Peptidoglycan Recognition Protein 1 Promotes House Dust Mite–Induced Airway Inflammation in Mice

Xianglan Yao¹, Meixia Gao¹, Cuilian Dai¹, Katharine S. Meyer¹, Jichun Chen², Karen J. Keeran³, Gayle Z. Nugent³, Xuan Qu⁴, Zu-Xi Yu⁴, Pradeep K. Dagur⁵, J. Philip McCoy⁵, and Stewart J. Levine¹

¹Cardiovascular and Pulmonary Branch, ²Hematology Branch, ³Laboratory of Animal Surgery and Resources Core Facility, ⁴Pathology Core Facility, and ⁵Flow Cytometry Core Facility, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland

Peptidoglycan recognition protein (Pglyrp) 1 is a pattern-recognition protein that mediates antibacterial host defense. Because we had previously shown that Pglyrp1 expression is increased in the lungs of house dust mite (HDM)-challenged mice, we hypothesized that it might modulate the pathogenesis of asthma. Wild-type and *Pglyrp1*^{−/−} mice on a BALB/c background received intranasal HDM or saline, 5 days/week for 3 weeks. HDM-challenged *Pglyrp1*^{−/−} mice showed decreases in bronchoalveolar lavage fluid eosinophils and lymphocytes, serum IgE, and mucous cell metaplasia, whereas airway hyperresponsiveness was not changed when compared with wild-type mice. T helper type 2 (Th2) cytokines were reduced in the lungs of HDM-challenged *Pglyrp1*^{−/−} mice, which reflected a decreased number of CD4⁺ Th2 cells. There was also a reduction in C-C chemokines in bronchoalveolar lavage fluid and lung homogenates from HDM-challenged *Pglyrp1*^{−/−} mice. Furthermore, secretion of CCL17, CCL22, and CCL24 by alveolar macrophages from HDM-challenged *Pglyrp1*^{−/−} mice was markedly reduced. As both inflammatory cells and airway epithelial cells express Pglyrp1, bone marrow transplantation was performed to generate chimeric mice and assess which cell type promotes HDM-induced airway inflammation. Chimeric mice lacking Pglyrp1 on hematopoietic cells, not structural cells, showed a reduction in HDM-induced eosinophilic and lymphocytic airway inflammation. We conclude that Pglyrp1 expressed by hematopoietic cells, such as alveolar macrophages, mediates HDM-induced airway inflammation by up-regulating the production of C-C chemokines that recruit eosinophils and Th2 cells to the lung. This identifies a new family of innate immune response proteins that promotes HDM-induced airway inflammation in asthma.

Keywords: asthma; house dust mite; innate immunity; pattern recognition proteins; peptidoglycan recognition protein 1

Airway inflammation is a key pathogenic feature of allergic asthma that is typified by T helper type 2 (Th2)-mediated adaptive immune responses (1). It has been increasingly recognized that allergic asthmatic airway inflammation also involves innate immune responses mediated by pattern recognition receptors, such as Toll-like receptors

CLINICAL RELEVANCE

Both innate and adaptive immune responses play an important role in the induction of house dust mite (HDM)-induced airway inflammation. This study shows that the pattern-recognition protein, peptidoglycan recognition protein 1, mediates HDM-induced eosinophilic and lymphocytic airway inflammation by promoting the expression of C-C chemokines from hematopoietic cells, such as alveolar macrophages. This identifies a new family of innate immune response proteins that facilitates the recruitment of eosinophils and T helper type 2 cells to the lung in HDM-induced asthma.

(TLRs), that bind pathogen-associated molecular patterns (1, 2). For example, extracts of house dust mites (HDMs), which are common aeroallergens that cause atopic asthma, contain a variety of gram-negative bacteria, including *Bartonella* species (3). Consistent with this, allergic sensitization to HDM requires the LPS-mediated activation of TLR4 signaling by bronchial epithelial cells that induces the release of IL-1α (4, 5). IL-1α then acts in an autocrine fashion to induce IL-33 and granulocyte/macrophage colony-stimulating factor, which mediate the recruitment of dendritic cells to the airway. Ligands for TLR2, TLR3, TLR4, and TLR5 can also induce the expression of granulocyte/macrophage colony-stimulating factor and CCL20 (macrophage inflammatory protein-3α) by bronchial epithelial cells, which mediate the recruitment and activation of immature dendritic cells (6, 7). The HDM protein, Der p2, also interacts with the TLR4 complex and LPS to facilitate dendritic cell activation (8). Similarly, binding of flagellin to TLR5 mediates eosinophilic airway inflammation and airway hyperresponsiveness (AHR) by priming allergic responses to HDM (9). Thus, innate immune responses mediated by TLRs expressed by airway epithelial cells and antigen-presenting cells play a central role in the initiation of allergic inflammation via the activation and recruitment of dendritic cells (4, 8).

To identify new mechanisms that mediate the pathogenesis of asthma, we previously used an experimental murine model where airway disease was induced by multiple nasal HDM challenges with the goal of identifying genes that have increased expression in the lung despite treatment with corticosteroids (10). A genome-wide analysis of the lung transcriptome identified 68 genes that met these criteria. These experiments identified an innate immunity gene, peptidoglycan recognition protein (Pglyrp) 1, which had not previously been known to modulate the pathogenesis of asthma, as having increased expression in the lungs of HDM-challenged mice. Pglyrp1 is a 19-kD member of a family of pattern-recognition proteins that are highly conserved from insects to mammals and mediate host defense against bacterial pathogens (11, 12). The mechanism by which Pglyrps kill bacteria involves activation of the CsrR-CsrS two-component

(Received in original form January 2, 2013 and in final form June 20, 2013)

This work was supported by the Division of Intramural Research, National Heart, Lung, and Blood Institute.

Author Contributions: Conception and design—X.Y., J.C., P.K.D., J.P.M., and S.J.L.; acquisition of data—X.Y., M.G., C.D., K.S.M., K.J.K., G.Z.N., X.Q., Z.-X.Y., and P.K.D.; analysis and interpretation—X.Y., M.G., Z.-X.Y., P.K.D., J.P.M., and S.J.L.; drafting and revising the manuscript for important intellectual content—X.Y., M.G., C.D., K.S.M., J.C., K.J.K., G.Z.N., X.Q., Z.-X.Y., P.K.D., J.P.M., and S.J.L.

Correspondence and requests for reprints should be addressed to Stewart J. Levine, M.D., Cardiovascular and Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 10, Room 6D03, MSC 1590, Bethesda, MD 20892-1590. E-mail: levines@nhlbi.nih.gov

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 49, Iss. 6, pp 902–911, Dec 2013

Published 2013 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2013-0001OC on June 28, 2013

Internet address: www.atsjournals.org

system that triggers cell death upon sensing of misfolded proteins (13). Pglyrp1 is bactericidal, but, similar to mammalian Pglyrp3 and Pglyrp4, lacks amidase activity that catalyzes the hydrolysis of amide bonds (12). Pglyrp1 is expressed by inflammatory cells, such as neutrophils and eosinophils, as well as by a variety of tissues, including the gastrointestinal system, salivary glands, and mammary glands (12). Pglyrp1 has been shown to have protective effects against experimental colitis by promoting normal gut flora, attenuating IFN- γ production, and reducing IFN-inducible gene expression (14). Pglyrp1 also has context-dependent effects on immune responses in experimental murine model systems. For example, Pglyrp1 promotes inflammation in models of atopic and contact dermatitis and psoriasis-like dermatitis, whereas it has anti-inflammatory effects in experimental peptidoglycan-induced arthritis (15–17). In addition, lymphokine-activated killer T cells produce Pglyrp1 that forms a complex with heat shock protein 70 and has cytotoxic activity toward tumor cells (18).

Based upon the up-regulated expression of Pglyrp1 in the lungs of HDM-challenged mice, we hypothesized that Pglyrp1 might modulate the pathogenesis of asthma. Here, we show that several of the key pathogenic manifestations of HDM-induced asthma, such as eosinophilic and lymphocytic airway inflammation, IgE production, and mucous cell metaplasia, are attenuated in *Pglyrp1*^{−/−} mice. This defines a novel function for the pattern-recognition protein, Pglyrp1, as a mediator of HDM-induced airway inflammation via its ability to promote C-C chemokine production by alveolar macrophages and the subsequent recruitment of eosinophils and Th2 lymphocytes to the lung.

MATERIALS AND METHODS

See the online supplement for additional methods.

Murine Model of HDM-Induced Airway Disease

Pglyrp1^{−/−} mice on a BALB/c background, which have previously been described, were generously provided by Dr. Roman Dziarski at Indiana University School of Medicine-Northwest (Gary, IN) and Dr. Tamas Oravecz, Lexicon Pharmaceuticals (The Woodlands, TX) (19). As was the case for the original colony founder, wild-type (WT) BALB/c mice were from Harlan Laboratories, Inc. (Indianapolis, IN), and were bred and housed in the same facility as the *Pglyrp1*^{−/−} mice. BALB/c and *Pglyrp1*^{−/−} mice (6–8 wk old) received daily intranasal challenges of 25 μ g of HDM (*Dermatophagoides pteronyssinus* extract; Greer Laboratories, Lenoir, NC) or normal saline, both in a volume of 10 μ l, 5 days/week for 3 weeks. Animals were killed for end-point analysis 72 hours after the last HDM administration. Experimental protocols were approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute.

Bronchoalveolar Lavage Fluid Cells

Bronchoalveolar lavage (BAL) was performed three times with 0.5 ml PBS (10, 20). Red blood cells were lysed with ACK buffer for 2 minutes at 4°C and BAL fluid (BALF) cells were resuspended in 0.3 ml RPMI-1640 containing 10% FBS. Total cell counts were performed using a hemocytometer and differential cell counts were performed using Diff-Quik–stained cytospin slides (Siemens, Deerfield, IL).

ELISAs

Quantification of cytokines and chemokines were performed using Duoset ELISA kits from R&D Systems (Minneapolis, MN). The limits of sensitivity were 9.3125 pg/ml for CCL11 and CCL22, 15.625 pg/ml for IL-4 and CCL24, 37.25 pg/ml for IL-5 and CCL17, and 62.5 pg/ml for IL-13.

Generation of Chimeric Mice by Bone Marrow Transplantation

Bone marrow cells were isolated by flushing the femurs and tibias of donor mice with improved minimum essential medium (Life Technologies, Grand Island, NY) and passing cells through a 100- μ m sterile cell strainer to obtain single-cell suspensions. Recipient mice were conditioned with total body irradiation administered as a single exposure at a dose of 800 rads using a cesium irradiator (ACL Gammacell 40; MDS Nordion, Ottawa, ON, Canada). Irradiated recipients received a single intravenous injection of 5×10^6 bone marrow cells in the lateral tail vein 4 hours after irradiation. Recipient mice did not receive HDM challenges until 12 weeks after the bone marrow transplant to allow engraftment. Chimerism was assessed by quantitative RT-PCR (qRT-PCR) analysis of Pglyrp1 mRNA levels in alveolar macrophages and peripheral blood mononuclear cells.

Statistical Analysis

Data are presented as means ($\pm$ SEM). Results were analyzed using a one-way ANOVA with Bonferroni's multiple comparison test, a two-way ANOVA with Bonferroni's post test, or an unpaired two-tailed *t* test for comparison of two experimental groups (GraphPad Prism version 5.0a; GraphPad Software, La Jolla, CA). A *P* value less than 0.05 was considered significant.

RESULTS

HDM Up-Regulates Pglyrp1 Expression in the Lung

To define the role of Pglyrp1 in the pathogenesis of asthma, WT and *Pglyrp1*^{−/−} mice received daily nasal HDM challenges, 5 days/week for 3 weeks. First, the effect of HDM challenges on Pglyrp1 expression in the lung was assessed. As shown in Figure 1, lung *Pglyrp1* mRNA levels were increased in response to HDM challenges in WT mice, whereas *Pglyrp1* mRNA was not detected in the lungs of *Pglyrp1*^{−/−} mice. Similarly, Pglyrp1 protein levels were increased in both BALF as well as lung tissue from HDM-challenged WT mice, whereas Pglyrp1 protein was not detected in the BALF or lungs from *Pglyrp1*^{−/−} mice. Next, lung mRNA was amplified by qRT-PCR to assess which members of the Pglyrp family demonstrated increased expression in response to HDM stimulation. As shown in Figure E1 in the online supplement, HDM challenge increased *Pglyrp2* mRNA levels in the lungs of WT and *Pglyrp1*^{−/−} mice, whereas mRNA levels of *Pglyrp3* and *Pglyrp4* were not modified.

Because a genome-wide analysis of the lung transcriptome had previously identified that *Pglyrp1* mRNA expression was increased in the lungs of A/J mice in response to HDM stimulation, and that mRNA levels remained greater than twofold increased despite treatment with corticosteroids (10), we next assessed the effect of corticosteroid treatment on Pglyrp1 protein levels in WT BALB/c mice, which is the genetic background of the *Pglyrp1*^{−/−} mice. As shown in Figure E2, corticosteroid treatment effectively suppressed the up-regulated expression of Pglyrp1 protein in response to HDM stimulation both in BALF and lung tissue. Furthermore, corticosteroid treatment significantly reduced HDM-induced increases in lung protein levels of CCL11 (eotaxin-1) and CCL24 (eotaxin-2), which mediate eosinophil chemotaxis via binding to CCR3, as well as CCL17 (TARC) and CCL22 (MDC), which mediate Th2 cell chemotaxis via binding to CCR4 (Figure E3) (21). Collectively, these data demonstrate that HDM-induced increases in the expression of Pglyrp1 and C-C chemokines in the lungs of BALB/c mice are significantly suppressed by corticosteroid treatment.

Experiments were next performed to assess which cell types express Pglyrp1 in the lung. As shown in Figure 2, confocal immunofluorescence microscopy of BALF cells from

Figure 1. House dust mite (HDM) challenge induces peptidoglycan recognition protein (Pglyrp) 1 expression in the lung. (A) Lung mRNA expression of *Pglyrp1* was assessed by quantitative RT-PCR (qRT-PCR) and is presented as relative mRNA expression ($n = 6$ mice; $*P < 0.001$, wild-type [WT] + saline vs. WT + HDM; one-way ANOVA with Bonferroni's multiple comparison test). Data are representative of two independent experiments. (B) Western blots of proteins present in bronchoalveolar lavage fluid (BALF; 15 μ l/lane) were reacted with antibodies directed against Pglyrp1. The polyvinylidene difluoride membrane was stained with Coomassie G-250 and a section with the most abundant proteins is shown for comparison of protein loading. Lung proteins from two mice are shown. This result is representative of four replicate blots. (C and D) Western blots of lung proteins (24 μ g/lane) were reacted with antibodies directed against Pglyrp1 or β -actin. Lung proteins from two mice are shown. This result is representative of four replicate blots. The ratio of Pglyrp1 to β -actin protein was quantified by densitometry ($n = 8$ mice, $*P < 0.0001$, one-way ANOVA with Bonferroni's multiple comparison test).

HDM-challenged WT mice demonstrated Pglyrp1 expression by eosinophils, neutrophils, and alveolar macrophages. Similarly, confocal immunofluorescence microscopy of lung sections from HDM-challenged WT mice showed that airway

epithelial cells express Pglyrp1. These findings demonstrate that Pglyrp1 expression in the lung is induced in response to HDM exposure, and that both inflammatory and structural cells express Pglyrp1.

Figure 2. Characterization of Pglyrp1 expression in the lung. Confocal immunofluorescence microscopy demonstrated that eosinophils, neutrophils, alveolar macrophages, and ciliated airway epithelial cells express Pglyrp1. (A) Cytospins of BALF cells from HDM-challenged WT BALB/c mice were reacted with antibodies against Pglyrp1 and either major basic protein for eosinophils (top panel), Gr-1 for neutrophils (middle panel), or CD68 for macrophages (bottom panel). (B) Confocal immunofluorescence microscopy was performed on lung tissue from HDM-challenged WT BALB/c mice that were reacted with antibodies against Pglyrp1 and acetylated α -tubulin. Secondary antibodies were conjugated with Alexa Fluor 488 (green) or Alexa Fluor 568 (red), and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue).

Figure 3. Airway inflammation is attenuated in HDM-challenged *Pglyrp1*^{-/-} mice. Number of total BALF inflammatory cells (A) and inflammatory cell types (B) ($n = 10$ mice, $*P < 0.01$, *Pglyrp1*^{-/-} + HDM vs. WT + HDM; one-way ANOVA with Bonferroni's multiple comparison test). Results are representative of three independent experiments. (C) Representative histologic sections of lungs from WT and *Pglyrp1*^{-/-} mice that had been challenged with saline or HDM and stained with hematoxylin and eosin (H&E; $\times 200$) or periodic acid-Schiff (PAS; $\times 200$ and $\times 1,000$).

Eosinophilic and Lymphocytic Airway Inflammation Are Attenuated in HDM-Challenged *Pglyrp1*^{-/-} Mice

BAL was performed on HDM-challenged WT and *Pglyrp1*^{-/-} mice to assess whether *Pglyrp1* modifies airway inflammatory responses. As shown in Figure 3, both the total number of BALF cells, as well as the number of BALF eosinophils and lymphocytes, were significantly reduced in HDM-challenged *Pglyrp1*^{-/-} mice compared with WT mice. Similarly, lung histology revealed a decrease in peribronchial inflammatory cell infiltrates in HDM-challenged *Pglyrp1*^{-/-} mice compared with WT mice (Figure 3C). Serum levels of total IgE, IgG₁, and IgG_{2a} (Figures 4A–4C), as well as HDM-specific IgE (Figure E4), were significantly decreased in HDM-challenged *Pglyrp1*^{-/-} mice compared with WT mice, whereas HDM-specific IgG₁ was not significantly reduced (Figure E4). There was no difference in the number of CD11b⁺/F4/80⁺/CD206⁺ alternatively activated macrophages that were present in BALF (Figure 4D), whereas airway remodeling

was significantly reduced in HDM-challenged *Pglyrp1*^{-/-} mice based upon a decrease in the percentage of airways with mucous cell metaplasia compared with WT mice (Figures 3C and 4E). Finally, there was no difference in AHR between HDM-challenged *Pglyrp1*^{-/-} and WT mice (Figure 4F). These results demonstrate that *Pglyrp1* mediates HDM-induced eosinophilic and lymphocytic airway inflammation, as well as increases in serum IgE and mucous cell metaplasia, but does not modulate AHR in a murine model of HDM-induced asthma.

Next, experiments were performed to confirm that the airway inflammation in HDM-challenged *Pglyrp1*^{-/-} mice reflected an allergen-mediated response to HDM rather than an isolated inflammatory response to a TLR ligand, such as LPS, which is a component of HDM extracts. The HDM extract that we used contained between 29.1 and 50 endotoxin units/mg of HDM protein, such that each mouse received between 73 and 125 pg of LPS per day when challenged with 25 μ g of HDM. Prior

Figure 4. Ig production and mucous cell metaplasia are attenuated in HDM-challenged *Pglyrp1*^{-/-} mice. (A–C) Serum levels of total IgE, IgG₁, and IgG_{2a} were quantified by ELISA. (*n* = 8–11 mice, **P* < 0.01, *Pglyrp1*^{-/-} + HDM vs. WT + HDM; one-way ANOVA with Bonferroni's multiple comparison test). (D) Quantification of the number of CD11b⁺/F4/80⁺/CD206⁺ alternatively activated macrophages in BALF by flow cytometry (*n* = 8 mice, *P* = nonsignificant, *Pglyrp1*^{-/-} + HDM vs. WT + HDM; unpaired two-tailed *t* test). (E) Mucous cell metaplasia is presented as the percentage of airways on lung histological sections that contained periodic acid–Schiff positive cells (*n* = 10 mice, **P* < 0.0001, *Pglyrp1*^{-/-} + HDM vs. WT + HDM; one-way ANOVA with Bonferroni's multiple comparison test). Airways (42.3 ± 1.4) were counted in each animal. (F) Airway resistance (cm H₂O/ml/s) was measured after nebulization of increasing doses of methacholine. (*n* = 19–20 mice, *P* = nonsignificant, *Pglyrp1*^{-/-} + HDM vs. WT + HDM; two-way ANOVA with Bonferroni's posttest). (A–E) Representative of two independent experiments; (F) pooled data from three independent experiments.

reports of experimental HDM-induced asthma have used HDM extracts that contained similar amounts of LPS (4). It has also been shown that exposure to low levels of LPS in this range did not generate pulmonary inflammation in sensitized animals in the absence of a concurrent allergen challenge (22). Similarly, we found that daily intranasal challenges of 100 pg of LPS to WT and *Pglyrp1*^{-/-} mice did not induce increases in BALF inflammatory cells (Figure E5A). Next, experimental asthma was induced by sensitization and challenge with a purified preparation of the major HDM allergen, Der p1, that contained a small amount of LPS (0.01 endotoxin units/mg), such that each mouse received 50 pg of LPS when challenged with 50 µg of Der p1. As shown in Figure E5B, WT mice that were sensitized and challenged with Der p1 had significant increases in the total number of BALF cells, which reflected increases in eosinophils and lymphocytes. Furthermore, the number of BALF eosinophils was significantly reduced in Der p1-challenged *Pglyrp1*^{-/-} mice compared with WT mice. Collectively, these results are consistent with the conclusion that daily administration of intranasal HDM model induced an allergic inflammatory response that was not solely mediated by the LPS component of HDM. Finally, experiments were performed to assess whether LPS or Th2 cytokines induce *Pglyrp1* expression in macrophages. As shown in Figure E6, bone marrow-derived macrophages that were stimulated *ex vivo* with IL-4 (20 ng/ml) had significant increases in *Pglyrp1* mRNA expression, whereas stimulation with either IL-13 (20 ng/ml) or LPS (100 pg/ml) had no effect. This demonstrates that the Th2 cytokine, IL-4, induces *Pglyrp1* mRNA expression in macrophages.

The Number of Th2 Cells Is Reduced in the Lungs of HDM-Challenged *Pglyrp1*^{-/-} Mice

Experiments were next conducted to assess whether *Pglyrp1* modulates Th2 cell numbers or cytokine production as a mechanism by which eosinophilic and lymphocytic airway inflammation are attenuated in HDM-challenged *Pglyrp1*^{-/-} mice. As shown in Figure 5, protein levels of Th2 cytokines, IL-4, IL-5, and IL-13, were reduced in lungs from HDM-challenged *Pglyrp1*^{-/-} mice as compared with HDM-challenged WT mice. Flow cytometry of BALF cells also showed that the number of CD4⁺ Th2 cells expressing IL-5 or IL-13 was decreased in the BALF and mediastinal lymph nodes from HDM-challenged *Pglyrp1*^{-/-} mice. In contrast, the level of expression of IL-5 and IL-13 by single T cells, as assessed by mean fluorescence intensity, was not reduced (Figures 5B–5E). Next, mediastinal lymph node cells isolated from HDM-challenged *Pglyrp1*^{-/-} and WT mice were restimulated with HDM to further assess Th2 cell function. Secretion of IL-4, IL-5, and IL-13 by mediastinal lymph node T cells from HDM-challenged *Pglyrp1*^{-/-} mice in response to HDM restimulation was similar to T cells isolated from HDM-challenged WT mice (Figure 5F). Collectively, these results demonstrate that the number of CD4⁺ Th2 cells is decreased in the lungs of HDM-challenged *Pglyrp1*^{-/-} mice, whereas their ability to produce cytokines is normal. Finally, we assessed whether the reduction in the number of Th2 cells in the lungs and mediastinal lymph nodes of HDM-challenged *Pglyrp1*^{-/-} mice was mediated at the level of CD4⁺/CD25⁺/Foxp3⁺ regulatory

Figure 5. The number of T helper type 2 (Th2) cells is reduced in the lungs of HDM-challenged *Pglyrp1*^{-/-} mice. (A) The expression of IL-4, IL-5, and IL-13 in lung homogenates was quantified by ELISA ($n = 10$ mice, $*P < 0.01$, *Pglyrp1*^{-/-} + HDM vs. WT + HDM; one-way ANOVA with Bonferroni's multiple comparison test). Data are representative of two independent experiments that showed similar results. (B–E) The percentage of CD3⁺/CD4⁺ T cells expressing IL-5 and IL-13 present in BALF (B) and mediastinal lymph nodes (C) ($n = 8–9$ mice, $*P < 0.05$, *Pglyrp1*^{-/-} + HDM vs. WT + HDM; unpaired two-tailed t test) were quantified by flow cytometry. The mean fluorescence intensity of CD3⁺/CD4⁺ T cells expressing IL-5 and IL-13 present in BALF (D) and mediastinal lymph nodes (E) ($n = 8–11$ mice, $P = NS$, *Pglyrp1*^{-/-} + HDM vs. WT + HDM; unpaired two-tailed t test) is also shown. (F) Cytokine secretion by ex vivo cultures of mediastinal lymph node cells that had been restimulated with or without HDM (100 μ g/ml) was quantified by ELISA ($n = 16–20$ mice, $P = NS$, *Pglyrp1*^{-/-} + HDM vs. WT + HDM; one-way ANOVA with Bonferroni's multiple comparison test). (G) The number of CD4⁺/CD25⁺/Fopx3⁺ cells present in BALF and mediastinal lymph nodes was quantified by flow cytometry ($n = 8–9$ mice, $P = NS$, *Pglyrp1*^{-/-} + HDM vs. WT + HDM; unpaired two-tailed t test). Results shown in (B–G) represent pooled data from two independent experiments.

T cells (Tregs). As shown in Figure 5G, the numbers of CD4⁺/CD25⁺/Fopx3⁺ Tregs in either BALF or mediastinal lymph nodes from HDM-challenged *Pglyrp1*^{-/-} mice were not different than those from WT mice. This shows that the reduction in the number of Th2 cells in the lungs of HDM-challenged *Pglyrp1*^{-/-} mice is not mediated by an increase in the number of Tregs.

C-C Chemokine Production by Alveolar Macrophages Is Attenuated in HDM-Challenged *Pglyrp1*^{-/-} Mice

The finding that the number of CD4⁺ Th2 cells was reduced in HDM-challenged *Pglyrp1*^{-/-} mice suggested that the production of C-C chemokines that possess chemotactic activity toward Th2 cells and eosinophils may be decreased as a mechanism by which

Figure 6. C-C chemokine secretion by alveolar macrophages is attenuated in HDM-challenged *Pglyrp1*^{-/-} mice. (A and B) The expression of CCL11, CCL24, CCL17 and CCL22 in lung homogenates (A) and BALF (B) was quantified by ELISA ($n = 8-10$ mice, $*P < 0.05$, *Pglyrp1*^{-/-} + HDM vs. WT + HDM; one-way ANOVA with Bonferroni's multiple comparison test). Data are representative of two independent experiments. (C) The amount of CCL17, CCL22, and CCL24 secreted by *ex vivo* cultures of alveolar macrophages isolated from HDM-challenged *Pglyrp1*^{-/-} and WT mice that had been restimulated with HDM was quantified by ELISA ($n = 10-16$ mice, $*P < 0.0001$, *Pglyrp1*^{-/-} + HDM vs. WT + HDM; one-way ANOVA with Bonferroni's multiple comparison test). Results represent pooled data from at least two independent experiments.

eosinophilic and lymphocytic airway inflammation is attenuated. Analysis of lung proteins and BALF from HDM-challenged *Pglyrp1*^{-/-} mice showed reductions of CCL11, CCL17, CCL22, and CCL24 (Figures 6A and 6B) (21). Next, we assessed whether the decrease in C-C chemokine production in the lungs of HDM-challenged *Pglyrp1*^{-/-} mice reflected reduced secretion by alveolar macrophages. *Ex vivo* cultures of alveolar macrophages isolated from the lungs of HDM-challenged *Pglyrp1*^{-/-} mice had significant reductions in the secretion of CCL17, CCL22, and CCL24 upon restimulation with HDM compared with alveolar macrophages from WT mice (Figure 6C). The amount of CCL11 produced by alveolar macrophages was below the limit of detection of the assay. This demonstrates that C-C chemokine production by alveolar macrophages from HDM-challenged *Pglyrp1*^{-/-} mice is reduced.

Pglyrp1 Expressed by Hematopoietic Cells Mediates HDM-Induced Airway Inflammation

Because both immune and structural cells express *Pglyrp1*, bone marrow transplantation experiments were performed to confirm that hematopoietic cells, such as alveolar macrophages, mediate *Pglyrp1*-dependent airway inflammation. Chimeric mice were generated that expressed either *Pglyrp1*-deficient hematopoietic cells and WT nonhematopoietic cells (*Pglyrp1*^{-/-} > WT mice), or WT immune cells and *Pglyrp1*-deficient nonhematopoietic cells (WT > *Pglyrp1*^{-/-} mice). WT > WT mice and *Pglyrp1*^{-/-} >

Pglyrp1^{-/-} mice served as controls. Chimerism was confirmed by qRT-PCR, which showed that *Pglyrp1* mRNA was expressed by peripheral blood leukocytes and alveolar macrophages from WT > WT and WT > *Pglyrp1*^{-/-} mice, but not by those from *Pglyrp1*^{-/-} > *Pglyrp1*^{-/-} and *Pglyrp1*^{-/-} > WT mice (Figure 7). Analysis of BALF showed that the total number of inflammatory cells, as well as the number of eosinophils and lymphocytes, was significantly decreased in HDM-challenged *Pglyrp1*^{-/-} > WT mice and *Pglyrp1*^{-/-} > *Pglyrp1*^{-/-} mice compared with HDM-challenged WT > *Pglyrp1*^{-/-} mice and WT > WT mice. Consistent with this finding, lung histology revealed that peribronchial inflammatory cell infiltrates were decreased in HDM-challenged *Pglyrp1*^{-/-} > WT and *Pglyrp1*^{-/-} > *Pglyrp1*^{-/-} mice as compared with WT > *Pglyrp1*^{-/-} and WT > WT mice. In addition, HDM-induced increases in mucous cell metaplasia were significantly decreased in *Pglyrp1*^{-/-} > WT mice and *Pglyrp1*^{-/-} > *Pglyrp1*^{-/-} mice compared with WT > *Pglyrp1*^{-/-} mice and WT > WT mice. Collectively, these data demonstrate that *Pglyrp1* expressed by hematopoietic cells mediates HDM-induced eosinophilic and lymphocytic airway inflammation.

DISCUSSION

*Pglyrp*s are a family of pattern-recognition proteins that mediate innate immunity to bacterial pathogens via binding peptidoglycan

Figure 7. Hematopoietic cells mediate *Pglyrp1*-dependent increases in airway eosinophils and lymphocytes in HDM-challenged mice. Chimeric mice were generated by bone marrow transplantation. (A and B) mRNA expression of *Pglyrp1* in peripheral blood leukocytes ($n = 9$ mice) and alveolar macrophages ($n = 4-5$ mice) from HDM-challenged mice were assessed by qRT-PCR and presented as relative mRNA expression. (C-E) Numbers of total BALF inflammatory cells (C), eosinophils (D), and lymphocytes (E) ($n = 10-18$ mice, $*P < 0.01$, vs. HDM-challenged WT $>$ WT; one-way ANOVA with Bonferroni's multiple comparison test) are shown. Data are representative of two independent experiments. (F) Mucous cell metaplasia is presented as the percentage of airways on lung histological sections that contained periodic acid-Schiff-positive cells ($n = 5-14$ mice, $*P < 0.0001$, vs. HDM-challenged WT $>$ WT; one-way ANOVA with Bonferroni's multiple comparison test). Airways (27.9 ± 0.7) were counted in each animal. Data are representative of two independent experiments. (G) Representative histologic sections of lungs from chimeric mice that had been challenged with saline or HDM and stained with H&E ($\times 200$) or PAS ($\times 200$ and $\times 1,000$).

moieties (12). Here, we show that *Pglyrp1* is expressed in the lung by eosinophils, neutrophils, alveolar macrophages, and airway epithelial cells. Furthermore, *Pglyrp1* expression at the mRNA and protein levels was significantly increased in the lung and BALF in response to multiple HDM challenges, whereas *Pglyrp1* expression by macrophages was significantly up-regulated by the Th2 cytokine, IL-4. *Pglyrp1*^{-/-} mice had attenuated airway

inflammatory responses to multiple nasal HDM challenges, with significant reductions in airway eosinophils and lymphocytes. The down-regulated airway inflammatory responses in HDM-challenged *Pglyrp1*^{-/-} mice were also associated with significant reductions in IgE production and mucous cell metaplasia. In contrast, AHR was not decreased. The finding that AHR was dissociated from airway inflammation, IgE production, and

mucous cell metaplasia in HDM-challenged *Pglyrp1*^{-/-} mice suggests that these cardinal pathogenic manifestations of asthma may be mediated by distinct pathways. The dissociation of AHR from airway inflammation has previously been reported. For example, we have previously shown that HDM-challenged *apoE*^{-/-} mice have significantly increased AHR and mucous cell metaplasia, whereas airway inflammation was not affected (10). In addition, treatment of WT A/J mice with corticosteroids significantly decreased airway inflammation and mucous cell metaplasia, but was associated with a small, albeit statistically significant, reduction in AHR. AHR has also been shown to be dissociated from airway inflammation after treatment with rapamycin (23). Ovalbumin-challenged transgenic mice in which NF- κ B activation is repressed specifically in the airway have decreases in allergic inflammation, IgE, and mucous cell metaplasia, but not AHR (24). Similarly, AHR has been reported to be dissociated from airway inflammation in patients with chronic asthma (25).

Th2 cells play a central role in the pathogenesis of asthma by their ability to secrete a canonical set of cytokines, including IL-4, IL-5, and IL-13, which mediate many of the key pathogenic manifestations of asthma (1, 21). We found that protein levels of Th2 cytokines were reduced in the lung of HDM-challenged *Pglyrp1*^{-/-} mice. The reduction in Th2 cytokine production, however, did not represent a decrease in cytokine production by CD4⁺ Th2 cells or an increase in number of CD4⁺/CD25⁺/Foxp3⁺ Tregs. Instead, the reduction in Th2 cytokines reflected a decrease in the number of CD4⁺ Th2 cells present in BALF and mediastinal lymph nodes from HDM-challenged *Pglyrp1*^{-/-} mice, which suggested that the defect in HDM-challenged *Pglyrp1*^{-/-} mice was primarily at the level of Th2 cell recruitment, not cytokine production.

We next assessed whether the attenuated eosinophilic and lymphocytic airway inflammation in HDM-challenged *Pglyrp1*^{-/-} mice represented defective production of C-C chemokines, leading to the reduced recruitment of Th2 cells and eosinophils to the lung (21). Chemokines are small, secreted polypeptides that regulate the trafficking of immune cells by signaling via chemokine receptors (26). CCR3, which is the receptor for CCL11 and CCL24, is highly expressed on eosinophils, whereas CCR4, which is the receptor for CCL17 and CCL22, is expressed on Th2 cells (26–28). Although CCR3 contributes to the early stages of allergen-induced Th2-mediated airway inflammation, it is CCR4 that is primarily responsible for the long-term recruitment of Th2 cells to the lung in response to chronic allergen stimulation (27, 29, 30). In contrast, CCR3 plays a critical role in the recruitment of eosinophils to the lung in experimental ovalbumin-induced asthma (31). Here, we show that HDM-mediated increases in C-C chemokines (CCL11, CCL17, CCL22, and CCL24) were significantly reduced in BALF and lung samples from HDM-challenged *Pglyrp1*^{-/-} mice. This finding is consistent with the conclusion that reduced C-C chemokine secretion in HDM-challenged *Pglyrp1*^{-/-} mice leads to the impaired recruitment of Th2 cells and eosinophils to the airway.

Macrophages have been shown to participate in Th2-mediated airway inflammation by producing C-C chemokines, such as CCL17 and CCL24, in response to IL-4 or IL-13 (32). Furthermore, CCL24, which is primarily produced by airway macrophages, is the critical STAT6-dependent chemotactic factor that recruits eosinophils to the lung in a cooperative fashion with CCL11 in response to ovalbumin or IL-13 (31, 33, 34). Therefore, we assessed whether alveolar macrophages mediate *Pglyrp1*-dependent, HDM-induced airway inflammation. Consistent with this, alveolar macrophages from HDM-challenged *Pglyrp1*^{-/-} mice had markedly reduced secretion of CCL17, CCL22, and CCL24 when restimulated with HDM as compared with cells isolated from HDM-challenged WT mice. Because both inflammatory cells and airway epithelial cells in

the murine lung express *Pglyrp1*, bone marrow transplantation experiments were performed to generate chimeric mice that were used to assess whether hematopoietic cells, such as alveolar macrophages, are required for *Pglyrp1*-dependent, HDM-induced eosinophilic and lymphocytic inflammation. These experiments showed that hematopoietic cells, not nonhematopoietic structural cells, mediated *Pglyrp1*-dependent increases in airway eosinophils and lymphocytes in HDM-challenged mice, as well as increases in mucous cell metaplasia.

A recent paper by Park and colleagues (35) has also shown that *Pglyrp1*^{-/-} mice have enhanced manifestations of HDM-induced experimental asthma, which provides additional evidence supporting the role of *Pglyrp1* in modulating the pathogenesis of asthma. Several important differences, however, exist between these two studies. Park showed an increase in the recruitment and retention of Tregs in the lungs of HDM-challenged *Pglyrp1*^{-/-} mice, which are proposed to down-regulate airway inflammatory responses. In contrast, our study did not identify an increase in CD4⁺/CD25⁺/Foxp3⁺ Tregs in either BALF or mediastinal lymph nodes, which shows that other mechanisms, aside from an increase in Tregs, exist to attenuate HDM-induced airway inflammation in *Pglyrp1*^{-/-} mice. Furthermore, we show that production of C-C chemokines (CCL17, CCL22, and CCL24) by alveolar macrophages isolated from HDM-challenged *Pglyrp1*^{-/-} mice is impaired. Therefore, we propose that the pathway by which *Pglyrp1* amplifies HDM-induced eosinophilic and lymphocytic airway inflammation involves the enhanced expression of C-C chemokines by alveolar macrophages that subsequently recruit increased numbers of eosinophils and lymphocytes to the lung. This identifies a new role for *Pglyrp1* in modulating airway inflammatory responses by regulating C-C chemokine production by alveolar macrophages. Park also showed that HDM-challenged *Pglyrp1*^{-/-} mice have reduced AHR, whereas, in our study, there were no differences in AHR between HDM-challenged *Pglyrp1*^{-/-} and WT mice. Therefore, we conclude that *Pglyrp1* is not required for maximal AHR in HDM-challenged mice, which is in contrast to its role in mediating amplified eosinophilic and lymphocytic inflammatory responses.

In summary, we have shown that *Pglyrp1*, a pattern recognition protein involved in host defense against bacterial infections, promotes airway inflammation, IgE production, and mucous cell metaplasia in an experimental murine model of HDM-mediated asthma. The pathway by which *Pglyrp1* amplifies HDM-induced eosinophilic and lymphocytic airway inflammation involves the enhanced expression of C-C chemokines by hematopoietic cells, such as alveolar macrophages. This identifies a new innate immune response to HDM that promotes the recruitment of eosinophils and Th2 cells to the lung in allergic asthma.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Dr. Roman Dziarski, Indiana University School of Medicine, and Dr. Tamas Oravecz, Lexicon Pharmaceuticals, for providing them with the *Pglyrp1*^{-/-} mice and Drs. Nancy and Jamie Lee, Mayo Clinic, Scottsdale, Arizona for the anti-major basic protein antibody. They are grateful for helpful discussions provided by Drs. Martha Vaughn and Joel Moss, and the support provided by the National Heart, Lung, and Blood Institute (NHLBI) Laboratory of Animal Surgery and Resources Core Facility and Drs. Christian A. Combs and Daniela Malide from the NHLBI Light Microscopy Core Facility.

References

- Holgate ST. Innate and adaptive immune responses in asthma. *Nat Med* 2012;18:673–683.
- Lambrecht BN,ammad H. The airway epithelium in asthma. *Nat Med* 2012;18:684–692.

3. Valerio CR, Murray P, Arlian LG, Slater JE. Bacterial 16s ribosomal DNA in house dust mite cultures. *J Allergy Clin Immunol* 2005;116:1296–1300.
4. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* 2009;15:410–416.
5. Willart MA, Deswarte K, Pouliot P, Braun H, Beyaert R, Lambrecht BN, Hammad H. Interleukin-1 α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J Exp Med* 2012;209:1505–1517.
6. Holtzman MJ. Asthma as a chronic disease of the innate and adaptive immune systems responding to viruses and allergens. *J Clin Invest* 2012;122:2741–2748.
7. Sha Q, Truong-Tran AQ, Plitt JR, Beck LA, Schleimer RP. Activation of airway epithelial cells by Toll-like receptor agonists. *Am J Respir Cell Mol Biol* 2004;31:358–364.
8. Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, Thorne PS, Wills-Karp M, Gioannini TL, Weiss JP, *et al.* Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 2009;457:585–588.
9. Wilson RH, Maruoka S, Whitehead GS, Foley JF, Flake GP, Sever ML, Zeldin DC, Kraft M, Garantziotis S, Nakano H, *et al.* The Toll-like receptor 5 ligand flagellin promotes asthma via a LDL receptor-mediated pathway. *Nat Med* 2012;18:1705–1710.
10. Yao X, Fredriksson K, Yu ZX, Xu X, Raghavachari N, Keeran KJ, Zywicke GJ, Kwak M, Amar MJ, Remaley AT, *et al.* Apolipoprotein E negatively regulates house dust mite-induced asthma via a LDL receptor-mediated pathway. *Am J Respir Crit Care Med* 2010;182:1228–1238.
11. Kang D, Liu G, Lundstrom A, Gelius E, Steiner H. A peptidoglycan recognition protein in innate immunity conserved from insects to humans. *Proc Natl Acad Sci USA* 1998;95:10078–10082.
12. Royet J, Gupta D, Dziarski R. Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. *Nat Rev Immunol* 2011;11:837–851.
13. Kashyap DR, Wang M, Liu LH, Boons GJ, Gupta D, Dziarski R. Peptidoglycan recognition proteins kill bacteria by activating protein-sensing two-component systems. *Nat Med* 2011;17:676–683.
14. Saha S, Jing X, Park SY, Wang S, Li X, Gupta D, Dziarski R. Peptidoglycan recognition proteins protect mice from experimental colitis by promoting normal gut flora and preventing induction of interferon- γ . *Cell Host Microbe* 2010;8:147–162.
15. Park SY, Gupta D, Kim CH, Dziarski R. Differential effects of peptidoglycan recognition proteins on experimental atopic and contact dermatitis mediated by Treg and Th17 cells. *PLoS ONE* 2011;6:e24961.
16. Park SY, Gupta D, Hurwich R, Kim CH, Dziarski R. Peptidoglycan recognition protein Pglyrp2 protects mice from psoriasis-like skin inflammation by promoting regulatory T cells and limiting Th17 responses. *J Immunol* 2011;187:5813–5823.
17. Saha S, Qi J, Wang S, Wang M, Li X, Kim YG, Nunez G, Gupta D, Dziarski R. PGLYRP-2 and Nod2 are both required for peptidoglycan-induced arthritis and local inflammation. *Cell Host Microbe* 2009;5:137–150.
18. Sashchenko LP, Dukhanina EA, Yashin DV, Shatalov YV, Romanova EA, Korobko EV, Demin AV, Lukyanova TI, Kabanova OD, Khaidukov SV, *et al.* Peptidoglycan recognition protein Tag7 forms a cytotoxic complex with heat shock protein 70 in solution and in lymphocytes. *J Biol Chem* 2004;279:2117–2124.
19. Dziarski R, Platt KA, Gelius E, Steiner H, Gupta D. Defect in neutrophil killing and increased susceptibility to infection with nonpathogenic gram-positive bacteria in peptidoglycan recognition protein-S (PGRP-S)-deficient mice. *Blood* 2003;102:689–697.
20. Yao X, Dai C, Fredriksson K, Dagur PK, McCoy JP, Qu X, Yu ZX, Keeran KJ, Zywicke GJ, Amar MJA, *et al.* 5A, an apolipoprotein A-I mimetic peptide, attenuates the induction of house dust mite-induced asthma. *J Immunol* 2011;186:576–583.
21. Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* 2008;118:3546–3556.
22. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, Toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 2002;196:1645–1651.
23. Mushaben EM, Kramer EL, Brandt EB, Khurana Hershey GK, Le Cras TD. Rapamycin attenuates airway hyperreactivity, goblet cells, and IgE in experimental allergic asthma. *J Immunol* 2011;187:5756–5763.
24. Poynter ME, Cloots R, van Woerkom T, Butnor KJ, Vacek P, Taatjes DJ, Irvin CG, Janssen-Heininger YM. NF- κ B activation in airways modulates allergic inflammation but not hyperresponsiveness. *J Immunol* 2004;173:7003–7009.
25. Crimi E, Spanevello A, Neri M, Ind PW, Rossi GA, Brusasco V. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. *Am J Respir Crit Care Med* 1998;157:4–9.
26. Panina-Bordignon P, Papi A, Mariani M, Di Lucia P, Casoni G, Bellettato C, Buonsanti C, Miotto D, Mapp C, Villa A, *et al.* The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J Clin Invest* 2001;107:1357–1364.
27. Lloyd CM, Delaney T, Nguyen T, Tian J, Martinez AC, Coyle AJ, Gutierrez-Ramos JC. CC chemokine receptor (CCR)3/eotaxin is followed by CCR4/monocyte-derived chemokine in mediating pulmonary T helper lymphocyte type 2 recruitment after serial antigen challenge *in vivo*. *J Exp Med* 2000;191:265–274.
28. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J Exp Med* 1998;187:875–883.
29. Perros F, Hoogsteden HC, Coyle AJ, Lambrecht BN, Hammad H. Blockade of CCR4 in a humanized model of asthma reveals a critical role for DC-derived CCL17 and CCL22 in attracting Th2 cells and inducing airway inflammation. *Allergy* 2009;64:995–1002.
30. Walsh ER, August A. Eosinophils and allergic airway disease: there is more to the story. *Trends Immunol* 2010;31:39–44.
31. Pope SM, Zimmermann N, Stringer KF, Karow ML, Rothenberg ME. The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. *J Immunol* 2005;175:5341–5350.
32. Kurowska-Stolarska M, Stolarski B, Kewin P, Murphy G, Corrigan CJ, Ying S, Pitman N, Mirchandani A, Rana B, van Rooijen N, *et al.* IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *J Immunol* 2009;183:6469–6477.
33. Pope SM, Fulkerson PC, Blanchard C, Akei HS, Nikolaidis NM, Zimmermann N, Molkenin JD, Rothenberg ME. Identification of a cooperative mechanism involving interleukin-13 and eotaxin-2 in experimental allergic lung inflammation. *J Biol Chem* 2005;280:13952–13961.
34. Zimmermann N, Hogan SP, Mishra A, Brandt EB, Bodette TR, Pope SM, Finkelman FD, Rothenberg ME. Murine eotaxin-2: a constitutive eosinophil chemokine induced by allergen challenge and IL-4 overexpression. *J Immunol* 2000;165:5839–5846.
35. Park SY, Jing X, Gupta D, Dziarski R. Peptidoglycan recognition protein 1 enhances experimental asthma by promoting Th2 and Th17 and limiting regulatory T cell and plasmacytoid dendritic cell responses. *J Immunol* 2013;190:3480–3492.