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Asthma is a common inflammatory disease of the airways characterized by airway hyperresponsiveness, increased mucus secretion, chronic inflammation, and airflow remodeling response. Asthma affects 7% of the United States population with sharply increasing prevalence and $20 billion annual healthcare costs. Sensitivity to asthma is determined by multiple genetic predisposition, aberrant immune response, and environmental factors, as ~50% of asthma cases are caused by allergy to environmental allergens. Thus, asthma may be classified as atopic (allergic or extrinsic) and nonatopic (nonallergic or intrinsic) (1).

Prevention of asthma is based on avoidance of allergens and the use of anti-inflammatory corticosteroids, whereas treatment of acute symptoms is usually accomplished with inhaled short-acting β2 agonists. Because both of these approaches have been used for decades with only partial success, new approaches to the prevention and treatment of asthma are needed, which can only come from better understanding of the etiology and pathogenesis of asthma (1).

Recent data indicate that innate immunity (in addition to acquired immunity) is crucial for triggering and maintaining allergic diseases, including asthma (2–5). One of the most frequent allergens involved in asthma is house dust mite (allergen) (HDM), because 10% of individuals with asthma are allergic to HDM (6). HDM can be used to induce experimental asthma in a mouse model that closely resembles human asthma (7–9). Recent genome-wide profiling of the lung transcriptome of mice with HDM-induced experimental asthma identified several genes that were involved in asthma (9). One of these genes was Pglyrp1 (9), which codes for antibacterial protein, peptidoglycan recognition protein 1.

Peptidoglycan recognition protein (Pglyrp1) belongs to a family of evolutionarily conserved innate immunity proteins, which in mammals also include Pglyrp2, Pglyrp3, and Pglyrp4. Pglyrp1, Pglyrp3, and Pglyrp4 are directly bactericidal (10–13) and bind to intact peptidoglycan (the main component of bacterial cell wall) and peptidoglycan fragments and also to other microbial cell wall components, including LPS and lipoteichoic acid (10–18). These Pglyrps kill bacteria by overactivating bacterial stress-response two-component systems and inducing lethal membrane depolarization and oxidative stress in bacteria (13). Pglyrp2 is an N-acetylMuramoyl-L-alanine amidase that hydrolyzes bacterial peptidoglycan and is also bactericidal (19). All mammalian Pglyrps are secreted proteins (15, 16). Pglyrp1 is highly expressed in neutrophils' and eosinophils' granules and to a lesser extent in epithelial and other cells (11, 14, 20, 21). Other Pglyrps are expressed in epithelial cells in the skin and mucous membranes, and Pglyrp2 is also expressed in the liver (10, 20, 22).

Because of their antibacterial activity, Pglyrps maintain beneficial healthy intestinal microbiome, which protects mice from...
experimentally induced ulcerative colitis (22). This protective effect is nonredundant, that is, each of the single Pglyrp knockout mice (Pglyrp1−/−, Pglyrp2−/−, Pglyrp3−/−, or Pglyrp4−/−) is more sensitive to colitis, likely because of the unique effect of each Pglyrp deficiency on gut microbiome (22). Mammalian Pglyrps also have unique nonredundant effects on intestinal, skin, and joint inflammation. All Pglyrps are anti-inflammatory in the intestine (22, 23), Pglyrp2 protects against psoriasis-like skin inflammation (24) and is required for the development of experimental arthritis (25), whereas Pglyrp3 and Pglyrp4 protect against atopic dermatitis (26). By contrast, Pglyrp1 has a proinflammatory effect in three mouse models of inflammatory skin diseases (atopic dermatitis, contact dermatitis, and psoriasis) (24, 26). Pglyrp1 also has anti-inflammatory effect in experimentally induced arthritis (25). Thus, Pglyrp1 is often proinflammatory, whereas other Pglyrps, depending on the type of inflammation and the type of Pglyrp, can have anti- or proinflammatory effects.

Many genes are associated with predisposition to allergic and inflammatory diseases, such as asthma, atopic dermatitis, psoriasis, and inflammatory bowel disease, and genetic predisposition for these diseases often overlaps (2, 27–29). However, all susceptibility genes for these diseases have not been yet identified. Thus, association of Pglyrp1 with experimental asthma (9) and changed sensitivity to colitis, psoriasis, atopic dermatitis, and contact dermatitis in Pglyrp-deficient mice raise a possibility of a similar genetic overlap of predisposition to these diseases in Pglyrp deficiencies.

Because of the overlapping predisposing factors and similar immunopathologic mechanisms, asthma is often associated with other allergic diseases, as about half of atopic dermatitis patients develop asthma later in life (1, 28, 30). On the basis of this association, we hypothesized that Pglyrps have similar effect on asthma as they do on atopic dermatitis. In this study, we tested the hypothesis that Pglyrp1 has a proinflammatory effect in experimentally induced asthma, similar to its proinflammatory effect in atopic dermatitis and other inflammatory skin diseases (24, 26). Consistent with this hypothesis we show here that Pglyrp1−/− mice develop less severe experimental asthma than wild-type (WT) mice following intranasal sensitization with HDM. This less severe asthma in Pglyrp1−/− mice is due to decreased Th2 and Th17 cell responses and increased regulatory T (Treg) cell and plasmacytoid dendritic cell (pDC) responses. Thus, our results suggest that blocking Pglyrp1 activity in the lungs may help to control development of allergic asthma.

Materials and Methods

Mice, HDM asthma model, and lung airway resistance

Female 8- to 10-wk-old Pglyrp1−/− and WT mice, both on BALB/c background, were generated and maintained under conventional pathogen-free conditions as described previously (21). 21, 24–26). mice were sensitized 5 d/wk with 3–5 wk with 10 μl per application of 2.5 mg/ml purified HDM allergen (from Dermatophagoides pteronyssinus, catalog number XB82D3A25, lot number 145793, endotoxin content 37 EU/mg protein; Greer Laboratories, Lenoir, NC) in PBS instilled into the nose under isoflurane anesthesia (7–9). Three days after the last sensitization, mice were evaluated for the extent of asthma and inflammatory response in the lungs, blood, mediastinal lymph nodes (MLN), and spleen. Untreated or PBS-treated mice were used as unsensitized controls. All experiments on mice were approved by the Indiana University School of Medicine–Northwest Institutional Animal Care and Use Committee.

Lung airway resistance was measured in response to aerosol of 1, 2, 4, and 8 mg/ml methacholine. Mice were anesthetized by ip injection of 0.2 ml of a mixture of 6.7 mg/ml ketamine hydrochloride and 1.3 mg/ml xylazine, an incision was made to access the trachea, and intratracheal cannula was connected to the respirometer, which maintained respirations at 140 respirations/min, delivered methacholine aerosol intratracheally, and measured lung airway resistance and compliance in response to methacholine (7–9). The results are resistance data presented as means ± SEM of 6–12 mice/group. The compliance data are not shown in Results, because they mirror the resistance with similar statistically significant differences.

To collect bronchoalveolar lavage (BAL) fluid, lungs were then lavaged for five times with 0.9 ml PBS. Total numbers of cells in the BAL fluid were then counted, followed by differential counts of inflammatory cells on H&E- or immunofluorescence-stained cytospin slides and by flow cytometry. Blood was collected for serum IgE and cytokine assays, and lungs, MLN, and spleen were collected for flow cytometry, RNA isolation, and IgE and cytokine assays.

Histology and immunofluorescence

For histological analysis, mice were anesthetized with isoflurane and exsanguinated, and the lungs were infused with 10% buffered protocol formalin (Fisher Scientific, Kalamazoo, MI) through a tracheal cannula at 20-cm hydrostatic pressure. The trachea was then ligated, and the lungs were placed in 10% buffered protocol formalin for 48 h and processed for paraffin embedding and periodic acid–Schiff (PAS) or H&E staining of longitudinal sections of the left lung lobe and evaluated microscopically. PAS+ mucus-producing goblet cells were counted on representative ×20 fields per lung along the bronchial tree. The results are reported as means ± SEM of six mice per group.

Cytospin slides of BAL cells were immediately fixed with methanol at 4°C for 5 min, dried, blocked with 5% mouse or rabbit serum in PBS, and incubated for 2 h at 20°C with 4 μg/ml rabbit anti-mouse Pglyrp1 or mouse monoclonal anti-mouse Pglyrp1 Abs (both specific to aa 123–182 from Santa Cruz Biotechnology) together with one of the following biotinylated Abs to mouse cell marker Ags: eosinophil catheptic protein (10 μg/ml rabbit Ab from Bios), neutrophil Ly6G (1.25 μg/ml rat mAb RB6-8C5 from eBioscience), macrophage CD68 (10 μg/ml rat mAb FA-11 from AbD Serotec), lung epithelial cell podoplanin (2.5 μg/ml hamster mAb 8.1.1 from eBioscience), or pDC CD317 (1.25 μg/ml rat mAb 927 from AbD Serotec). The slides were washed with PBS, incubated for 1 h at 20°C with either anti-rabbit IgG biotinylated mAb or anti-mouse IgG FITC mAb, reverse-translated rabbit Ab (non–cross-reactive with other species from Sigma-Aldrich) and with streptavidin–allophycocyanin (eBioscience), washed with PBS, and observed in Olympus Fluoview FV300 confocal microscope. The excitation and emission spectra were set such that there was no “cross-bleeding” of the signal between FITC and allophycocyanin. Individual slices were examined and the pictures shown represent merged stacks.

RNA and quantitative real-time RT-PCR

RNA was isolated from right lobes of unsensitized or sensitized lungs using the TRIzol method (Invitrogen), followed by digestion with RNase-free DNase (Qiagen) and purification on RNasey spin columns using RNasey Minikit (Qiagen) (22, 24–26). Quantitative real-time RT-PCR (qRT-PCR) was used to quantify the amounts of mRNA in the lungs using custom RT2 Profiler PCR Arrays designed by us and manufactured by Qiagen/Saint Biosciences (22, 24–26). The arrays typically included 40 assay genes (listed in the figures), five housekeeping genes, and reverse transcription efficiency and DNA contamination controls. All primer sets were from Qiagen/Saint Biosciences, except for the Pglyrp1 primers (24). cDNA was synthesized from 2 μg RNA using RT2 PCR Array First Strand Kit (Qiagen/Saint Biosciences), and the arrays were performed according to the manufacturer’s instructions using Qiagen/Saint Biosciences MasterMix. The expression levels were performed on RNA contamination controls, reverse transcription controls, and aliquot of each gene from two arrays: ΔCt = ΔCt1 – ΔCt2, where ΔCt1 is the HDM-sensitized mice group, and ΔCt2 is the unsensitized mice group, using the program provided by Qiagen/Saint Biosciences. This calculation gives the fold increase in expression of each gene in the sensitized mice versus unsensitized mice per microgram RNA and does not include in the calculation the increased amount of RNA obtained from the lungs of sensitized with unsensitized mice (and thus the increases in gene expression would be greater if calculated per lung, rather than per microgram RNA). The genomic DNA contamination controls, reverse transcription controls, and positive PCR controls were included in each array and were all passed. Additional control to assure amplification from RNA, but not from possible contaminating DNA, included parallel reaction sets

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from which reverse transcriptase was omitted and which showed no amplification. To compare baseline gene expression in untreated mice, ∆Ct1 was from untreated Pglyrp1−/− mice, and ∆Ct2 was from untreated WT mice.

The results are reported as mean fold increases after HDM sensitization (HDM/PBS) or ratios of fold increases in Pglyrp1−/− mice to WT mice, calculated as follows: ([Pglyrp1−/− HDM]/[Pglyrp1−/− PBS])/(WT HDM)/[WT PBS]) and presented as heat maps generated using Java TreeView after converting fold differences (ratios) of [WT PBS]/([WT HDM]/[WT PBS]); p ≤ 0.05 was considered significant.

**Isolation of cells and flow cytometry**

Mouse lungs were minced into ∼1-mm pieces in RPMI 1640 medium with 5% FBS with 3 mg/ml collagenase D and 0.15 μg/ml DNAse I and digested for 2 h at 37°C (31). Cells were washed twice with RPMI 1640 medium with 5% FBS. RBCs were removed with a lysis buffer (BioLegend), and the cells were incubated for 14 h in the same medium at 37°C. Cells were then strained through a 40-μm filter and resuspended in 2× 10^6 cells/ml in RPMI 1640 medium with 5% FBS. Single cell suspensions from MLN and the spleen were obtained by mincing and passing the tissue through a 40-μm filter; RBCs were removed from the spleen cells with a lysis buffer (BioLegend), and the cells were suspended at 2× 10^5 cells/ml in RPMI 1640 medium with 5% FBS. Incubated for 14 h in the same medium at 37°C in 5% CO2, and then processed for flow cytometry staining. Fourteen-hour incubation improved recovery of lung cells and did not significantly affect expression of the assayed markers on lung, MLN, and spleen lymphocytes and pDCs.

Cells (1× 10^7) were stained with CD4-allophycocyanin (clone RM4-5; BioLegend) mAb for 20 min at 4°C. CD4-stained cells were then stained for Foxx3-PE (clone FJK-16s; eBioscience) or for cytokines IFN-γ-P (clone XMG1.2), IL-4-PE (clone IB11) and IL-17-PE (clone TC11-18H10.1) with mAbs from BioLegend, all used at 2 μg/ml, according to BioLegend protocols using BioLegend buffers. Prior to staining for cytokines, CD4+allophycocyanin–stained cells were activated with 12-O-tetradecanoylphorbol 13-acetate (25 ng/ml) and ionomycin (250 ng/ml) in the presence of the Golgi inhibitor, monensin (BioLegend), for 4 h at 37°C in 5% CO2. Cells were analyzed by flow cytometry using MACSQuant (Miltenyi Biotec) cytometer. Foxx3, IFN-γ, IL-4, and IL-17–positive cells were measured within the CD4+ gate (shown in the figures) and also within the entire lymphocyte gate (with similar results; data not shown). For pDC staining, cells were first treated with FC blocking reagent (Miltenyi Biotec), followed by one of two protocols. The standard protocol used a mixture of PE-anti-calves serum (CALS) (Miltenyi Biotec) mAb for 20 min at 4°C, followed by sonication and centrifugation (26).

**Depletion of pDC**

pDC were depleted in vivo by i.v. injections of anti-CD317 mAb (clone 927) (33–35) or control rat IgG2b (both functional grade from eBioscience), 50 μg twice a week for 5 wk into Pglyrp1−/− mice, given 1 h before sensitization with HDM. Significant decreases in the numbers of pDC in the lungs, MLN, and spleen were determined by flow cytometry and are shown in Results.

**Statistical analysis**

All quantitative results are presented as means ± SEM, with statistical significance of the differences between groups determined by the two-sample one-tailed Student t test; p ≤ 0.05 was considered significant.

**Results**

Pglyrp1−/− mice have reduced response in HDM-induced asthma model

To determine the role of Pglyrp1 in allergic asthma, we selected the HDM-induced asthma model in mice, because this model most closely mimics human allergic asthma (7–9) and because HDM is one of the most frequent allergens associated with human asthma (6). Repeated intranasal sensitization with HDM for 3–5 wk induced severe asthmatic-like response in WT mice and significantly attenuated response in Pglyrp1−/− mice. Compared with WT mice, after 3 and 5 wk of HDM sensitization, Pglyrp1−/− mice had significantly reduced lung airway resistance, which is a measure of hyperresponsiveness of airway smooth muscle cells to methacholine, a muscarinic receptor agonist, which induces bronchoconstriction, characteristically more severe in asthma (Fig. 1). There was no significant difference in the response to methacholine in unsensitized WT and Pglyrp1−/− mice. These results indicate that deletion of Pglyrp1 diminishes bronchial hyperresponsiveness in sensitized mice and suggest that in WT mice Pglyrp1 plays a role in the development of allergic asthma upon HDM sensitization.

We then compared the numbers and types of inflammatory cells in BAL fluid and in dissociated lungs in unsensitized and HDM-sensitized WT and Pglyrp1−/− mice, because allergic asthma is characterized by pronounced inflammatory response in the lungs. Inflammatory cells were mostly undetectable in BAL fluid and lungs in unsensitized WT and Pglyrp1−/− mice. HDM sensitization induced high numbers of inflammatory cells in WT mice after 3 wk of HDM sensitization, and the numbers of these cells further increased after 5 wk of HDM sensitization (Fig. 2). The inflammatory cells in WT mice in BAL fluid were dominated by eosinophils, with many lymphocytes, neutrophils, and macrophages also present, whereas in dissociated lung cells, the most numerous inflammatory cells were lymphocytes, the numbers of eosinophils and neutrophils were also high. This prominent eosinophilic response is highly characteristic of allergic asthma. HDM-sensitized Pglyrp1−/− mice, compared with WT mice, had significantly lower total numbers of cells and the numbers of eosinophils in BAL fluid and lungs both at 3 and 5 wk of sensitization, and the numbers of macrophages at 3 wk and neutrophils and lymphocytes at 5 wk of sensitization (Fig. 2). These results demonstrate significantly lower cellular infiltration of HDM-sensitized lungs in Pglyrp1−/− than in WT mice and indicate that in WT mice Pglyrp1 is required for high recruitment of inflammatory cells to the lung during HDM sensitization.

We then compared lung histology of unsensitized and HDM-sensitized WT and Pglyrp1−/− mice to determine the pathologic basis of this lower response to HDM sensitization in Pglyrp1−/− mice. Unsensitized WT and Pglyrp1−/− mice both showed normal histology and no positive cells on PAS staining (Fig. 3A). HDM-sensitized WT mice showed massive inflammatory cell infiltrates mainly around bronchi and also around bronchioles and pulmonary arteries and veins along the bronchial tree (Fig. 3A). The inflammatory infiltrates contained mononuclear cells and numerous eosinophils (Fig. 3B). HDM-sensitized WT 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. There was also smooth muscle hypertrophy and fibrosis, also indicative of remodeling. All of these histopathologic changes are highly characteristic of allergic asthma (1, 7–9). HDM-sensitized Pglyrp1−/− mice, compared with WT mice, had substantially smaller cellular infiltrates with fewer eosinophils (consistent with lower inflammatory cell counts in Fig. 2) and smaller and significantly fewer PAS+ mucin-producing goblet cells (Fig. 3A–C). These results demonstrate substantially less severe histopathologic changes characteristic of asthma in HDM-sensitized lungs in Pglyrp1−/− than in WT mice. These results indicate that in WT mice Pglyrp1 is required for the development of severe inflammatory cell infiltrates and airway remodeling with goblet cell hyperplasia and metaplasia following HDM sensitization.

The above results demonstrate development of less severe allergic asthma in Pglyrp1−/− mice compared with WT mice, which indicates that in WT mice Pglyrp1 is required for full manifestation of allergic asthma.

Pglyrp1 is expressed in eosinophils, neutrophils, macrophages, and epithelial cells

We determined the expression of Pglyrp1 in the cells from the BAL fluid from asthmatic lungs using immunofluorescence and confocal microscopy, to identify the cellular sources of Pglyrp1 in HDM-sensitized mice. Pglyrp1 was highly expressed in eosinophils (identified by morphology and expression of eosinophil cationic protein), neutrophils (identified by morphology and expression of Ly-6G), macrophages (identified by morphology and expression of CD68), and lung epithelial cells (identified by morphology and expression of...
expression of podoplanin) in the lungs of HDM-sensitized WT mice (Fig. 4). pDC (identified by morphology and expression of CD317) expressed low levels of Pglyrp1 (Fig. 4), whereas lymphocytes did not express Pglyrp1 (data not shown), consistent with previous results (14). Eosinophils and neutrophils are known to constitutively express Pglyrp1 (11, 14). However, lung epithelial cells in unsensitized mice did not express Pglyrp1 (data not shown). Thus, strong expression of Pglyrp1 in epithelial cells from HDM-sensitized mice indicates that HDM sensitization induces expression of Pglyrp1 in lung epithelial cells. These results demonstrate abundant expression of Pglyrp1 by inflammatory cells in HDM-sensitized lungs.

\[ \text{Pglyrp}^{1/-} \text{mice have reduced levels of IgE, eotaxins, IL-4, IL-5, and IL-17 in the lungs} \]

We then compared the levels of IgE in the lungs and serum in unsensitized and HDM-sensitized WT and \( \text{Pglyrp}^{1/-} \) mice, because atopic allergies, including atopic (allergic) asthma and atopic dermatitis, are characterized by increased local and systemic production of IgE. IgE was undetectable in the BAL fluid, lung homogenates, and serum of unsensitized WT and \( \text{Pglyrp}^{1/-} \) mice (Fig. 5). WT mice sensitized with HDM for 3 and 5 wk had high levels of IgE in BAL fluid, lung homogenates, and serum, whereas similarly sensitized \( \text{Pglyrp}^{1/-} \) mice had significantly lower levels of IgE in BAL fluid, lungs, and serum (Fig. 5). These results are consistent with the lower asthmatic responses of \( \text{Pglyrp}^{1/-} \) mice and indicate that in WT mice Pglyrp1 is required for high IgE production following HDM sensitization. These results also suggest that \( \text{Pglyrp}^{1/-} \) mice have lower Th2 response to HDM, because high IgE production in WT mice is the result of high Th2 polarization of T cell responses.

We also compared the levels of CCL-11 and CCL-24 (eotaxins 1 and 2), IL-4, IL-5, and IL-17 in the lungs and serum in unsensitized and HDM-sensitized WT and \( \text{Pglyrp}^{1/-} \) mice, because of high eosinophil and neutrophil infiltrations in the lungs in HDM-sensitized WT but not \( \text{Pglyrp}^{1/-} \) mice (Figs. 2, 3). CCL-11 and CCL-24 are the main eosinophil-attracting chemokines. IL-4 and IL-5 are the main Th2 cytokines and Th2 polarization promotes IgE responses and type I hypersensitivity. IL-17 promotes Th17 response, which then results in production of neutrophil-attracting chemokines. CCL-11, CCL-24, IL-4, IL-5, and IL-17 were undetectable in the lung homogenates and serum of unsensitized WT and \( \text{Pglyrp}^{1/-} \) mice (Fig. 5). WT mice sensitized with HDM for 3 and 5 wk had high levels of CCL-11, CCL-24, IL-4, IL-5, and IL-17 in the lung homogenates and of IL-4, CCL-
11, and CCL-24 also in the serum. However, similarly sensitized Pglyrp1/2 mice had significantly lower levels of CCL-11, CCL-24, IL-4, IL-5, and IL-17 in the lungs at 3 and/or 5 wk but similar levels in the serum (except IL-4, which was lower both in the lungs and serum, and IL-5 and IL-17, which were not detectable in the serum) (Fig. 5). These results are consistent with the cell counts and histopathological findings (Figs. 2, 3) and suggest reduced local (in the lung) eosinophil- and neutrophil-attracting proinflammatory responses in Pglyrp1−/− mice but no general systemic defect in these responses. These results indicate that in WT mice Pglyrp1 is required for full production of chemokines and cytokines in the lungs.

We also assayed the levels of TSLP in the lungs and serum of HDM-sensitized mice, because TSLP is produced by keratinocytes in allergic individuals, is secreted into the serum, and enhances sensitivity to asthma (36, 37). However, TSLP was not detectable in the serum or lungs in WT or Pglyrp1−/− mice sensitized with HDM for 3 or 5 wk (<5 pg/ml serum or <100 pg/lung, using ELISA). TSLP mRNA expression in the lungs was also not increased after 3 or 5 wk of HDM sensitization in WT and Pglyrp1−/− mice (determined by qRT-PCR; data not shown). Thus, TSLP does not play a role the HDM-induced asthma.

Pglyrp1−/− mice have decreased Th2 and Th17 responses in the lungs

We then determined which types of responses and cell subpopulations significantly differed between WT and Pglyrp1−/− mice to determine the cellular basis for the differences in the inflammatory responses in the lungs of these mice. This was first accomplished by measuring the amounts of mRNA for several marker genes characteristic of the responses generated by various immune and inflammatory cell types in the unsensitized and sensitized lungs. To determine which marker genes are increased or decreased in Pglyrp1−/− mice compared with WT mice (and thus the type of response and the cell type), we calculated how many times higher or lower these genes were induced in Pglyrp1−/− mice than in WT mice (fold induction in Pglyrp1−/− mice/fold induction in WT mice).

Expression of the majority of the genes that we studied was lower in the lungs of unsensitized Pglyrp1−/− mice compared with WT mice (Fig. 6, left panel, Supplemental Table I). HDM sensitization of WT mice highly induced the expression in the lungs of several marker genes characteristic of Th2 (Il4, Il13, Il33, and Tnfrsf4)
and Th17 (Cxcl1, Cxcl2, Cxcl5, Il17a, Il21, Il23a, and Rorgt) responses, and also of alternative macrophage activation (Arg1, Chi3l3, and Fizz1), conventional (myeloid) dendritic cell (cDC) response (Cd273 and Tnfsf4), eosinophil attraction (Ccl11), and goblet cell activation and mucin production (Clca3 and Muc5ac) (Fig. 6, middle panels, Supplemental Table I). These results are consistent with the high allergic asthmatic response in WT mice. Expression of Pglyrp1 was also increased in the lungs in HDM-sensitized WT mice.

Similarly sensitized Pglyrp1−/− mice had significantly lower expression than WT mice of genes characteristic of Th2, Th17, macrophage (both alternatively and classically activated), cDC, eosinophil, and goblet cell responses at both 3 and 5 wk of sensitization (Fig. 6, right panel, Supplemental Table I). These results are consistent with lower allergic asthmatic response in Pglyrp1−/− mice. At 3 wk of sensitization, Pglyrp1−/− mice had significantly higher expression than WT mice of genes characteristic of Th1 responses (Il2, Ifng, Cxcl9, and Cxcl10), which may reflect Th1 polarization due to decreased Th2 and Th17 responses. These results suggest that Th2, Th17, alternatively activated macrophage, and cDC responses drive high proinflammatory allergic asthmatic response in the lungs of HDM-sensitized WT mice and that these responses are attenuated in Pglyrp1−/− mice.

We then used flow cytometry to directly measure T cell types in the BAL fluid, lungs, draining MLN, and spleen to further investigate the role and the location of Th cell types in the decreased sensitivity of Pglyrp1−/− mice to HDM-induced asthmatic response. CD4+ T cells were undetectable in the BAL fluid and barely detectable in the lungs of unsensitized mice but were highly increased in both the BAL fluid and the lungs after 3 and 5 wk of HDM sensitization in WT mice (Fig. 7A). Similarly sensitized Pglyrp1−/− mice had significantly lower numbers of CD4+ T cells than WT mice in both BAL fluid and the lungs. Th17 (CD4+IL-17+) cells were undetectable in the lungs of unsensitized mice, and they were highly increased at 3 and 5 wk of sensitization in WT but not in Pglyrp1−/− mice (Fig. 7A).

Flow cytometry also revealed that the percentages of Th2 (CD4+IL-4+) and Th17 cells after 3 and 5 wk of HDM sensitization were significantly higher in the lungs and MLN in WT than in Pglyrp1−/− mice (Fig. 7B, 7C). In addition, the percentage of Th1 (CD4+IFN-γ+) cells in the lungs was also significantly higher in WT than in Pglyrp1−/− mice at 5 wk of sensitization. These results confirm and extend the gene expression results (Fig. 6) and show high local Th2 and Th17 responses in the lungs in sensitized WT but not Pglyrp1−/− mice. Similar percentages of Th1, Th2, and Th17 cells in the spleen in sensitized WT and Pglyrp1−/− mice indicate that Pglyrp1−/− mice do not have systemically defective Th cell responses and suggest diminished local recruitment and retention of these cells in the lungs and draining lymph nodes of Pglyrp1−/− mice. These results are consistent with lower CCL-11, CCL-24, IL-4, IL-5, and IL-17 levels in the lungs in HDM-sensitized Pglyrp1−/− mice (Fig. 5). Altogether, these results indicate that in WT mice.
Pglyrp1 is required for high allergic inflammatory cell response to HDM.

Pglyrp1−/− mice have increased Treg and pDC responses in the lungs

Because Pglyrp1−/− mice were able to limit lung inflammation following HDM sensitization more effectively than WT mice, we then tested whether this difference was due to more efficient generation or function of Treg cells in Pglyrp1−/− mice. Sensitized Pglyrp1−/− mice had higher expression than WT mice of genes characteristic of Treg cell response (Foxp3 and Il10) at both 3 and 5 wk of HDM sensitization (Fig. 6, right panel, Supplemental Table I), suggesting higher numbers and/or activation of Treg cells in the sensitized lungs of Pglyrp1−/− than WT mice.

To further verify these findings, we then used flow cytometry to directly measure Treg cells (CD4+Foxp3+) in the BAL fluid and lungs in HDM-sensitized WT and Pglyrp1−/− mice. The percentages of Treg cells were significantly higher in the BAL fluid and lungs in Pglyrp1−/− mice compared with WT mice after 3 and 5 wk of sensitization with HDM (Fig. 7B, 7C). Two possible reasons for this higher percentage of Treg cells in the lungs of Pglyrp1−/− mice could be either more efficient recruitment of Treg cells to the sensitized lungs and/or higher generation of Treg cells in all lymphoid organs in Pglyrp1−/− mice. Our data support more efficient recruitment of Treg cells to the lungs in Pglyrp1−/− mice for two reasons. First, the lungs of HDM-sensitized Pglyrp1−/− mice had higher expression of Treg-attracting chemokines (Ccl1, Ccl17, and Ccl27a) than WT mice (Fig. 6, right panel, Supplemental Table I). Second, WT mice had similar percentage of Treg cells in the spleen and higher percentage in the MLN than Pglyrp1−/− mice at 3 wk of HDM sensitization (Fig. 7B), which indicates sufficient systemic generation of Treg cells in WT mice in lymphoid organs and argues against the possibility of higher systemic capacity for generation of Treg cells in Pglyrp1−/− mice than in WT mice.

We next investigated possible reasons for more efficient recruitment and retention of Treg cells in the lungs of Pglyrp1−/− than WT mice. We first compared the expression of marker genes for various cell types that regulate T cells, including alternatively and classically activated macrophages, cDC, and pDC in WT and
Pglyrp1−/− mice. After both 3 and 5 wk of HDM sensitization, Pglyrp1−/− mice had higher expression of pDC signature genes (Aldh1a1, Aldh1a2, and Idol) than WT mice (Fig. 6, right panel, Supplementary Table I). These results are consistent with the known capacity of pDC to promote generation of Treg cells (32, 34, 35, 38–40) and suggest pDC-mediated generation of Treg cells as a possible mechanism for the more efficient generation of Treg cells by Pglyrp1−/− mice in the sensitized lungs. By contrast, expression of signature genes for cDC and macrophages, which promote Th2 and Th17 responses, was lower in the lungs of HDM-sensitized Pglyrp1−/− than WT mice (Fig. 6, Supplementary Table I), which is consistent with the higher Th2 and Th17 responses in WT mice (Figs. 5, 6).

To verify the above gene expression results, we performed flow cytometry assays, which also revealed significantly higher numbers of pDC in the lungs of Pglyrp1−/− than in WT mice after 5 wk of HDM sensitization (Fig. 8A). We then determined which subpopulations of pDC were increased in HDM-sensitized Pglyrp1−/− mice, because CD8α+β+ and CD8α−β− pDC, but not CD8α−β+ pDC, were recently shown to induce Foxp3+ Treg cells and prevent induction of airway hyperreactivity in OVA-sensitized mice (32). Using flow cytometry, we detected significantly higher percentages of CD8α+β+ and CD8α−β− pDC (I-A/I-E+CD317+) in the lungs and spleens of HDM-sensitized Pglyrp1−/− mice than WT mice (Fig. 8B). These results indicate that in WT mice Pglyrp1 promotes generation or retention of CD8α+β+ and CD8α−β− pDC in asthmatic lungs and suggest that these higher numbers of pDC may be responsible for generation of higher numbers of Treg cells and lower inflammatory responses of Pglyrp1−/− mice.

**Depleting pDC reverses attenuated asthmatic phenotype in Pglyrp1−/− mice**

To directly test the role of pDC in the attenuated asthmatic response of Pglyrp1−/− mice, we depleted pDC in vivo with anti-pDC mAb (anti-CD317 clone 927) (33–35) during 5 wk of sensitization of Pglyrp1−/− mice with HDM. Treatment with anti-pDC mAb significantly reduced the percentages of pDC in the lungs, MLN, and spleen in HDM-sensitized Pglyrp1−/− mice, compared with similarly sensitized WT mice because of their increased pDC responses, which generate increased numbers of Treg cells in the lungs.

**Discussion**

Allergic asthma is a complex disease, which in addition to the well-known Th2 bias and overproduction of IgE, also involves interactions of many other cell types. Thus, the pathogenesis of allergic asthma involves overactivation of Th2 cells, which results in eosinophilic inflammation, and also overactivation of both Th17 cells and Th1 cells, which enhance neutrophilic infiltration (a hallmark of severe asthma) by inducing increased production of neutrophil-attracting chemokines in the lungs (2, 41). Th17 cells also indirectly promote eosinophilic infiltration by enhancing Th2 responses and differentiation of B cells (42, 43). Moreover, although asthma is classically considered an inflammatory allergic disease, cross-talk between innate and adaptive immune systems is crucial for the initiation and propagation of asthma (2–4), because many allergens, such as HDM or cockroach allergen, are also similarly sensitized mice treated with an isotype control IgG (Fig. 9A). Anti-pDC mAb treatment during HDM sensitization also significantly increased the total numbers of inflammatory cells and the numbers of eosinophils, neutrophils, lymphocytes, and macrophages in the BAL fluid and lungs of Pglyrp1−/− mice (Fig. 9B). Anti-pDC mAb treatment during HDM sensitization also significantly increased the lung airway resistance (Fig. 9C) and resulted in higher numbers of CD4+ cells in the BAL fluid and lungs, higher numbers of Th1, Th2, and Th17 cells in the lungs, and reduced percentages of Treg cells in the BAL fluid and lungs, compared with similarly sensitized Pglyrp1−/− mice treated with isotype control IgG (Fig. 9D). Thus, reducing the numbers of pDC reverses the low responsive phenotype of Pglyrp1−/− mice in the HDM asthma model to resemble the more proinflammatory WT phenotype. These results indicate that pDC play a significant role in the generation of Treg cells and low responsiveness of Pglyrp1−/− mice to HDM sensitization.

Altogether, our results indicate that Th2, Th17, alternatively activated macrophage, and cDC responses drive high proinflammatory allergic asthmatic response in the lungs of HDM-sensitized WT mice, and that this response is attenuated in Pglyrp1−/− mice because of their increased pDC responses, which generate increased numbers of Treg cells in the lungs.

**FIGURE 8.** Increased percentages of pDC and CD8α+β+ and CD8α−β− pDC in the lungs in HDM-sensitized Pglyrp1−/− mice. WT and Pglyrp1−/− pDC were sensitized with HDM for 5 wk, and the percentages of pDC in their lungs, MLN, and spleens were measured by flow cytometry. (A) B220+CD11c+CD317+ pDC are shown as CD317+ pDC within B220+ gate. (B) CD8α+β+ and CD8α−β− I-A/I-E+CD317+ pDC are shown within I-A/I-E+CD317+ gate. The results are means ± SEM of six mice per group or representative dot plots for pDC in the lungs; *p < 0.05; Pglyrp1−/− versus WT mice.
strong activators of innate immunity TLRs (44, 45). Thus, the presence or absence of TLR activation modulates sensitization in allergic asthma (40).

Regulation of immune and inflammatory responses in asthma is equally complex. cDC promote development and activation of Th2, Th17, and Th1 cells and in general have proinflammatory effects, which promote inflammation in asthma (2, 46). By contrast, pDC have an opposite anti-inflammatory effect in asthma, because depletion of pDC enhances the numbers of eosinophils, neutrophils, lymphocytes, and macrophages in BAL cells and lungs (B), increased lung airway resistance (C), increased numbers of CD4+ cells in BAL cells and CD4+, Th1, Th2, and Th17 cells in the lungs, and decreased the percentage of Treg cells in BAL cells and the lungs (D), measured by flow cytometry, with gating as shown in Figs. 7, 8A; means ± SEM of six mice per group; *p < 0.05; **p < 0.005; anti-pDC versus IgG.

FIGURE 9. Depletion of pDC in vivo reverses attenuated asthma phenotype in HDM-sensitized Pglyrp1−/− mice. Pglyrp1−/− mice were treated with anti-pDC (anti-CD317) mAb or isotype control IgG during sensitization with HDM for 5 wk. Anti-pDC mAb reduced the numbers of pDC in vivo (A). Depletion of pDC increased the numbers of eosinophils, neutrophils, lymphocytes, and macrophages in BAL cells and lungs (B), increased lung airway resistance (C), increased numbers of CD4+ cells in BAL cells and CD4+, Th1, Th2, and Th17 cells in the lungs, and decreased the percentage of Treg cells in BAL cells and the lungs (D), measured by flow cytometry, with gating as shown in Figs. 7, 8A; means ± SEM of six mice per group; *p < 0.05; **p < 0.005; anti-pDC versus IgG.

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to, maintained, and activated at the mucosal sites and prevent and control inflammation (2, 41). Thus, genetic predisposition to asthma may be associated with genes that affect any of the above aspects of adaptive and innate immunity.

In this study, we reveal a novel innate immunity mechanism that is involved in the development of allergic asthma. We demonstrate that HDM-sensitized Pglyrp1−/− mice develop less severe asthma than WT mice. The mechanism underlying this decreased sensitivity of Pglyrp1−/− mice to allergic asthma is increased generation and activation of CD8αβ+ and CD8αβ- pDC and increased recruitment and activity of Treg cells in the lungs. These cells then decrease production and activation of Th2 and Th17 cells in the lungs and result in attenuated asthmatic response. Thus, our results reveal a previously unknown mechanism of regulation of pDC and Treg cells by Pglyrp1, a member of a family of innate immunity proteins. In WT mice, Pglyrp1 affects
the functions of both innate and adaptive immune cells by decreasing the responses of pDC cells in the lungs, which results in reduced generation of Treg cells and increased Th2 and Th17 responses and a more severe asthmatic phenotype. Our results extend recent findings that pDC (especially CD8αβ+ and CD8αβ- pDC) enhance generation, expansion, and maintenance of Treg cells in the asthmatic lungs (32, 34, 35). Pglyrp1 regulates local proinflammatory responses not only in the lungs (reported in this paper) but also in the intestine (22), skin (24, 26), and joints (25), with little effect on the systemic inflammatory responses, because the most pronounced changes in the Treg and Th cells in Pglyrp1−/− mice occur in the local inflammatory sites.

How do pDC in Pglyrp1−/− mice induce higher numbers of Treg cells in asthma? Our results suggest three possible mechanisms. First, pDC express a tryptophan-degrading enzyme, IDO, which is known to induce Foxp3 expression in naive CD4+ T cells, to generate Treg cells, and to activate the existing Treg cells, both in vitro and in vivo (38). Consistent with this model, our HDM-sensitized Pglyrp1−/− mice express higher level of IDO (Idol; Fig. 6) in their lungs than WT mice, which correlates with the increased numbers of Treg cells in Pglyrp1−/− mice and their attenuated asthma phenotype. Second, generation and maintenance of Treg cells, especially by CD8αβ+ and CD8αβ- pDC, is also promoted by all-trans-retinoic acid, which is produced from retinaldehyde by aldehyde dehydrogenases Aldh1a1, Aldh1a2, and Aldh1a3 (32, 47). Consistent with this model, both the expression of Aldh1a1 and Aldh1a2 and the percentages of CD8αβ+ and CD8αβ- pDC are increased in the lungs of HDM-sensitized Pglyrp1−/− mice compared with WT mice (Figs. 6, 8B). Third, in addition to inducing Treg cells, pDC can also suppress inflammation by downregulating cDC (35), which promote airway inflammation. Indeed, the expression of Cd273, a marker gene for cDC, and the expression of Tnfsf4 (also known as OX40L, which is produced by cDC and amplifies Th2 cell differentiation) are both reduced in HDM-sensitized Pglyrp1−/− mice compared with WT mice (Fig. 6). Consistent with this mechanism, the expression of Tnfsf4 (Tnfsf4 receptor, also known as OX40, which is produced by Th2 cells) is also reduced in HDM-sensitized Pglyrp1−/− mice compared with WT mice (Fig. 6).

There are also other mechanisms of generating and maintaining Treg cells, which are, however, less likely targets for up-regulation in Pglyrp1−/− mice. Generation of IL-10–secreting Treg cells by pDC can be mediated through the ICOS ligand (39). This mechanism is less likely in our model, because the expression of Icosl was not up-regulated in the lungs of HDM-sensitized Pglyrp1−/− mice compared with WT mice (data not shown). Another molecule that is required for the induction of Foxp3 expression and that enhances generation and maintenance of Treg cells is programmed death ligand 1 (PD-L1, also known as CD274) (48–50). However, generation of increased numbers of Treg cells in HDM-sensitized Pglyrp1−/− mice through the increased expression of PD-L1 is unlikely, because the expression of PD-L1 (Cd274) is not increased in HDM-sensitized Pglyrp1−/− mice compared with WT mice (Fig. 6).

The classical activation of macrophages and cDC, which both promote inflammation, is decreased in HDM-sensitized Pglyrp1−/− mice compared with WT mice (reduced Nos2, C2d273, and Tnfsf4 expression in Fig. 6), consistent with the attenuated asthma phenotype of Pglyrp1−/− mice. Expression of marker genes for the alternative activation of macrophages (Arg1, Chi3l1, Chi3l3, Fizz1, and Mrcl), which may either promote inflammation or promote healing (51–54), is also mostly decreased in HDM-sensitized Pglyrp1−/− mice compared with WT mice (Fig. 6), consistent with the total decrease in the numbers of macrophages in the lungs of HDM-sensitized Pglyrp1−/− mice compared with WT mice (Fig. 2).

How are pDC recruited into the lungs in HDM-sensitized Pglyrp1−/− mice? Local allergen sensitization recruits pDC into the lungs from MLN as a feedback mechanism to inhibit inflammation (35). The expression of Cxcl9 and Cxcl10 is increased in Pglyrp1−/− mice (Fig. 6) and pDC express CXCR3 (55), which is the receptor for these chemokines. CXCL9 and CXCL10 are also likely candidates for preferential recruitment of pDC to the sensitized lungs in Pglyrp1−/− mice. As significantly more pDC accumulate in the lungs, more Treg cells are generated, which results in greatly diminished numbers of Th2, Th17, and Th1 cells in Pglyrp1−/− mice than in WT mice, which increases the generation of Treg cells and pDC (56–58). Especially relevant to our results are recent findings showing that germ-free mice have decreased numbers of pDC and enhanced sensitivity to asthma, which can be reversed by colonization with certain bacteria (59), and that some commensal bacteria induce Treg cells through their effect on pDC (60). Moreover, metabolites from commensal bacteria, such as fatty acids (61) or ATP (62), greatly affect not only the metabo-


lism of the host but also its immune system. For example, generation of Treg cells is promoted by fatty acids, whereas generation of Th cells is promoted by glucose (63). Thus, changes in commensal bacteria (such as those in Pglyrp1−/− mice), could influence the development of pDC or Treg cells. Another possibility is that induction of Pglyrp1 expression in lung epithelial cells or delivery of Pglyrp1 by eosinophils, neutrophils, and macrophages (Fig. 4) (11, 14, 20, 21) have direct effects on the secretion of chemokines and cytokines by epithelial cells in the lungs, creating a proinflammatory microenvironment. These possibilities will be investigated in future studies.

Peptidoglycan is the ligand for several pattern recognition molecules and a potent stimulus of innate immunity (15, 17). Pglyrp1 can inhibit some of these immunostimulatory activities by binding to peptidoglycan and preventing its recognition by innate immunity receptors (14, 18). Pglyrp2 can also hydrolyze peptidoglycan and eliminate some of its immunostimulatory activities (15, 19). However, these effects of Pglyrp1 and other Pglyrps on peptidoglycan are probably unrelated to their ability to modulate inflammation, because Pglyrps modulate inflammatory responses induced not only by peptidoglycan (14, 18, 25) but also by unrelated allergens and Ags (22–26). Therefore, as discussed above, modulation of inflammation by Pglyrps is most likely indirect through the effects of Pglyrps on the microbiome and local cytokine-secreting cells.

Our results on the HDM mouse model are relevant to human asthma for several reasons. First, HDM is a frequent allergen in human asthma (6). Second, asthma is associated with atopic dermatitis in 50% of patients (30), and our previous (26) and current results reveal a role for Pglyrp1 in both atopic dermatitis and asthma. These results suggest Pglyrp1 as a possible candidate for susceptibility to both lung and skin inflammatory diseases (24, 26). Third, increased numbers of cDC (which are proinflammatory and oppose anti-inflammatory effects of pDC) are present in BAL fluid in patients with asthma (64), whereas these patients have low numbers of pDC (65) or impaired functions of pDC (66, 67). And fourth, deficiency of circulating pDC during infancy is a risk factor for more frequent and more severe respiratory tract infections, wheezing, and diagnosis of asthma, whereas infants with higher numbers of pDC are protected against these outcomes (68). Thus, our results are consistent with these clinical observations that increasing numbers are protected against these outcomes (68). Thus, our results are consistent with these clinical observations that increasing numbers are protected against these outcomes (68). Thus, our results are consistent with these clinical observations that increasing numbers of pDC modulate the inflammatory activity of pDC to decreasing its expression in the lungs may be a new approach to prevention of asthma; Chang H. Kim (Purdue University) for advice on T cell subpopulations; and Julie Cook for maintaining and breeding mice.

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Disclosures

The authors have no financial conflicts of interest.

References


Corrections


In Fig. 9D, the descriptions for the y axes should read 10^{-4} instead of 10^{-6}. The corrected Fig. 9 is shown below. The figure legend is correct as published and is shown below for reference.

**FIGURE 9.** Depletion of pDC in vivo reverses attenuated asthma phenotype in HDM-sensitized Pglyrp1^{-/-} mice. Pglyrp1^{-/-} mice were treated with anti-pDC (anti-CD317) mAb or isotype control IgG during sensitization with HDM for 5 wk. Anti-pDC mAb reduced the numbers of pDC in vivo (A). Depletion of pDC increased the numbers of eosinophils, neutrophils, lymphocytes, and macrophages in BAL cells and lungs (B), increased lung airway resistance (C), increased numbers of CD4^{+} cells in BAL cells and CD4^{+}, Th1, Th2, and Th17 cells in the lungs, and decreased the percentage of Treg cells in BAL cells and the lungs (D), measured by flow cytometry, with gating as shown in Figs. 7, 8A; means ± SEM of six mice per group; *p < 0.05; **p < 0.005; anti-pDC versus IgG.

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