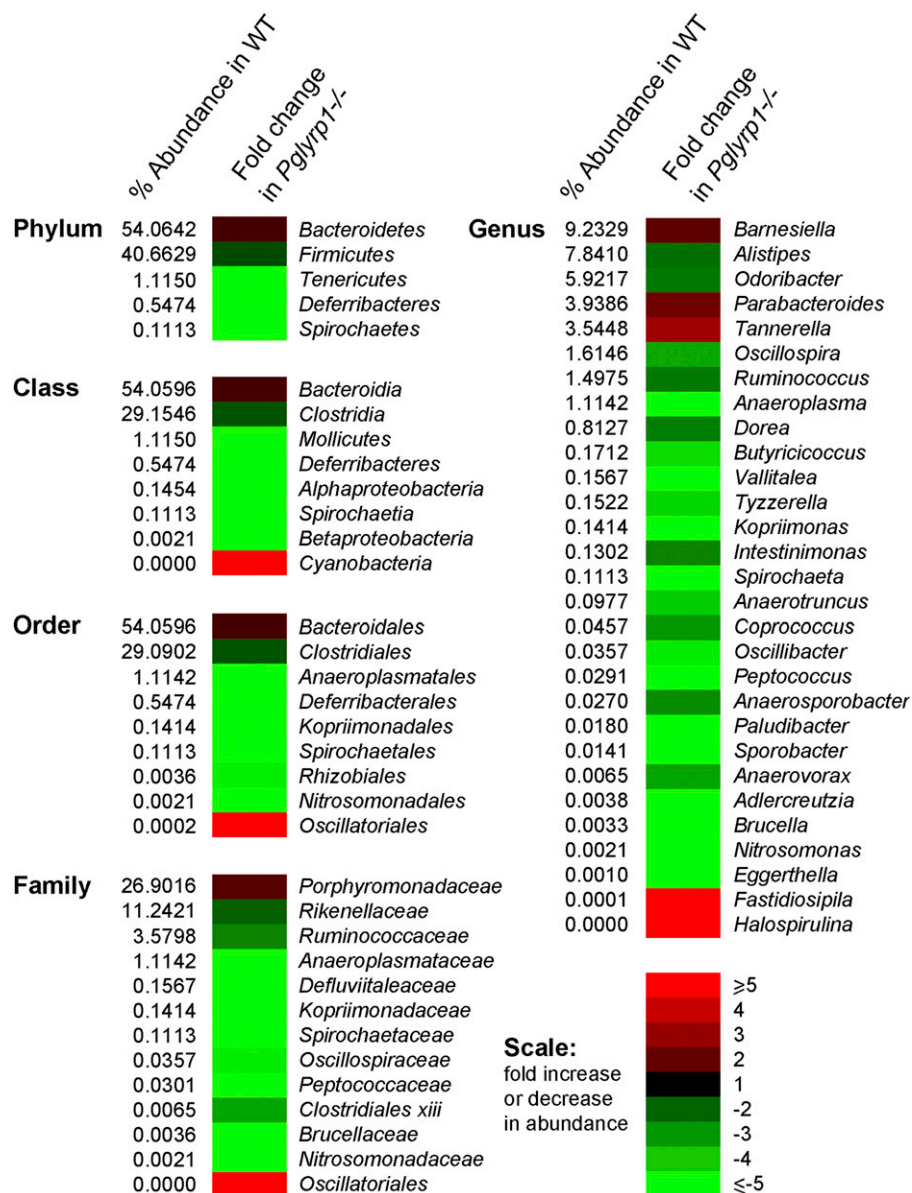
Correlations between changes in microbiome and asthmatic response

Several changes in fecal microbiome showed high correlation ($r \geq 0.6$) with several characteristics of the decreased asthmatic inflammation in germ-free mice colonized with microbiota from *Pglyrp1*^{-/-} mice (Fig. 6). Overall, lower total numbers of inflammatory cells, lymphocytes, and macrophages in the BAL fluid showed the highest correlation with changes in fecal microbiota (Fig. 6), consistent with the most significant decrease in these cells in Fig. 1C. Low levels of IL-13, IL-5, CCL22, and CCL24 and low air resistance in the lungs also frequently correlated with several changes in fecal microbiota.

These low levels of inflammatory cells, cytokines, and air resistance in the lungs most often correlated with lower abundance in the feces of several *Firmicutes* (*Vallitalea*, *Tyzzerella*, *Oscillibacter*, *Peptococcus*, *Sporobacter*) and some *Tenericutes* (*Anaeroplasma*), *Proteobacteria* (*Kopriimonas*, *Nitrosomonas*), *Spirochaetes* (*Spirochaeta*), *Bacteroidetes* (*Paludibacter*), and *Actinobacteria* (*Eggerthella*) (Fig. 6). Therefore, decreased abundance of these bacteria in the feces correlated with decreased asthmatic responses.

These low asthmatic responses also correlated with increased abundance in the feces of one genus of *Bacteroidetes* (*Tannerella*), one genus of *Firmicutes* (*Fastidiosipila*), and one genus of *Cyanobacteria* (*Halospirulina*) and with increased abundance in the oropharynx of one genus of *Proteobacteria* (*Pasteurella*) (Fig. 6). Therefore, increased abundance of these bacteria in the

FIGURE 5. Differences in β diversity of fecal microbiomes in germ-free WT BALB/c mice colonized with microbiota from WT or *Pglyrp1*^{-/-} mice. Heatmap representation shows fold increase (red) or decrease (green) in bacterial taxa with significantly different abundance (FDR $q < 0.05$ for WT versus *Pglyrp1*^{-/-} groups). Mice were colonized as shown in Fig. 1A, and microbiome samples were collected at the time of lung function assays; $n = 12$ –14 mice per group.



feces or oropharynx (respectively) correlated with decreased asthmatic responses.

Low numbers of neutrophils and eosinophils both in BAL fluid and lungs and low levels of IgE, CCL11, and CCL17 did not correlate or infrequently correlated with the changes in fecal and oropharyngeal microbiota (Fig. 6). These results suggest that not all aspects of inflammatory asthmatic response are equally affected by the composition of microbiome.

Altogether, our microbiome analysis shows that the composition of primarily fecal microbiome significantly correlates with most aspects of the inflammatory asthmatic response in the lungs. Our results also identify several bacteria whose abundance may either increase or decrease asthmatic responses.

Discussion

Several clinical studies showed correlation between changes in intestinal or respiratory microbiomes, especially early in life, and increased sensitivity to asthma (34, 36–39, 41–44). However, there is little experimental evidence that changes in microbiome cause changes in the susceptibility to asthma or its severity, and even less is known about the host genes that regulate asthma-promoting

and asthma-protective bacteria in the host microbiome. This study identifies *Pglyrp1* as one of the host innate immunity genes that regulates allergic asthmatic responses through its effect on the host microbiome.

In this study, we have established that decreased responsiveness to allergic asthma in *Pglyrp1*^{-/-} mice is to a large extent determined by the composition of their intestinal microbiome. Our results show that decreased responsiveness to allergic asthma could be transferred to germ-free WT mice by colonization of mothers and newborn mice with microbiota from *Pglyrp1*^{-/-} mice. This decreased responsiveness to asthma was manifested by decreased airway resistance, lower numbers of inflammatory cells in the lungs, less-severe histopathological changes in the lungs, lower levels of IgE in the lungs and serum, and lower levels of proallergic cytokines and chemokines in the lungs.

We also established that the effect of microbiome on the responsiveness to allergic asthma depends on the genetic background of the host, the method of microbiome depletion before colonization with the new microbiome, and the site of microbiome (intestinal tract, oropharynx, lungs). The microbiome-dependent decreased responsiveness to allergic asthma was most pronounced in BALB/c

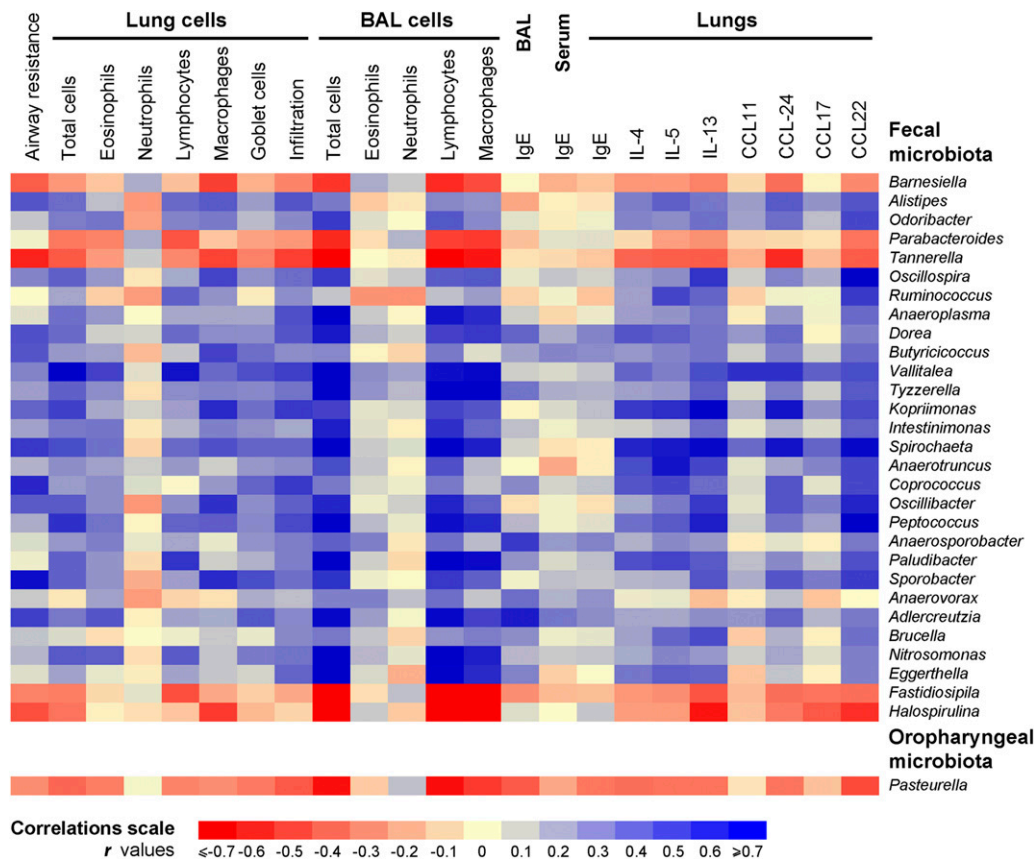


FIGURE 6. Correlation between the abundance of fecal and oropharyngeal bacterial genera and changes in the airway resistance, cell numbers in lungs and BAL, and IgE, cytokine, and chemokine levels in germ-free WT BALB/c mice with HDM-induced asthma, colonized with microbiota from WT or *Pglyrp1*^{-/-} mice. Mice were colonized as shown in Fig. 1A, and samples were collected at the time of lung function assays. Changes in bacterial abundance are shown in Fig. 5. The r values were calculated by Spearman rank test; $n = 12$ –14 mice per group.

mice, which are genetically predisposed to asthma and Th2-dependent responses, and was only partially manifested in outbred Swiss Webster mice, which have no Th2 bias and are not predisposed to allergic responses. Whereas all clinical manifestations of decreased responsiveness to asthma seen in *Pglyrp1*^{-/-} mice were manifested in WT BALB/c mice colonized with microbiota from *Pglyrp1*^{-/-} mice, only decreased airway resistance and lower numbers of inflammatory cells in the lungs were seen in Swiss Webster mice colonized with microbiota from *Pglyrp1*^{-/-} mice; their IgE, cytokine, and chemokine responses were unchanged or unexpectedly dysregulated.

Our results further show that the full effect of microbiome-dependent decreased responsiveness to allergic asthma in genetically predisposed BALB/c mice requires germ-free conditions before colonization with microbiota from *Pglyrp1*^{-/-} mice and could not be reproduced by colonization of conventional WT BALB/c mice following microbiome depletion by treatment with antibiotics, which demonstrates resilience of the established WT microbiome to subsequent changes. This resilience, however, was not complete because antibiotic-treated conventional BALB/c mice colonized with microbiota from *Pglyrp1*^{-/-} mice still showed lower IgE, CCL11, and CCL17 production and strong correlations between several aspects of decreased asthmatic responses in individual mice.

Mice with a low asthmatic response colonized with microbiota from *Pglyrp1*^{-/-} mice had many significant differences in their intestinal (fecal) microbiomes, compared with mice with a high asthmatic response colonized with microbiota from WT mice. Germ-free mice colonized with microbiota from *Pglyrp1*^{-/-} mice

had increased abundance of *Bacteroidetes* and decreased abundance of *Firmicutes*, *Tenericutes*, *Deferribacteres*, and *Spirochaetes* phyla in the feces and increased abundance of *Pasteurella* (*Proteobacteria*) in the oropharynx, compared with mice colonized with microbiota from WT mice. Many of these changes in bacterial abundance correlated with decreased responsiveness to allergic asthma, including 1) decreased abundance in the feces of five genera from *Firmicutes* and six other genera (from phyla *Tenericutes*, *Proteobacteria*, *Spirochaetes*, *Bacteroidetes*, and *Actinobacteria*), 2) increased abundance in the feces of three other genera (from phyla *Bacteroidetes*, *Firmicutes*, and *Cyanobacteria*), and 3) increased abundance in the oropharynx of one genus of *Proteobacteria* (*Pasteurella*). Thus, our results show the dominant effect of intestinal microbiome on the responsiveness to allergic asthma and identify several bacteria that may increase or decrease these allergic responses.

Our results are consistent with several clinical observations showing increased abundance of various *Proteobacteria* (30–33) in the lower and upper respiratory tract and oropharynx in patients with asthma and higher abundance in the feces or higher exposure to *Bacteroides* and lower abundance of *Firmicutes* in infants and children with lower risk of asthma and atopic allergy later in life (37–40). Our results are also consistent with experimental models of asthma showing increased abundance of *Bacteroidetes* and decreased abundance of *Firmicutes* in the stools (54, 56) of mice with increased resistance to asthma. However, the effects of microbiome on asthma are complex, as there are also several studies showing the opposite effects of changes in the gut (fecal) microbiota early in life. For example, lower abundance of

Firmicutes (entire phylum or some of its members, e.g., genus *Roseburia*, *Faecalibacterium*, and *Ruminococcus*, or family *Lachnospiraceae*) or genus *Alistipes* (*Bacteroidetes*) in the feces of young infants correlated with increased incidence of asthma later in life (41–43). The reasons for these discrepancies are not clear, but they may be due to the dynamic nature of intestinal and respiratory microbiomes, which are very different in young infants, children, and adults, as these microbiomes have different effects at different stages of infant and child development (57). The effects of microbiome on asthma also depend on the host genetic background, as they often manifest only in children of asthmatic mothers (43). This observation is consistent with our results showing the strongest effect of microbiome on the responsiveness to asthma in genetically susceptible asthma-prone BALB/c mice and only partial effect in asthma-resistant Swiss Webster mice.

Pglyrp1 is directly bactericidal for both Gram-positive and Gram-negative bacteria, has high constitutive expression in neutrophils and eosinophils, and its expression is also induced in macrophages and epithelial cells, including oropharynx, airways, and intestinal tract (7, 10–12, 19–21, 28). Thus, Pglyrp1 can directly exert its antibacterial effects not only on bacteria that invade the body but also on bacteria on mucous membranes and affect the composition of microbiome. Efficient killing of bacteria by Pglyrps requires oxygen and induction of oxidative stress in bacteria (15, 16), which is consistent with the overall decrease of the abundance of anaerobic bacteria in *Pglyrp1*^{−/−} mice or in germ-free recipients of microbiota from *Pglyrp1*^{−/−} mice (possibly due to increases in abundance of aerobic and facultative bacteria). However, Pglyrps are still bacteriostatic under anaerobic conditions (15, 16) and thus can potentially control both aerobic and anaerobic bacteria, and different species of commensal microbiota have differential sensitivity to Pglyrp1 killing (11, 13). It is also likely that Pglyrp1 works together with other Pglyrps and also antimicrobial peptides secreted on mucous membranes to control the overgrowth of commensal bacteria and control the composition of mucous membrane microbiome because Pglyrps and antimicrobial peptides are synergistically bactericidal (13). *Pglyrp1* could also affect microbiome indirectly through its stimulatory effect on neutrophils, monocytes, and macrophages because of Pglyrp1 interaction with triggering receptor expressed on myeloid cells-1 (TREM-1), which results in activation of these cells and increased production of proinflammatory cytokines (58). Future research will focus on the exact mechanism of microbiome control by *Pglyrp1*.

In conclusion, our results show that decreased responsiveness to allergic asthma in *Pglyrp1*^{−/−} mice is to a large degree dependent on the changed microbiome because this decreased responsiveness can be transferred to germ-free WT mice by colonization with microbiota from *Pglyrp1*^{−/−} mice. Our results also demonstrate that *Pglyrp1* is one of the host genes that promote proasthmatic host microbiome and identify specific groups of *Pglyrp1*-regulated bacteria in intestinal and oropharyngeal microbiomes that are associated with changed responsiveness in allergic asthma. These results may help in the future development of new ways to prevent or control asthma by modulating the expression of *Pglyrp1* and/or adjusting the composition of bacterial microbiome.

Acknowledgments

We are grateful to Julie Cook for maintaining and breeding mice.

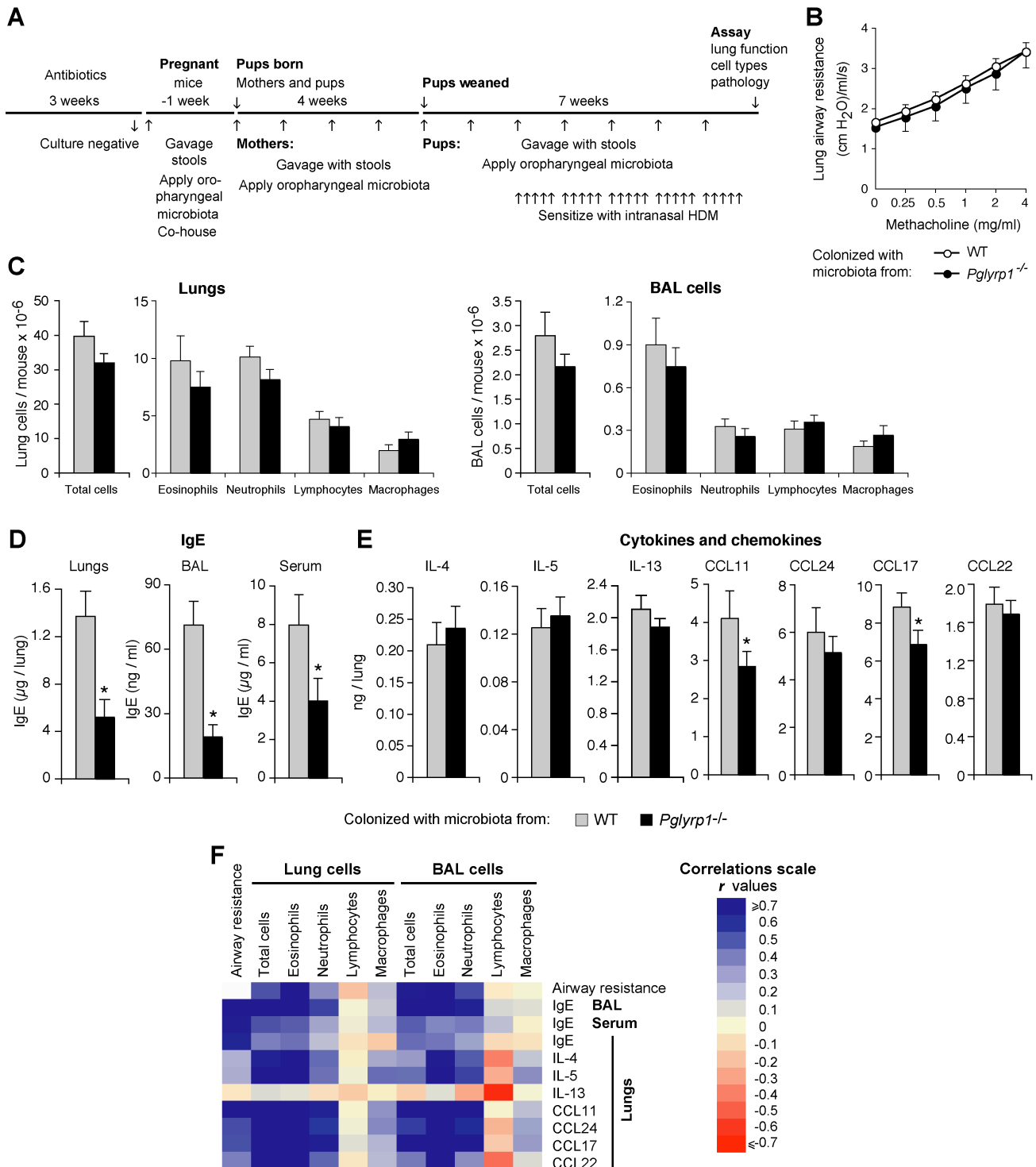
Disclosures

The authors have no financial conflicts of interest.

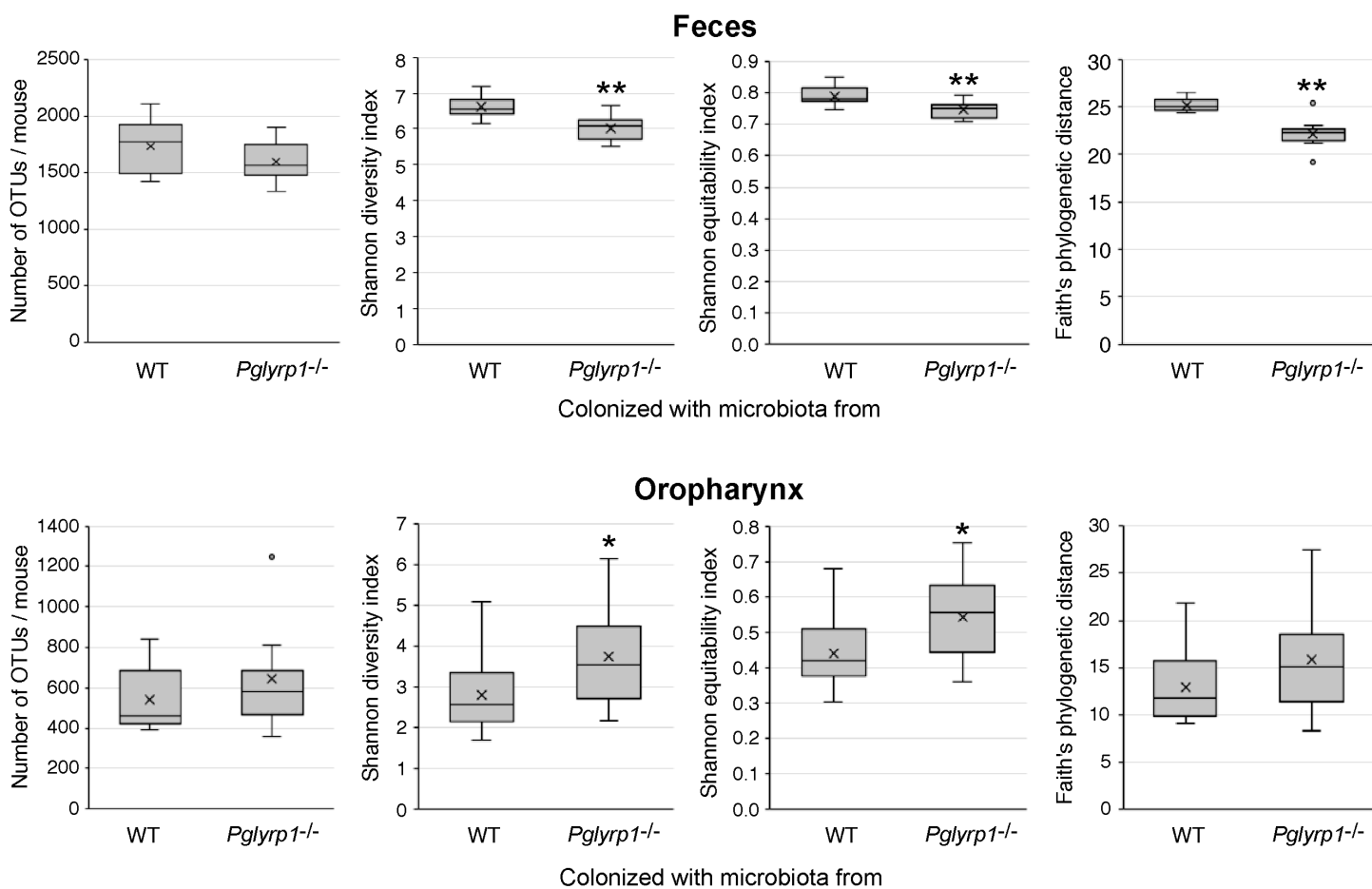
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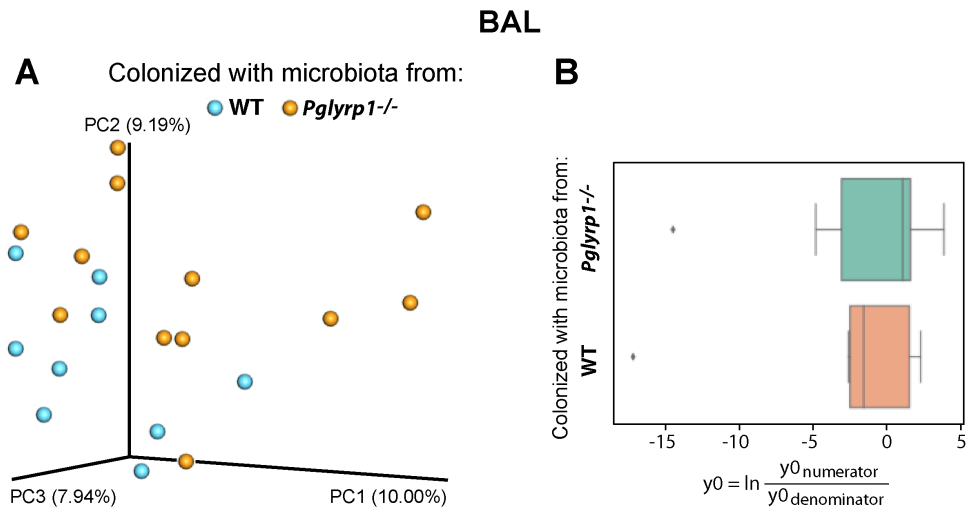
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Supplemental Fig. 1. BALB/c mice depleted of microbiota with antibiotics and colonized with microbiota from *Pglyrp1*^{-/-} mice have normal lung airway resistance and numbers of inflammatory cells in the lungs, but low IgE, CCL-11, and CCL17 response following HDM sensitization. (A) Experimental timeline for depletion of microbiota in BALB/c mice with oral antibiotics and colonization of pregnant mice and pups with fecal and oropharyngeal microbiota from WT or from *Pglyrp1*^{-/-} mice, sensitization of pups with HDM, and asthma assays. (B) Lung airway resistance in response to methacholine, (C) inflammatory cells in lungs and BAL fluid, (D) IgE levels, (E) IL-4, IL-5, IL-13, CCL11 (eotaxin 1), CCL24 (eotaxin 2), CCL17, and CCL22 levels in the lungs, and (F) correlation between the airway resistance and cell numbers in lungs and BAL, and IgE and cytokine and chemokine levels in antibiotic-treated WT BALB/c mice colonized with microbiota from WT or from *Pglyrp1*^{-/-} mice and sensitized with HDM. The results are means $\pm$ SEM (B-E); * $P < 0.05$ for WT versus *Pglyrp1*^{-/-} groups (t -test); the r values were calculated by Spearman's rank test (F); $N = 12$ mice/group.



Supplemental Fig. 2. α -diversity of fecal and oropharyngeal microbiomes in germ-free BALB/c mice colonized with microbiota from WT or *Pglyrp1^{-/-}* mice. Mice were colonized as shown in Fig. 1A and microbiome samples were collected at the time of lung function assays. Each box spans the interquartile range with median marked by a horizontal line and mean by an X inside the box, whiskers mark the range, except for outliers marked with dots; * $P < 0.05$; ** $P < 0.001$ for WT versus *Pglyrp1^{-/-}* groups (Kruskal-Wallis test); $N = 12-14$ mice/group.



Supplemental Fig. 3. β -diversity of BAL microbiomes in germ-free WT BALB/c mice colonized with microbiota from WT or *Pglyrp1*^{-/-} mice. Unweighted Unifrac PCoA analysis (A) and Gneiss analysis (B). Mice were colonized as shown in Fig. 1A and BAL microbiome samples were collected at the time of lung function assays; $N = 9$ (WT) or 12 (*Pglyrp1*^{-/-}) mice/group.

Supplemental Table I. Summary of parameters for microbiome β diversity in germ-free BALB/c mice colonized with microbiota from WT or *Pglyrp1*^{-/-} mice. ^a

Sample (<i>N</i>)	Parameter ^b	Jaccard distance	Bray Curtis	Unweighted Unifrac	Weighted Unifrac
Feces (26)	Pseudo F	12.552	12.281	13.005	11.389
	<i>P</i> value	0.001	0.001	0.001	0.002
	<i>q</i> value	0.001	0.001	0.001	0.002
Oropharynx (26)	Pseudo F	2.173	5.048	1.465	4.169
	<i>P</i> value	0.004	0.002	0.090	0.010
	<i>q</i> value	0.004	0.002	0.090	0.010
BAL (21)	Pseudo F	1.113	1.338	1.445	2.147
	<i>P</i> value	0.226	0.089	0.075	0.035
	<i>q</i> value	0.226	0.089	0.075	0.035
Lungs (25)	Pseudo F	1.226	1.282	1.733	0.741
	<i>P</i> value	0.075	0.117	0.029	0.676
	<i>q</i> value	0.075	0.117	0.029	0.676

^a Mice were colonized as shown in Fig. 1A and microbiome samples were collected at the time of lung function assays.

^b PERMANOVA, number of permutations: 999.