ORIGINAL RESEARCH

Identification of Epithelial Phospholipase A_2 Receptor 1 as a Potential Target in Asthma

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Abstract

Secreted phospholipase A_2s ($sPLA_2$s) regulate eicosanoid formation and have been implicated in asthma. Although $sPLA_2$s function as enzymes, some of the $sPLA_2$s bind with high affinity to a C-type lectin receptor, called PLA2R1, which has functions in both cellular signaling and clearance of $sPLA_2$s. We sought to examine the expression of *PLA2R1* in the airway epithelium of human subjects with asthma and the function of the murine *Pla2r1* gene in a model of asthma. Expression of *PLA2R1* in epithelial brushings was assessed in two distinct cohorts of children with asthma by microarray and quantitative PCR, and immunostaining for PLA2R1 was conducted on endobronchial tissue and epithelial brushings from adults with asthma. C57BL/129 mice deficient in *Pla2r1* (*Pla2r1*$^{-/-}$) were characterized in an ovalbumin (OVA) model of allergic asthma. *PLA2R1* was differentially overexpressed in epithelial brushings of children with atopic asthma in both cohorts. Immunostaining for PLA2R1 in endobronchial tissue localized to submucosal glandular epithelium and columnar epithelial cells. After OVA sensitization and challenge, *Pla2r1*$^{-/-}$ mice had increased airway hyperresponsiveness, as well as an increase in cellular trafficking of eosinophils to the peribronchial space and bronchoalveolar lavage fluid, and an increase in airway permeability. In addition, *Pla2r1*$^{-/-}$ mice had more dendritic cells in the lung, higher levels of OVA-specific IgG, and increased production of both type-1 and type-2 cytokines by lung leukocytes. *PLA2R1* is increased in the airway epithelium in asthma, and serves as a regulator of airway hyperresponsiveness, airway permeability, antigen sensitization, and airway inflammation.

Keywords: asthma; allergy; phospholipase A_2; phospholipase A_2 receptor 1; C-type lectin

Eicosanoids, such as cysteinyl leukotrienes, are elevated in the airways of patients with asthma (1, 2) and contribute to the development of allergic inflammation in murine models (3–5). The rate-limiting step in eicosanoid formation is phospholipase A_2 (PLA_2)–mediated release of arachidonate from the *sn*-2 position of membrane phospholipids. A total of 10 mammalian secreted PLA_2s ($sPLA_2$s) have been identified that may serve as key regulators of eicosanoid synthesis (6–8), often acting in concert with the well described group IVA cytosolic PLA_2 (i.e., $cPLA_2\alpha$) (9, 10).

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Clinical Relevance

The present study identifies a novel C-type lectin receptor, called phospholipase A_2 receptor 1 (PLA2R1), that is overexpressed in the airway epithelium of children with asthma and localizes to airway epithelial cells and submucosal glandular epithelium in adults with asthma. Functional evaluation of the murine homolog, *Pla2r1*, in a model of allergic inflammation found that the receptor serves as a regulator of allergic inflammation, airway permeability, and airway hyperresponsiveness.

We have identified $sPLA_2$ group 2A ($sPLA_2$-IIA), 5 ($sPLA_2$-V), and 10 ($sPLA_2$-X) in the airways of patients with asthma (11, 12). It is clear that the majority of the $sPLA_2$ activity in the airways of humans is mediated by $sPLA_2$-IIA and $sPLA_2$-X (12), and that the amount of $sPLA_2$-X protein is increased in asthma (13). In murine models of asthma, $sPLA_2$-X plays a key role in the ovalbumin (OVA) with aluminum potassium sulfate (alum) adjuvant model of allergic inflammation (14, 15), and $sPLA_2$-V is involved in the OVA model (16) as well as house dust mite–mediated allergic inflammation (17, 18). Although the $sPLA_2$s act as enzymes with varying affinity for the release of free fatty acids from mammalian cells (19, 20), a receptor has also been identified that binds to $sPLA_2$s.

PLA_2 receptor 1 (PLA2R1) is a 180-kD type 1 or integral transmembrane receptor with a large extracellular domain and a short cytoplasmic domain (21). The receptor has varying affinities for the different $sPLA_2$s, and may also act as a pleotropic receptor by binding non-$sPLA_2$ ligands (22). In fact, the receptor belongs to the superfamily of C-type lectins, and is a paralog of the macrophage mannose receptor and other receptors within this superfamily (23). The protein is transcribed as either a transmembrane form or a shortened secreted form (24), suggesting functions in both cellular signaling (25–27) as well as clearance of $sPLA_2$s (28–30). The soluble form of the receptor can also be generated by proteolytic cleavage (27). A prior investigation of the function of murine *Pla2r1* in a low-dose OVA model found that the clearance of $sPLA_2$ group IB ($sPLA_2$-IB) from the lung was reduced in *Pla2r1*-deficient ($Pla2r1^{-/-}$) mice, and that airway inflammation was increased, but airway hyperresponsiveness (AHR) and quantitative lung morphometry were not reported (31).

In a genome-wide expression study of epithelial cells, we identified increased expression of the human *PLA2R1* gene in the epithelium of children with allergic asthma. We then characterized the expression of *PLA2R1* in a second cohort of children with and without asthma, and characterized the location of *PLA2R1* immunostaining in endobronchial airway biopsies of adults with asthma. We examined the effects of the murine *Pla2r1* gene deletion in an OVA model with an exogenous adjuvant and assessed the effects on AHR, airway morphometry, trafficking of leukocytes to the airways and lung, airway permeability, production of key cytokines by lung leukocytes, and allergen-specific IgE and IgG.

Materials and Methods

Epithelial Microarray and Quantitative PCR Analysis

We initially conducted an analysis of PLA_2-related epithelial gene expression using microarrays that we performed on epithelial brushings from children with and without mild atopic asthma (AA) (32). In this cohort, children were recruited before elective surgery and characterized based on the presence or absence of a physician diagnosis of asthma and a radioallergosorbent result to a panel of common allergens. Children with asthma had not taken inhaled or oral corticosteroids for at least 1 month before surgery. Epithelial cells were collected by nonbronchoscopic cytology brushings collected at the time of surgery. For the microarray analysis, RNA from epithelial brushings of seven children with AA and nine healthy, nonatopic (HNA) control subjects was hybridized to the Affymetrix Human Genome U133A Array (Affymetrix, Santa Clara, CA). Differential gene expression between the groups was assessed after normalization by GC robust multiarray algorithm based on the adjusted *P value* and false discovery rate.

To further corroborate these results, we used quantitative PCR to assess the gene expression of *PLA2R1* from epithelial brushings from a distinct cohort of nine children with AA and nine HNA children as control subjects. TaqMan primer probe sets (Thermo Fisher Scientific, Waltham MA) were used to assess the expression of *PLA2R1* (Hs00234853_m1) relative to the expression of *PPIA* (cyclophilin A; Hs99999904_ml) as an endogenous control. The internal review board of the University of Western Australia (Perth, WA, Australia) approved the pediatric airway epithelial studies.

Immunohistochemistry of Endobronchial Biopsies

To localize the expression of PLA2R1 protein in the airways, we conducted immunostaining using endobronchial biopsies and epithelial brushings from a cohort of subjects with asthma who had undergone research bronchoscopy (13). Endobronchial biopsies were obtained from second- to fifth-generation carina of the

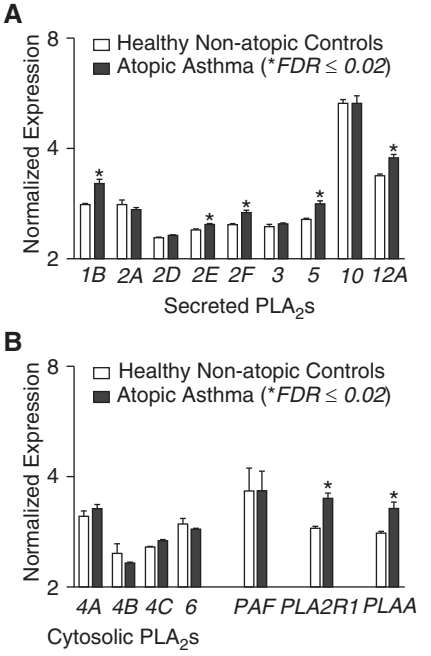

Figure 1. Differential expression of phospholipase A_2s (PLA_2s) and related genes in the airway epithelium of asthma. Microarrays assessed the expression of PLA_2-related genes from epithelial brushings of children with atopic asthma (AA) relative to healthy, nonatopic (HNA) control subjects. (*A*) Expression of secreted PLA_2 groups *1B*, *2E*, *2F*, *5*, and *12A* were differentially expressed at a false discovery rate (FDR) of 0.02 or less. (*B*) Cytosolic PLA_2s and platelet activating factor acetylhydrolase (PAF) were not differentially expressed, whereas *PLA2R1* and PLA_2-activating protein (*PLAA*) were differentially expressed (*FDR ⩽ 0.02). Data are represented as means ± SEM.

right lower and middle lobes using 1.8-mm forceps. The biopsies were fixed in methyl Carnoy's solution before embedding in paraffin. Immunostaining for PLA2R1 was localized using a rabbit polyclonal anti-PLA2R1 antibody that was generated in the laboratory of G.L. (33). The biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) was visualized with 3,3′-diaminobenzidine with nickel chloride enhancement. No immunostaining was seen with a rabbit polyclonal isotype control antibody that was used as a negative control (34). The internal review board of the University of Washington (Seattle, WA) approved the research bronchoscopy studies to acquire these samples.

OVA with Exogenous Adjuvant Model of Asthma

C57BL/129 *Pla2r1*$^{-/-}$ mice and wild-type (WT) *Pla2r1*$^{+/+}$ controls received intraperitoneal sensitization with 100 μg of endotoxin-free OVA (vac-pova; InvivoGen, San Diego, CA) complexed with alum (Thermo Scientific, Waltham, MA) on Days 0 and 14. The mice received an intranasal dose of 100 μg OVA on Day 14, and 50 μg OVA on Days 26–28. Control groups received 0.2 ml normal saline with alum intraperitoneally on Days 0 and 14 and saline without intranasal alum on Days 14 and 26–28. On Day 29, mice were intubated and ventilated using a flexiVent ventilator (SCIREQ Inc., Montreal, PQ, Canada), and lung function assessed after increasing concentrations of inhaled methacholine. Dynamic lung resistance was calculated using the single-frequency forced oscillation technique based on a 1-second broadband perturbation (35). The University of Washington Institutional Animal Care and Use Committee approved the murine studies.

Assessment of Allergen-Induced Inflammation

Bronchoalveolar lavage (BAL) fluid was collected and the cell differential was enumerated by flow cytometry. The left lung was digested in collagenase D and DNase, and, after magnet-assisted positive selection, CD45-positive leukocytes were further enumerated by flow cytometry (Figure E1). Suspensions of lung leukocytes were placed in 96-well cell culture plates for 24 hours either with/or without antigen stimulation with OVA (100 μg/ml). Cell culture supernatants were assayed for the levels of cytokines by murine V-plex assay (Meso Scale Diagnostics, Rockville, MD). Mouse plasma samples were assayed for OVA-specific IgE and IgG1 by ELISA (Cayman, Ann Arbor, MI). Detailed methods for the murine model are presented in the online supplement.

Mouse Lung Histopathology

The right lung was inflated and fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections for histopathology analyses. The sections were immunostained for eosinophils using a rat anti–major basic protein (MBP) antibody that was generously provided by J. J. Lee (Mayo Clinic, Phoenix, AZ) (36, 37). To quantify the density of eosinophils in the lung, deconvolution analysis was conducted using the Visiopharm system (Visiopharm, Hoersholm, Denmark) to quantify total MBP immunostaining in the lung. We used segmentation to determine the number of eosinophils in the lung. To quantify eosinophils surrounding the airways, we outlined the submucosal space surrounding the airways as the region of interest to define the peribronchial area. Within this peribronchial area, we quantified the total MBP immunostaining using deconvolution, and the number of eosinophils using segmentation. To quantify the density of mucus-producing cells, we quantified the amount of Alcian

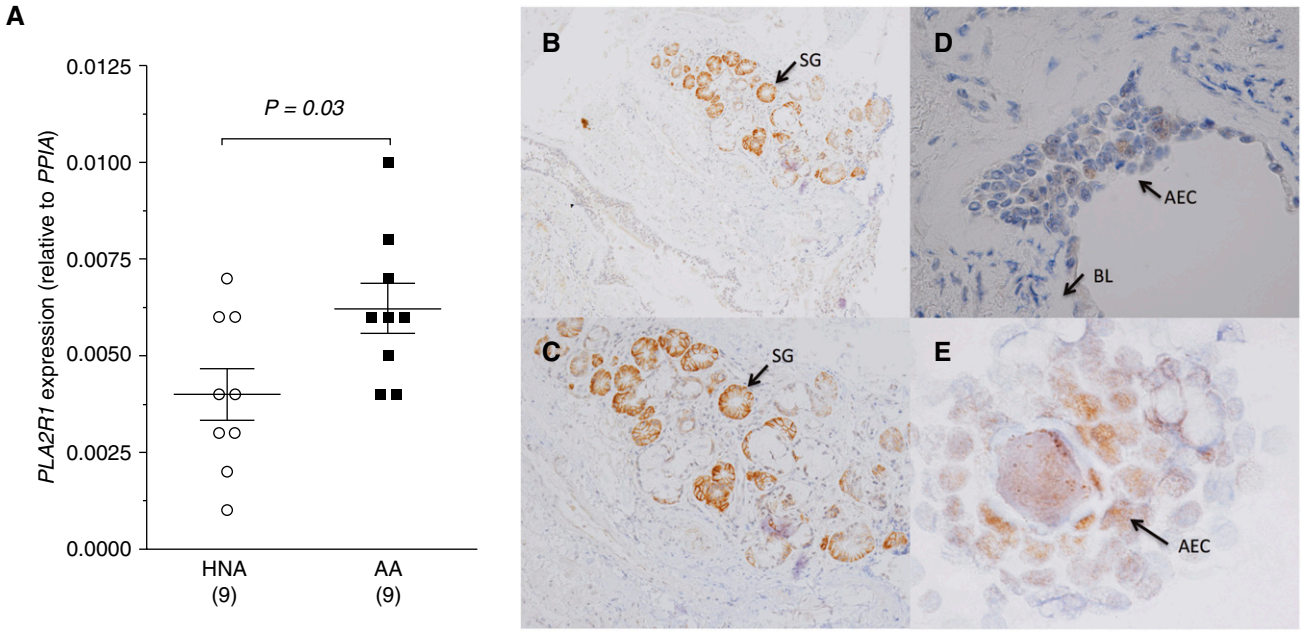

Figure 2. Localization of PLA2R1 in endobronchial airway tissue. (*A*) Differential expression of *PLA2R1* in epithelial brushings was confirmed in a distinct cohort of children with and without asthma. Data are represented as means ± SEM. (*B–D*) Immunostaining for PLA2R1 in endobronchial tissue shows prominent immunostaining in submucosal glandular epithelium shown at (*B*) 10× brightfield and (*C*) 40× brightfield. (*D*) Immunostaining is also evident in columnar airway epithelial cells at 60× oil. (*E*) Epithelial cytospin preparations at 100× oil further demonstrate immunostaining for PLA2R1 in columnar epithelial cells. *Arrows* indicate submucosal glands (SG, panels *B* and *C*), basal lamina (BL, panel *D*), and airway epithelial cell (AEC, panel *E*).

blue/periodic acid–Schiff (PAS) staining relative to the area of the epithelium and relative to the length of the basal lamina.

Statistical Analysis

Details of the statistical analysis of the microarray were reported previously (32). We assessed the relationship between the expressions of the different PLA_2-related genes using linear regression analyses. Comparison of epithelial gene expression based on PCR was assessed with an unpaired *t* test. Alterations in AHR to methacholine were assessed with a two-way ANOVA with contrasts between methacholine dose and genotype. *Post hoc* tests of each dose step were assessed after correction for multiple comparisons with Bonferroni's test. Differences in features of inflammation, including the differential cells counts, cytokine production by antigen-stimulated leukocytes, and quantification of immunostaining, were assessed with a two-way ANOVA with contrasts of allergen treatment and genotype. *Post hoc* comparisons for inflammatory parameters were made with Fisher's least significant difference.

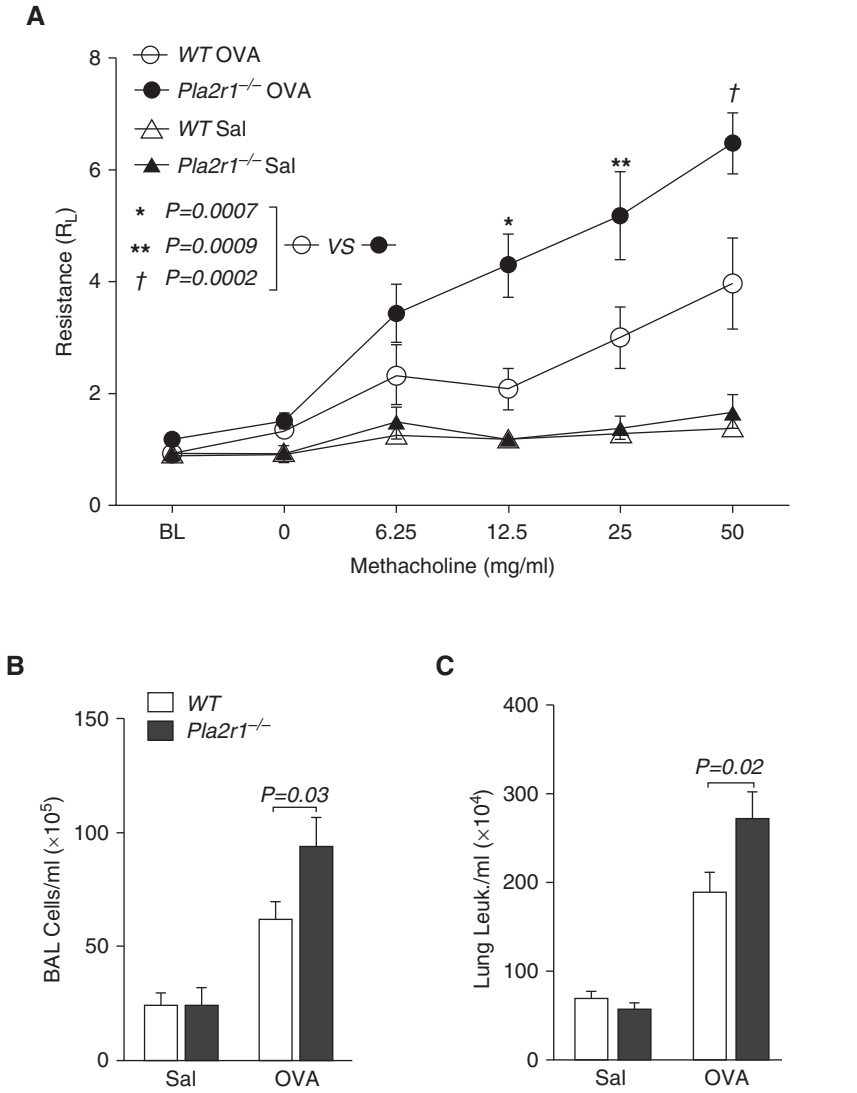

Figure 3. Changes in airway mechanics and airway inflammation in *Pla2r1*-deficient (*Pla2r1*$^{-/-}$) mice. (*A*) Airway hyperresponsiveness (AHR) to increasing doses of inhaled methacholine revealed increased AHR in *Pla2r1*$^{-/-}$ mice relative to wild-type (WT) mice after ovalbumin (OVA) sensitization and challenge ($P = 0.0005$ overall genotype effect, two-way ANOVA). (*B*) Bronchoalveolar lavage (BAL) cells (*C*) and lung leukocytes (Leuk.) were increased in *Pla2r1*$^{-/-}$ mice relative to WT mice after OVA treatment. Data are represented as means ± SEM. *$P = 0.0007$, **$P = 0.0009$, †$P = 0.0002$. R_L, lung resistance; Sal, saline.

Results

Among the PLA_2-Related Genes, *PLA2R1* Is the Most Differentially Expressed in Epithelial Brushings of Children with and without Asthma

We assessed differences in the expression of PLA_2s and PLA_2-related genes in the epithelium in asthma using oligonucleotide array data that we collected on a cohort of children with AA relative to HNA control subjects (Figure 1). The results of this focused analysis reveal that secreted PLA_2 groups *1B*, *2E*, *2F*, *5*, and *12A* were each differentially expressed in asthma at a false discovery rate 0.02 or less, but the magnitude of these differences was modest (*see* additional details in Table E1). We found no differences in the expression of any of the cytosolic PLA_2s, including the calcium-dependent groups *4A*, *4B*, and *4C* or the calcium-independent group 6. Similarly, there was no alteration in the expression of $sPLA_2$ group 7, also known as platelet-activating factor acetylhydrolase. The genes with the greatest differential gene expression in this group based on the *P* value and fold change were *PLA2R1* and PLA_2-activating protein. We found that there were strong positive correlations between the expression of *PLA2R1* and the expression levels of *PLA2G1B*, the group II $sPLA_2$s, *PLA2G5*, *PLA2G12A*, and PLA_2-activating protein, whereas *PLA2G10* had a weak inverse correlation with the expression of *PLA2R1* (Figure E2 and Table E2).

Differential Expression of *PLA2R1* Was Confirmed in a Distinct Cohort and Localized to Glandular and Nonciliated Epithelium

To further corroborate the differences in gene expression of *PLA2R1* in the epithelium, we assayed the gene expression by quantitative PCR in a new cohort of children with and without asthma. The epithelial gene expression of *PLA2R1* relative to *PPIA* (cyclophilin A) was increased in children with AA relative to HNA control subjects by approximately 1.5 fold (Figure 2A).

To further define the location of epithelial immunostaining in asthma, we performed immunostaining on endobronchial biopsies and epithelial brushings from a repository of samples from adults with asthma (13). On a low-power brightfield image (10×), immunostaining is readily apparent in submucosal glandular

epithelium, with fainter immunostaining in the airway epithelium (Figure 2B). At higher power (40× brightfield), immunostaining is readily apparent in the submucosal glandular epithelium (Figure 2C). At higher power (60× oil) focused on the airway epithelium, staining for PLA2R1 is identified in the columnar epithelium (Figure 2D). Immunostaining in columnar epithelial cells was also identified on cytospin preparations of epithelial cells from bronchial brushings (100× oil, Figure 2E).

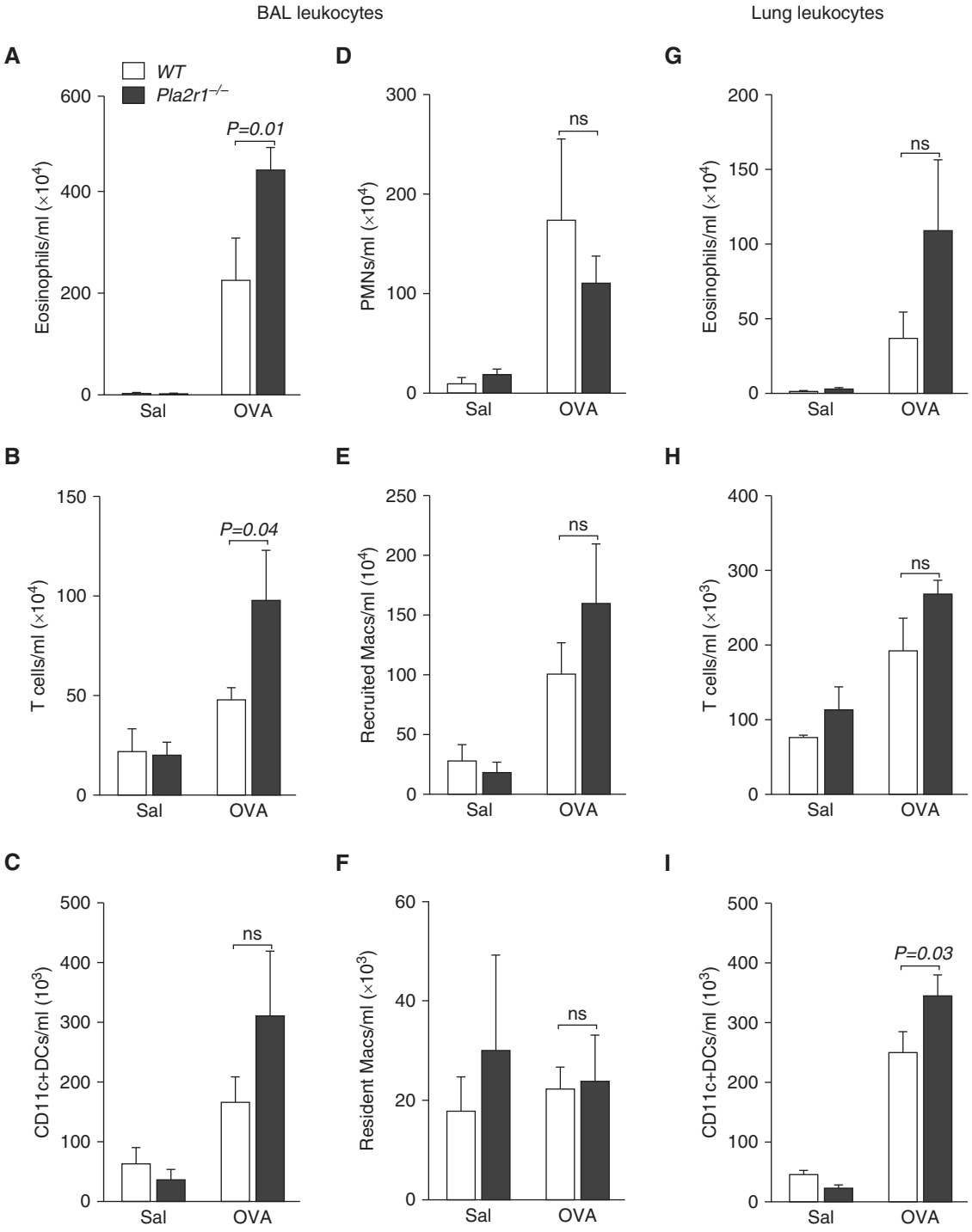

Figure 4. Alterations in leukocyte trafficking in the BAL fluid and lung tissue in *Pla2r1*$^{-/-}$ mice. Concentrations of (*A*) eosinophils and (*B*) T cells were increased in *Pla2r1*$^{-/-}$ mice in the BAL fluid after OVA, whereas there were no differences in the concentrations of (*C*) dendritic cells (DCs), (*D*) polymorphonuclear neutrophils (PMNs), (*E*) recruited macrophages (Macs), or (*F*) resident macrophages relative to WT mice exposed to OVA. Although the concentrations of (*G*) eosinophils and (*H*) T cells were unchanged, there was an increase in the concentration of (*I*) DCs in lung tissue from *Pla2r1*$^{-/-}$ mice exposed to OVA relative to WT controls. ns, not significant. Data are represented as means ± SEM.

Deletion of *Pla2r1* Increases AHR and Eosinophilic Airway Inflammation, and Alters the Levels of sPLA$_2$-X in a Mouse Asthma Model

To examine the function of Pla2r1, we examined the effects of a global deletion of the *Pla2r1* gene in a murine model of sensitization with OVA and alum, followed by airway challenge with OVA. Prior work demonstrated that mice express *Pla2r1* in the airway epithelium and airway smooth muscle (31). We found that *Pla2r1*$^{-/-}$ mice have increased AHR to methacholine after OVA sensitization and challenge ($P = 0.0005$ overall genotype effect two-way ANOVA; Figure 3A), and specifically at the final three methacholine dose steps ($P = 0.0002$–0.0009). After OVA sensitization and challenge, the concentration of cells in BAL fluid was greater in *Pla2r1*$^{-/-}$ mice relative to WT controls ($P = 0.03$, Figure 3B). The number of BAL CD45$^+$ leukocytes was similarly increased in the *Pla2r1*$^{-/-}$ mice ($P = 0.01$, data not shown). We also examined the total concentration of CD45$^+$ leukocytes in the lung after BAL and removal of circulating cells, and found that the concentration of leukocytes was increased in the lungs of the *Pla2r1*$^{-/-}$ mice after sensitization and challenge relative to WT controls ($P = 0.02$, Figure 3C). As we have previously identified a major role for sPLA$_2$-X in the OVA model of allergic inflammation (14, 15), we measured the levels of sPLA$_2$-X by Western blot in BAL fluid, and found that the amount of sPLA$_2$-X in saline-treated mice was markedly increased in *Pla2r1*$^{-/-}$ mice relative to WT mice ($P = 0.0006$), but this difference was no longer apparent after OVA sensitization and challenge (Figure E3).

We assessed the concentration of leukocyte subsets in BAL fluid by flow cytometry and found that the concentration of eosinophils and T cells were increased in the *Pla2r1*$^{-/-}$ mice after sensitization and challenge, whereas there were no significant differences in the concentration of neutrophils, recruited macrophages, resident macrophages, or dendritic cells (DCs) (Figures 4A–4F). We similarly characterized leukocyte trafficking to the lung using flow cytometry of lung leukocytes isolated from lung tissue after both BAL and perfusion of the lung with saline to remove circulating leukocytes. We found that, although eosinophils, T cells, and CD11C$^+$ DCs were increased after OVA treatment (Figures 4G–4I), only the number of DCs was significantly increased in the *Pla2r1*$^{-/-}$ mice. There were also no differences in the concentrations of neutrophils, recruited macrophages, or resident macrophages between the WT and *Pla2r1*$^{-/-}$ mice (data not shown).

Alterations in the Airway Wall and Increased Airway Permeability in *Pla2r1*$^{-/-}$ Mice

We quantified the number of eosinophils in the peribronchial space using immunohistochemistry for the eosinophil granule protein, MBP. Representative images are shown in Figure 5A. Segmentation analysis of the peribronchial space, using an unbiased automated system that counts MBP-positive cells, demonstrated that the total number of eosinophils surrounding the airways was increased in *Pla2r1*$^{-/-}$ mice relative to WT mice after OVA (Figure 5B). We also quantified the submucosal space surrounding the airways using image analysis; this analysis revealed that the peribronchial area was increased in the

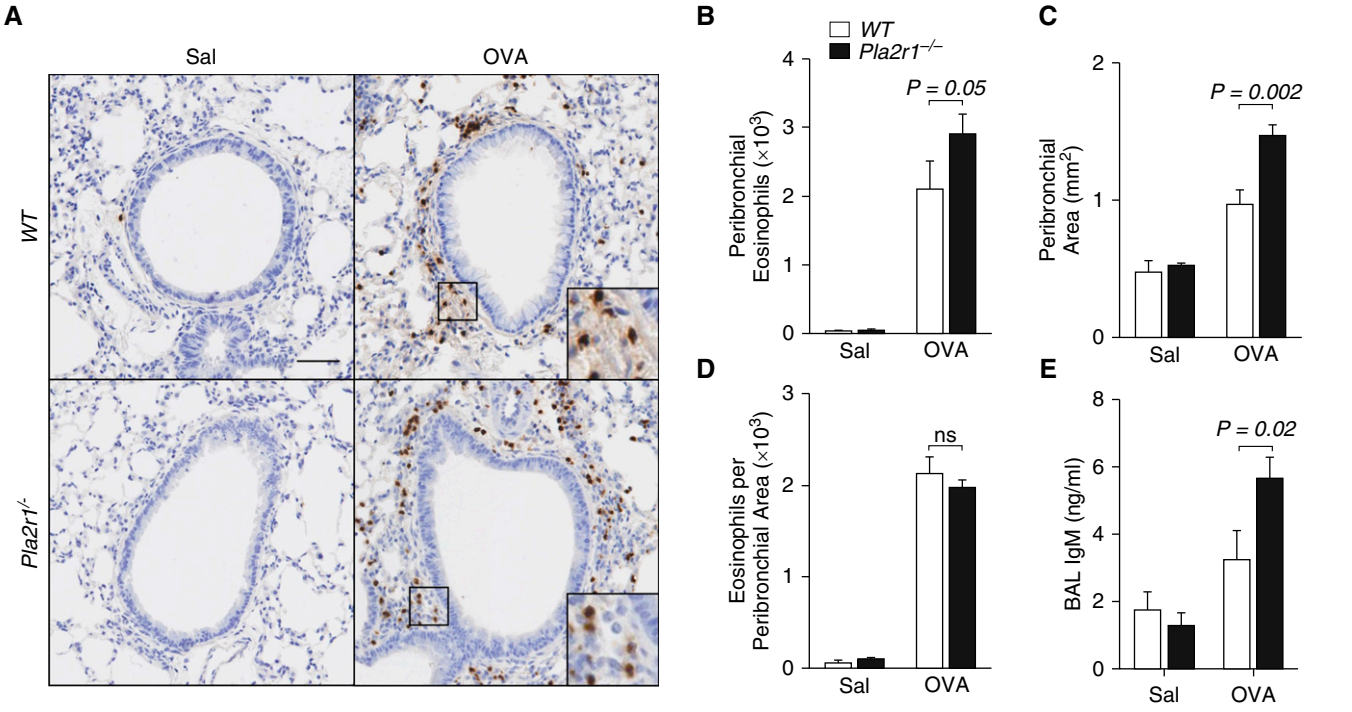

Figure 5. Deficiency of *Pla2r1* alters peribronchial area, eosinophilic inflammation, and airway permeability. (*A*) Representative images of eosinophil major basic protein (MBP) staining of lung tissue from saline- and OVA-exposed WT and *Pla2r1*$^{-/-}$ mice (original magnification, 20×; *scale bar*: 50 μM). *Insets* show detail of focal MBP staining in OVA-treated WT mice, representing peribronchial eosinophils. (*B*) Total peribronchial eosinophils and (*C*) the peribronchial area were increased in *Pla2r1*$^{-/-}$ mice after OVA challenge; however, the number of eosinophils per peribronchial area was unchanged (*D*). (*E*) Airway permeability based on the transit of IgM to the airway after methacholine challenge was increased in *Pla2r1*$^{-/-}$ mice exposed to OVA. Data are represented as means ± SEM.

Pla2r1$^{-/-}$ mice relative to WT mice after OVA (Figure 5C). Because the area surrounding the airways was increased in the *Pla2r1*$^{-/-}$ mice, there was no difference between the WT and *Pla2r1*$^{-/-}$ mice for the number of eosinophils per area surrounding the airways (Figure 5D). Although we did not further characterize the composition of the space surrounding the airways, we examined the degree of airway permeability after methacholine challenge using the large protein IgM, and found that the level of IgM in the BAL fluid was markedly elevated in the *Pla2r1*$^{-/-}$ mice relative to WT mice (Figure 5E). These results suggest that peribronchial remodeling contributes to AHR through increased airway permeability.

Through the use of image analysis, we also quantified the total percent area of MBP staining, representing the amount of MBP within eosinophils as opposed to counting discrete cells. The total amount of MBP staining was actually decreased in the *Pla2r1*$^{-/-}$ mice, suggesting that the amount of MBP within the eosinophils was decreased (Figure E4A), consistent with increased degranulation and release of the granule protein MBP in these mice. To further corroborate this finding, we examined the amount of MBP in BAL fluid by Western blot and found that there was a notable trend toward an increase in MBP in the BAL fluid of the *Pla2r1*$^{-/-}$ mice relative to WT mice ($P = 0.08$, Figure E4B). We also quantified the total number of eosinophils in the lung by segmentation analysis and found that total lung eosinophils was not altered in the *Pla2r1*$^{-/-}$ mice after OVA (Figure E4C), consistent with our results by flow cytometry of eosinophils. The amount of MBP in the lung eosinophils tended to be decreased in the lungs of *Pla2r1*$^{-/-}$ mice ($P = 0.13$, Figure E4D), consistent with increased eosinophil degranulation in the *Pla2r1*$^{-/-}$ mice. Overall, these results demonstrate that there are more eosinophils surrounding the airways in *Pla2r1*$^{-/-}$ mice in association with enlargement of the peribronchial space, increased airway permeability, and increased eosinophil degranulation. These findings provide important insights into the mechanism of elevated AHR in the *Pla2r1*$^{-/-}$ mice.

The Amount of Mucin in the Airway Epithelium after Methacholine Challenge Is Reduced in *Pla2r1*$^{-/-}$ Mice

We quantified the amount of mucin staining in the epithelium using segmentation analysis, demonstrating that, with sensitization and challenge with OVA, there was a marked increase in the percent of mucin staining relative to the area of the epithelium, and the percent of mucin staining relative to the length of the basal lamina ($P < 0.0001$ for both measures). We conducted the PAS staining for mucin after

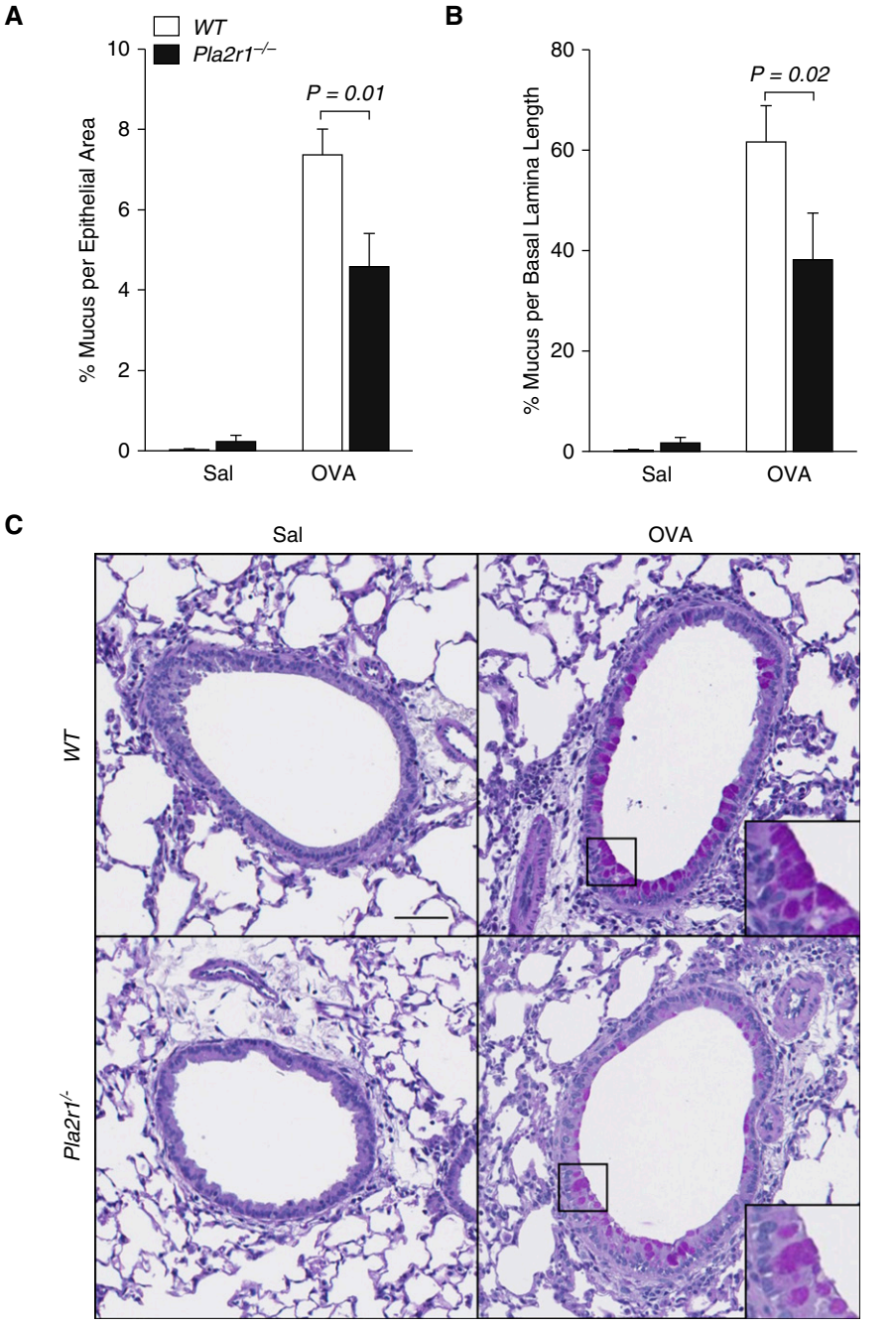

Figure 6. Mucin content in the airway epithelium is altered in *Pla2r1*$^{-/-}$ mice after methacholine challenge. Mucin content in lung tissue was determined by periodic acid–Schiff staining and quantified by segmentation analysis. The mucin content was increased after OVA, and (*A*) percentage of mucus per total epithelial area and (*B*) percentage of mucus per BL length were decreased in *Pla2r1*$^{-/-}$ after methacholine challenge, consistent with greater mucin release. (*C*) Representative images of mucin staining of lung tissue from WT and *Pla2r1*$^{-/-}$ mice exposed to OVA or saline (*original magnification*, 20×; *scale bar*: 50 μM). *Insets* show detail of mucin staining. Data are represented as means ± SEM.

BAL and after methacholine challenge. In this context, it was previously demonstrated, using quantitative morphometry, that there is an acute decrease in mucin content in the epithelium that represents the release of mucin from the epithelium (38). We found that there was a significant decrease in mucin staining relative to the epithelial area in the *Pla2r1*$^{-/-}$ mice relative to WT controls ($P = 0.01$, Figure 6A). Similarly, we found that the percentage of mucin staining relative to the length of the basal lamina was decreased in *Pla2r1*$^{-/-}$ mice relative to WT controls ($P = 0.02$, Figure 6B). These results are consistent with greater epithelial mucin release during methacholine challenge in the *Pla2r1*$^{-/-}$ mice. Representative PAS images are shown in Figure 6C.

Cytokine Production by Lung Leukocytes and Allergen-Specific IgG Are Increased in *Pla2r1*$^{-/-}$ Mice

We isolated lung leukocytes after sensitization and challenge and stimulated these cells *in vitro* for 24 hours with saline control or OVA. We found that there was an OVA-induced increase in the generation of the cytokines, IL-2, IL-4, and IL-17 (Figure 7). The levels of IL-13 were below the level of detection in our assay. The OVA-induced generation of IL-2, IL-4, and IL-17 in OVA-sensitized mice was lower in the *Pla2r1*$^{-/-}$ mice relative to WT mice (Figure 7). In contrast, the generation of multiple cytokines that were not OVA induced *in vitro* was markedly higher in *Pla2r1*$^{-/-}$ mice relative to WT mice after sensitization and challenge with OVA (Figure 8). These cytokines included both type-1 and type-2 cytokines, including IFN-γ, IL-1β, IL-5, IL-6, IL-10, IL-12p70, chemokine (C-X-X motif) ligand 1 (CXCL1)/keratinocyte-derived chemokine (KC), and TNF-α.

We found a significant increase in OVA-specific IgE in OVA-treated mice in both genotypes, and, although the OVA-specific IgE tended to be higher in the OVA-treated *Pla2r1*$^{-/-}$ mice, it did not reach statistical significance ($P = 0.34$, Figure 9A). There was a marked increase in OVA-specific IgG1 in OVA-treated mice ($P < 0.01$), and a significant increase in OVA-specific IgG1 in *Pla2r1*$^{-/-}$ mice relative to WT mice after treatment with OVA ($P = 0.0003$, Figure 9B).

Discussion

We identified human *PLA2R1* as an epithelial gene that is overexpressed in asthma. Within the airways of patients with asthma, the receptor is abundantly expressed in the submucosal glands and in columnar epithelial cells. In a model of asthma, absence of *Pla2r1* increased AHR to methacholine after sensitization and challenge, and increased eosinophil trafficking to the peribronchial space and BAL fluid. We identified an increase in the peribronchial area, a marked increase in airway permeability, increased eosinophil degranulation and greater mucin release that may contribute to this increase in AHR in the absence of Pla2r1. Our results further demonstrate that, in the absence of Pla2r1, there are marked alterations in leukocyte function notable for the increased production of a broad range of cytokines by lung leukocytes, including a notable increase in IL-5, as well as an increase the number of CD11c^{+} DCs in the lung and an increase OVA-specific IgG. These findings may be related to an increase in the level of sPLA$_2$-X in the airways, which was markedly increased in *Pla2r1*$^{-/-}$ mice without allergic sensitization. These results suggest that Pla2r1 serves as a key regulator of allergic inflammation and AHR in mice, and that the overexpression of this receptor in the epithelium of human subjects with asthma could serve as a mechanism to limit airway inflammation.

Our study is the first to identify increased expression of *PLA2R1* in the airway epithelium of subjects with asthma; however, it is known that several different high-affinity ligands for PLA2R1, including sPLA$_2$-IIA, sPLA$_2$-V, and sPLA$_2$-X, are abundantly expressed in the skin (39, 40),

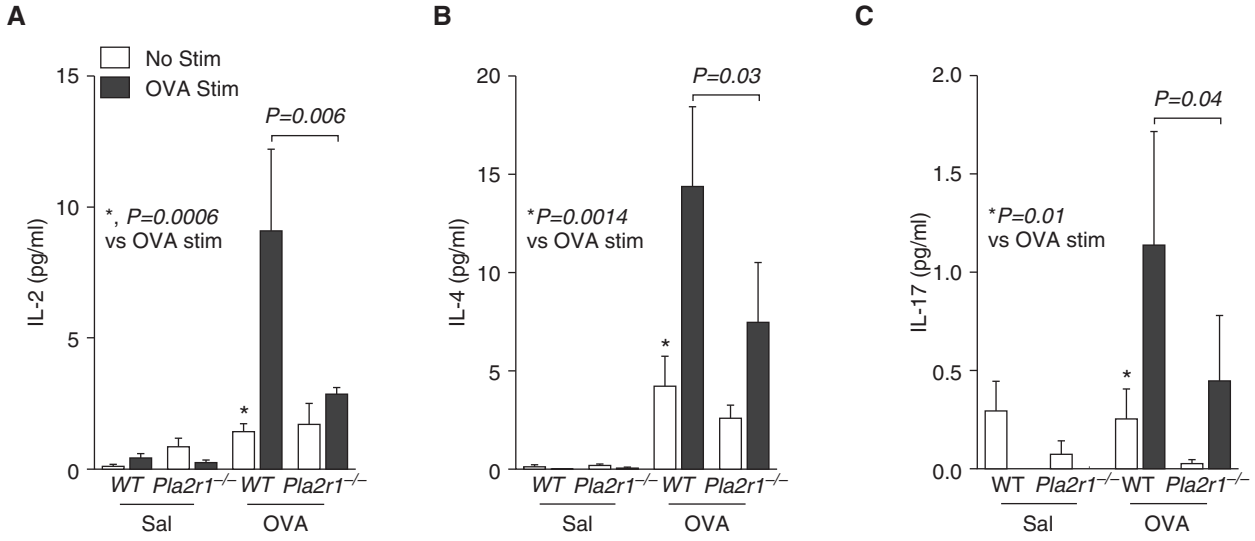

Figure 7. Differential antigen-dependent production of proinflammatory cytokines in *Pla2r1*$^{-/-}$ mice. Isolated lung leukocytes were cultured in the presence of OVA or saline for 24 hours and cell culture supernatant was assessed for cytokine release. There was an OVA-induced production of the cytokines, IL-2, IL-4, and IL-17, in WT mice exposed to OVA. The production of (*A*) IL-2, (*B*) IL-4, and (*C*) IL-17 were significantly decreased in OVA-restimulated cells isolated from *Pla2r1*$^{-/-}$ mice relative to OVA-restimulated cells from WT mice. (*A*) *$P = 0.0006$ versus OVA stimulated (Stim); (*B*) *$P = 0.0014$ versus OVA stimulated; (*C*) *$P = 0.01$ versus OVA stimulated.

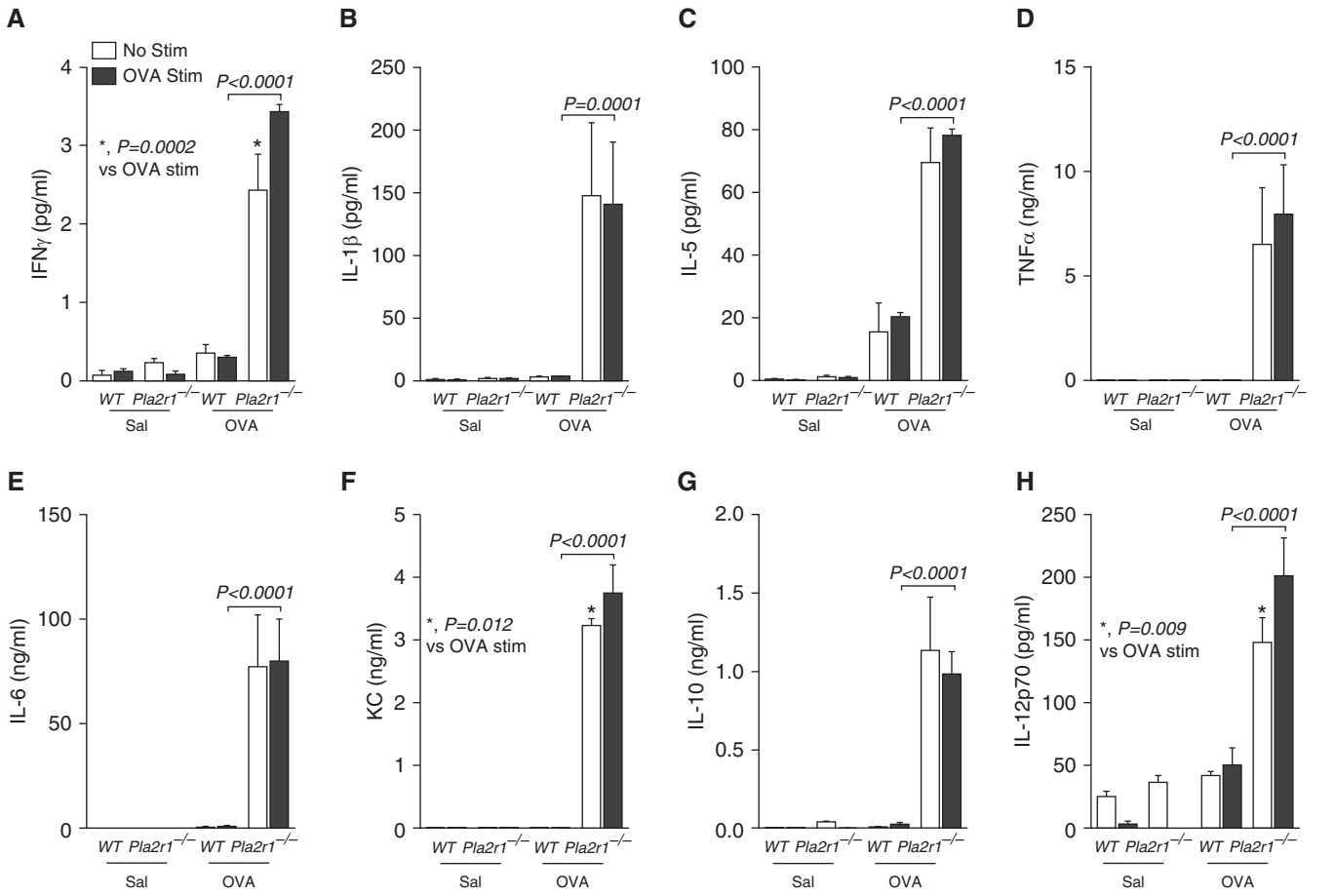

Figure 8. Antigen-independent production of proinflammatory cytokines is augmented in *Pla2r1*$^{-/-}$ mice. Isolated lung leukocytes were cultured in the presence of OVA or saline for 24 hours and cell culture supernatant was assessed for cytokine release. The levels of cytokines (*A*) IFN-γ, (*B*) IL-1β, (*C*) IL-5, (*D*) TNF-α, (*E*) IL-6, (*F*) chemokine (C-X-X motif) ligand 1 (CXCL1)/keratinocyte-derived chemokine (KC), (*G*) IL-10, and (*H*) IL-12p70 were significantly elevated in *Pla2r1*$^{-/-}$ mice relative to WT mice, although there was no evidence of an *ex vivo* increase in the production of these cytokines by OVA treatment. (*A*) **P* = 0.0002 versus OVA stimulated; (*F*) **P* = 0.012 versus OVA stimulated; (*H*) **P* = 0.009 versus OVA stimulated. Data are represented as means ± SEM.

and we have found that *PLA2G10* is expressed at higher levels than either *PLA2G2A* or *PLA2G5* in the airway epithelium (12). In the skin, the transgenic expression of sPLA$_2$-IIA increases skin carcinogenesis (41), whereas *Pla2r1*$^{-/-}$ mice have increased sensitivity to carcinogen-induced skin tumorigenesis (42). As such, the marked immunostaining in glandular epithelium suggests that the release of the soluble form of the receptor could serve in the clearance of sPLA$_2$s in the airways. The immunostaining for Pla2r1 in mice also indicates localization to the airway epithelium (31), implicating the epithelial expression of this receptor in the *in vivo* findings in this study; however, the immunostaining in the airway smooth muscle that was identified in mice is not readily apparent in our human endobronchial biopsy specimens.

Our results are consistent with a prior study by Tamaru and colleagues (31) that demonstrated an increase in BAL eosinophils in *Pla2r1*$^{-/-}$ mice; however, the effects on AHR, leukocyte trafficking to the lung, leukocyte function, and antigen-specific Igs were not previously assessed. Our results are critical to understanding the function of Pla2r1 because of the opposing functions of the receptor. The receptor is a C-type lectin receptor that is a paralog of the mannose receptor. Ligation of this receptor by high-affinity PLA$_2$ ligands initiates cellular signaling events in cells such as macrophages (25–27). The soluble form of the receptor can be generated via alternatively transcribed forms (24), and also by proteolytic cleavage (27), although the specific protease has not been identified. The soluble form of PLA2R1 is involved in clearance of sPLA$_2$s, particularly groups IB, IIA, IIE, IIF, and X, that have high affinity for this receptor (22), but enhanced clearance from the lung has only been demonstrated for sPLA$_2$-IB (31), an enzyme with uncertain relevance to asthma pathogenesis. In addition, the transmembrane form of the receptor also mediates the internalization and removal of sPLA$_2$s via the lysosomal pathway (28–30).

The widespread proinflammatory effect of the *Pla2r1* deletion in the murine model suggests that the predominant effect was through the loss of the clearance mechanism mediated by this receptor. In fact, we found that the level of sPLA$_2$-X in the BAL fluid was markedly elevated in *Pla2r1*$^{-/-}$ mice before sensitization and challenge. The one area where the loss of the receptor appeared to

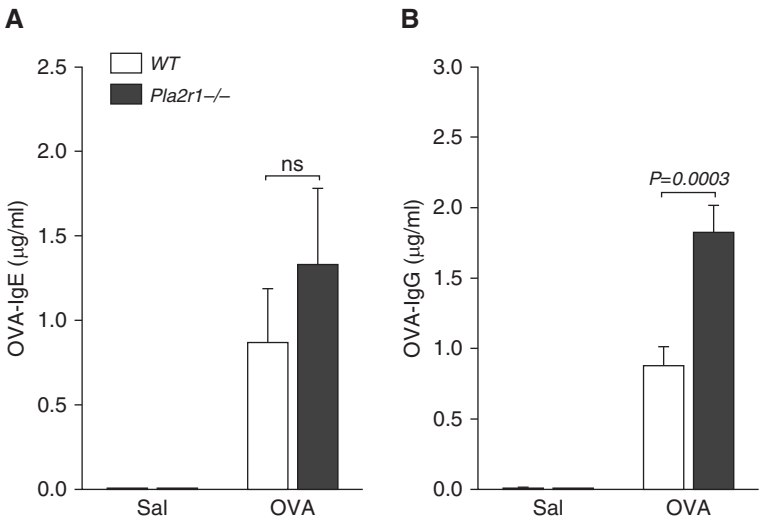

Figure 9. Effects of *Pla2r1* deficiency on allergen-specific Igs. The levels of (*A*) OVA-specific IgE were not significantly elevated in *Pla2r1*$^{-/-}$ mice relative to WT controls; however, the levels of OVA-specific IgG were increased in *Pla2r1*$^{-/-}$ mice after OVA (*B*). Data are represented as means ± SEM.

reduce leukocyte activation was on the OVA-induced production of IL-2, IL-4, and IL-17 by lung leukocytes, which were reduced in the *Pla2r1*$^{-/-}$ mice. Regardless, the overall effects of the *Pla2r1* deletion were proinflammatory, including the widespread overproduction of cytokines by lung leukocytes, increased DCs in the lung, and higher levels of antigen-specific IgG1. As cytokines associated with activation of the innate immune system were increased in the *Pla2r1*$^{-/-}$ mice, it is notable that a recent study showed that bee venom PLA_2, an enzyme that functions much like endogenous $sPLA_2$s, was involved in generating Th2 type T cell responses in the periphery in a process that was dependent upon the generation of IL-33 and innate lymphoid cells (43).

A key finding in the present study was the marked increase in AHR in mice deficient in *Pla2r1*. The most plausible explanation for these findings is the increase in the peribronchial area, increased airway permeability, and increased degranulation of eosinophils in the airways noted in *Pla2r1*$^{-/-}$ mice. Studies using cationic proteins, such as poly-l-lysine, that mimic eosinophil cationic proteins and cause airway edema, substantially increase AHR to methacholine by increasing the thickness of the airway wall (35, 44). Another plausible explanation is a change in the permeability of endothelial cells in the *Pla2r1*$^{-/-}$ mice, as PLA2R1 is the major autoantigen in membranous nephropathy, a disease with increased permeability of highly specialized epithelial cells called podocytes. In the present study, we found that the large protein IgM transited into the BAL fluid in increased amounts in the *Pla2r1*$^{-/-}$ mice. In membranous nephropathy, the receptor itself may be involved in the development of the disease, as gene variants of the human *PLA2R1* are associated with the development of the disease (45). Finally, we showed that the amount of mucin in the epithelium was decreased after methacholine challenge in the *Pla2r1*$^{-/-}$ mice, consistent with increased mucin release that contributes to AHR (38).

Our study is limited by the inability to determine cell-specific effects to further understand the function of epithelial *Pla2r1* relative to leukocyte *Pla2r1* on allergic sensitization, airway inflammation, airway edema, and AHR. The effects on allergic sensitization also need further exploration in models of allergic sensitization that have greater dependence on innate cells such as macrophages. The PLA2R1 receptor is a paralog of the mannose receptor, and has carbohydrate recognition domains that could be important in the recognition of components of common antigens, such as house dust mites. Effects on airway remodeling should also be explored in future model systems, as the expression of this receptor on myofibroblasts is important in the remodeling of the myocardium after an ischemic event (46), and we found that the peribronchial area is altered in *Pla2r1*$^{-/-}$ mice.

We conclude that *PLA2R1* is overexpressed in the airway epithelium in asthma, and likely serves in the airways to reduce the effects of $sPLA_2$s. In a murine model of asthma, the absence of the receptor led to a predominance of effects that increased cellular inflammation, peribronchial remodeling, and allergic sensitization. Furthermore, the absence of the receptor had pronounced effects on AHR. These results suggest that PLA2R1 plays an important role in the regulation of airway inflammation and airway edema relevant to asthma pathogenesis. ■

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

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