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Deletion of Secretory Group V Phospholipase A2 Attenuates Cell Migration and Airway Hyperresponsiveness in Immunosensitized Mice

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We investigated the role of group V phospholipase A2 (gVPLA2) in OVA-induced inflammatory cell migration and airway hyperresponsiveness (AHR) in C57BL/6 mice. Repeated allergen challenge induced biosynthesis of gVPLA2 in airways. By aerosol, gVPLA2 caused dose-related increase in airway resistance in saline-treated mice; in allergic mice, gVPLA2 caused persistent airway narrowing. Neither group IIa phospholipase A2, a close homolog of gVPLA2, nor W31A, an inactive gVPLA2 mutant with reduced activity, caused airway narrowing in immune-sensitized mice. Pretreatment with MCL-3G1, a blocking Ab against gVPLA2, before OVA challenge blocked fully gVPLA2-induced cell migration and airway narrowing as marked by reduction of migrating leukocytes in bronchoalveolar lavage fluid and decreased airway resistance. We also assessed whether nonspecific AHR caused by methacholine challenge was elicited by gVPLA2 secreted from resident airway cells of immune-sensitized mice. MCL-3G1 also blocked methacholine-induced airway bronchoconstriction in allergic mice. Blockade of bronchoconstriction by MCL-3G1 was replicated in allergic pla2g5−/− mice, which lack the gene encoding gVPLA2. Bronchoconstriction caused by gVPLA2 in pla2g4−/− mice was comparable to that in pla2g4+/− mice. Our data demonstrate that gVPLA2 is a critical messenger enzyme in the development of AHR and regulation of cell migration during immunosensitization by a pathway that is independent of group IVa phospholipase A2.

Bronchial asthma is a chronic inflammatory disease of airways (1, 2) characterized by eosinophilic infiltrates (3, 4), mucus hypersecretion (5, 6), and hyperreactivity to inhaled allergen (7–9) and to nonspecific stimuli (10, 11). Phospholipases A2 (PLA2s)3 are a superfamily of enzymes that hydrolyze the sn-2 acyl ester of phospholipids to generate fatty acids and lysophospholipids. Because the products of PLA2 hydrolysis, arachidonic acid in particular, serve as precursors for potent mediators of airway inflammation, leukotrienes, PLA2 has been implicated in the pathogenesis of asthma. Among multiple forms of mammalian PLA2, the involvement of two isoforms, 85-kDa cytosolic group IVa PLA2 (gIVaPLA2) and 14-kDa secretory group IVa PLA2 (gVPLA2), in inflammation has been shown by gene knockout studies.

GIVaPLA2 is the only PLA2 known to have genuine specificity for phospholipids containing sn-2 arachidonyl group. Prior in vivo and in vitro studies have demonstrated that blockade of GIVaPLA2 inhibits 1) cellular adhesion/migration of eosinophils and lymphocytes (12–14), 2) synthesis of arachidonate metabolites (15–20), and 3) airway narrowing (21–23). Mammalian secretory 14-kDa PLA2s, e.g., gVPLA2, GHaPLA2, and GXLPLA2, are inflammatory enzymes that are involved directly and indirectly in inducing inflammatory and allergic processes; however, the mode of action of these sPLA2 isoforms has not been fully established. GHaPLA2 is a close homolog of gVPLA2; however, unlike gVPLA2, GHaPLA2 has low affinity for zwitterionic phosphatidylycholine-rich outer plasma membranes and little ability to generate free fatty acids and lysophospholipids (24–27). Although the relative contributions of cytosolic gIVaPLA2 and secretory PLA2s differ between cells and tissues, different PLA2s may act synergistically in producing proinflammatory lipid mediators (28–30). Among the secretory PLA2s, gVPLA2 is a unique enzyme because it: 1) possesses interfacial binding properties and high affinity/activity and hydrolyzes for phosphatidylcholine-rich outer cell plasma membrane to see generate lysophospholipids and free fatty acids (24, 31–33); 2) can enter mammalian cells in a heparan sulfate proteoglycan-dependent manner and acts intracellularly by transmembrane transport caused by binding to surface heparan sulfate proteoglycan (25, 33, 34); 3) acts on the perinuclear membrane to generate bioactive lipid mediators through both gIVaPLA2-dependent (32, 33) and -independent mechanisms (34, 35); and 4) up-regulates eosinophil surface CD11b/CD18 expression (35) causing adhesion to the endothelial counterligand, ICAM-1 (35, 36).

We have shown previously that blockade of eosinophil migration caused by inhibition of GIVaPLA2 corresponded to attenuation

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3 Abbreviations used in this paper: PLA2, phospholipase A2; GVPLA2, group V phospholipase A2; GIVaPLA2, cytosolic group IVa PLA2; AHR, airway hyperresponsiveness; pla2g5−/−; gVPLA2−/− mice; WT, wild type; pla2g5+/−; gVPLA2 WT mice; pla2g4−/−; cytosolic gIVaPLA2−/− mice; pla2g4+/−, cytosolic gIVaPLA2 WT mice; Rrs, airway lung resistance; MCh, methacholine; BALF, bronchoalveolar lavage fluid.

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of airway hyperresponsiveness (AHR) in allergic guinea pigs (21). Other studies have demonstrated substantially greater AHR in immunosensitized gIVαPLA₂ wild-type (WT) mice than in gIVαPLA₂ knockout mice (23, 34, 37). Although much attention has been focused on gIVαPLA₂ as the critical regulating enzyme in integrin adhesion (12–14) and eicosanoid generation (15–20), the signal initiating stimulus that triggers the activation of inflammatory cells to cause cell migration and AHR in chronic asthma has not been defined. We have demonstrated that gIVαPLA₂ can trigger the biosynthesis and release of LTB₄ from human neutrophils (32, 33) in a gIVαPLA₂-dependent manner and LTC₄ from human eosinophils in a gIVαPLA₂-independent manner (34, 35). We and others have also reported recently that epithelial cells are a natural source of gIVαPLA₂ (35, 36) and that in vitro transcellular migration of gVPLA₂ secreted from activated epithelial cells causes secretion of LTC₄ from adherent eosinophils by a pathway that is independent of gIVαPLA₂ (35, 36).

In this study, we hypothesized that gVPLA₂ is an intercellular messenger protein for induction of airway inflammation in vivo and subsequent AHR. To test this hypothesis, we measured the effect of MCL-3G1, a blocking mAb against gVPLA₂ (38, 39), in cell migration and AHR in immunosensitized airways. We assessed whether non specific AHR elicited by methacholine (MCh) challenge was caused by endogenous gVPLA₂ secreted from airways of immunosensitized mice. In additional studies, we used allergic gVPLA₂null mice to confirm the contributory role of gVPLA₂ in cell migration and AHR. Finally, we determined whether gVPLA₂-induced AHR and airway inflammation were independent of gIVαPLA₂ activation. Our data are the first demonstration that 1) gVPLA₂ regulates AHR in immunosensitized mice and 2) induction of AHR by gVPLA₂ is dependent of cytosolic gIVαPLA₂.

Materials and Methods

Isolation of secretory PLA₂s

The mutagenesis of gVPLA₂ to the W31A mutant was performed as described previously (32–35). gIIαPLA₂, gVPLA₂, and W31A were expressed in Escherichia coli and refolded as described previously (32–35). Mouse mAb directed against human gVPLA₂, MCL-3G1, was produced from the Hybridoma Facility of the University of Chicago (Chicago, IL) (38–39). We have shown previously that MCL-3G1 is a functional blocking Ab (35) that prevents the effects of secreted gVPLA₂ from cultured epithelial cells on adjacent granulocytes. We further have shown that in cell-free systems in vitro, MCL-3G1 inhibits hydrolysis of phosphatidylcholine, which is its target on the plasma membrane (34).

Animals

Homozygous gVPLA₂-deficient mice (pla2g5⁻/⁻) and littermate control (pla2g5⁺/⁻) mice were derived as described (40–41). Heterozygous mice were bred to a C57BL/6 background and were bred to derive N11 C57BL/6 pla2g5⁻/⁻, pla2g5⁺/⁻, pla2g4⁻/⁻, and pla2g4⁺/⁺ mice, and littermate control mice.

Immunosensitization

Animals were sensitized by i.p. injection of 10 µg of chicken egg OVA (Sigma-Aldrich) and 1.125 mg of alum in 0.2 ml of sterile saline at days 0, 7, and 14. On days 21–23, animals were placed in an incubation chamber and were challenged with 1% OVA for 40 min using a DeVelbiss nebulizer (39). Similar procedures were performed in pla2g5⁻/⁻, pla2g4⁻/⁻, and littermate control mice.

Secretory gVPLA₂ expression

Immunohistochemistry. Tissue sections were examined for the presence of gVPLA₂ by using MCL-3G1 mAb (38, 39). Frozen mouse tissues ~5- to 6-µm-thick sections were subsequently cut and fixed in 10% formalin solution. Endogenous peroxidase activity and nonspecific Ab binding were prevented by incubating the sections for 15 min in 0.5% H₂O₂, methanol (vol/vol) and normal goat serum (1/5 dilution). Sections were incubated for 1 h in primary Ab (MCL-3G1) diluted in normal saline solution. Ab binding was localized with a biotinylated secondary Ab, avidin-conjugated HRP, and diaminobenzidine chromogenic substrate (Vector Laboratories).

Immunoblotting analysis. Lung protein extracts (40 µg/tube) were run on SDS-PAGE and transferred to a nitrocellulose membrane. gVPLA₂ expression was probed with MCL-3G1, a mAb directed against gVPLA₂, and secondary goat anti-mouse IgG1 (HRP conjugated) as previously described (38–39).

PCR. An ~2-mm tail was cut from each weaning. DNA was isolated, and genotyping was performed using PCR. Mice with the required genotype, pla2g5⁻/⁻, pla2g5⁺/⁻, pla2g4⁻/⁻, and pla2g4⁺/⁺ were kept until immunologically mature. Primers used for gVPLA₂ are: forward, 5’-GAGTCA CGACCGTTGGTTATG-3’; reverse, 5’-TAATCTCAATGAGAACGGTCT AGGT-3’. Primers for neo are: forward, 5’-GCTGTGCTGACGTTG TCACT-3’. Primers used for gIVαPLA₂ genotyping are: number 1, 5’-CG ACTCTACAGTGCCTTCATCAC; number 2, 5’-GGAAACCTTCCTGAC TAGG; number 3, 5’-TGTGTAACATCTTGTGTTTCC.

Preparation of animals

Mice were anesthetized with 30 mg/ml xylazine plus 80 mg/ml ketamine i.p. and were ventilated quasissinusoidally at a frequency of 150 breaths/min (tidal volume, 10 ml/kg) as described previously (42). Increasing concentrations of sPLA₂ and MCh were delivered by Aeroneb, an ultrasonic nebulizer device attached to the ventilator. All outcome parameters of the flexivent (SCIREQ) are calculated using well-validated mathematical models with a sound physical basis (42). The change in airway resistance (Rrs) was expressed as centimeters of H₂O per milliliter per second.

Measurement of AHR

Concentration-response curve. Rrs as a function of airway narrowing was measured using a computer-controlled small-animal ventilator as described previously (42). MCh (0–10 mg/ml) or 14–1400 ng/ml gVPLA₂, gIIαPLA₂, or W31A were given via Aeroneb to mice. Rrs was measured during continuous nebulization (15 s) of MCh, gVPLA₂, gIIαPLA₂, or W31A. MCh concentration-response curves were generated in treated pla2g5⁻/⁻ and pla2g5⁺/⁻ mice, and similar measurements were performed. Nebulization of gVPLA₂ was performed in allergic pla2g4⁻/⁻ mice, which lack genes encoding gVPLA₂ and littermate control mice.

Blockade of gVPLA₂ or MCh-induced bronchoconstriction by MCL-3G1. Treatment 1 was an i.p. injection of 20 µg of MCL-3G1 daily (days 21, 22, and 23) and 60 min of OVA challenge by aerosol (after phase I, before phase II; see Fig. 3A).

Treatment 2 was an i.p. injection of 20 µg of MCL-3G1 24 h after the last OVA challenge; 60 min later, gVPLA₂ or MCh inhalation was performed (after phase II; see Fig. 3A).

Increase in Rrs was measured for all treated mice. Either saline or iso-type-matched control (IgG1) was used as negative control.

Inflammatory cell count

The cell pellet was collected from bronchoalveolar lavage fluid (BALF), and differential cell counts were performed with Wright-Giemsa method.

Lung histology

Treated lungs were inflated to 20–25 cm of H₂O before inflation of 10% formalin solution and were sliced longitudinally and embedded in paraffin. Histological sections, 3 µm thick, of transverse cross-sections of peripheral airways were cut and stained with H&E for assessment of peribronchial inflammation and of structural alterations of bronchial airways.

Statistical analysis

Data are presented as mean ± SEM. Variation between three or more groups was analyzed by ANOVA followed by Fisher’s protected least significant difference. Variation between two groups was tested using a two-tailed Student t test. Statistical significance was claimed whenever p was <0.05.
specimens. However, full thickness airways demonstrated bronchoconstriction in allergic mice but not phocytes. The H&E-stained transverse cross-sections of peripheral challenged with OVA (allergic mice), the lungs were heavily infiltrated in nonallergic (E), and allergic (F) mice. * p < 0.001 compared with each cell before challenge.

Results

Validation studies of immunosensitization

The efficacy of the protocol for sensitization with OVA was demonstrated in four mice (Fig. 1). In OVA-sensitized mice acutely challenged with OVA (allergic mice), the lungs were heavily infiltrated with inflammatory cells, particularly, eosinophils and lymphocytes. The H&E-stained transverse cross-sections of peripheral airways demonstrated bronchoconstriction in allergic mice but not in saline-treated mice (n = 4 nonallergic mice); luminal narrowing was accompanied by 1) epithelial enfolding, 2) cellular infiltrates, and 3) thickening of basement membrane compared with nonallergic airway.

Ag-up-regulated gVPLA2 expression

We and others have identified that gVPLA2 is highly expressed in isolated heart (43, 44), epithelial cells (35–36, 38), macrophages (40, 43), mast cells (20, 28), neutrophils (17), and T cells (45) but is not contained in eosinophils (34, 35, 38). Preliminary studies revealed expression of gVPLA2 in abundant quantities in micro-sections of human asthmatic airways and minimal or no expression of gVPLA2 in nonasthmatic airways as determined by immunohistological staining (data not shown). However, full thickness airway section can be obtained only from pneumonectomy or autopsy specimens.

In this study, expression of gVPLA2 first was analyzed in airway microsections obtained from nonallergic and allergic mice. The histoslides were stained with isotype-matched control, IgG1 (Fig. 2, A and B) and MCL-3G1 (Fig. 2, C and F), mAb directed against gVPLA2 (34, 35, 38, 39). By light microscopy, expression of gVPLA2 was identified in abundant quantities in airway micro-sections of allergic mice (Fig. 2, D and F, inset) compared with nonallergic mice (Fig. 2, C and E, inset). Secretory gVPLA2 expression (dark brown stain) was evident in epithelial cells, airway smooth muscle, and inflammatory cells, mostly macrophages (see red arrow), surrounding the bronchial airways (Fig. 2F). Immunoblotting analysis showed that gVPLA2 was highly expressed in lungs of allergic mice (Fig. 2G); modest expression of gVPLA2 was observed in lungs of nonallergic mice. Excised heart from allergic mice was used as positive control because gVPLA2 is highly expressed in this organ (43, 44). These data demonstrate that gVPLA2 is inducible in murine airways by Ag-induced immune sensitization.

Secretory gVPLA2-induced bronchoconstriction in nonallergic and allergic mice

We next examined the effect of gVPLA2 in causing airway narrowing in nonallergic and allergic mice. Inhalation of gVPLA2 caused concentration-related increase in airway lung resistance (Rrs; cm H2O/ml/s) in nonallergic mice (Fig. 3A). By contrast, maximal bronchoconstriction was elicited at 14 ng/ml gVPLA2 and was sustained thereafter in allergic mice. Baseline Rrs was 0.48 ± 0.08 cm H2O/ml/s and increased to 1.44 ± 0.30 cm H2O/ml/s in nonallergic mice (p < 0.05); in allergic mice, Rrs increased from 1.27 ± 0.0968 cm H2O/ml/s at baseline to 2.4 ± 0.188 cm H2O/ml/s after 14 ng/ml gVPLA2 (p < 0.05). Greater concentrations of gVPLA2 had no further effect on Rrs in allergic mice.

To test the specificity of airway responses elicited by gVPLA2, we next examined the effect of gIIaPLA2, a close homolog of gVPLA2 (25, 31–35), and W31A, which has a point mutation at the putative interfacial binding site of gVPLA2 and much reduced hydrolytic activity (32–35), in causing bronchoconstriction in allergic mice (Fig. 3B). Neither gIIaPLA2 nor W31A was effective in causing an increase in Rrs at any concentration tested indicating the specificity of gVPLA2 and the requirement for interfacial unique membrane binding property in eliciting airway narrowing in immune-sensitized mice.

MCL-3G1, a gVPLA2 blocking Ab, attenuated gVPLA2-induced bronchoconstriction and cell migration in allergic mice

To determine whether inhibition of endogenous gVPLA2 by MCL-3G1 would block AHR caused by immunosensitization, 1) isotype matched-control (IgG1) or 2) 20 μg of MCL-3G1 (after phase I) was administered to mice for 3 consecutive days before OVA challenge (Fig. 4A, treatment 1). The change in Rrs caused by gVPLA2 for control mice receiving isotype-matched-control (IgG1) or 2) 20 μg of MCL-3G1 after phase I was injected 24 h after phase II (Fig. 4B, ). The contractile response to increasing concentrations of gVPLA2 was attenuated by MCL-3G1 to baseline level (Fig. 4B, ). The contractile response to increasing concentrations of gVPLA2 was attenuated by MCL-3G1 to baseline level (Fig. 4B, ). In separate studies, MCL-3G1 was injected 24 h after phase II (Fig. 4A, treatment 2), and the mice were challenged with gVPLA2 (Fig. 4B, ). Bronchoconstriction elicited by gVPLA2 also was blocked fully in those mice receiving MCL-3G1 24 h after the last OVA challenge and gVPLA2 inhalation.

The total BALF cell count was 1240 ± 98.6 × 103 cells in mice receiving 20 μg of MCL-3G1 after phase II compared with 153 ± 14.6 × 103 cells for mice receiving 20 μg of MCL-3G1 after phase I and before each of three inhalation challenges with OVA in phase...
The total inflammatory cell count decreased from 1100 to 120 cells for eosinophils (p < 0.001), from 160 to 23 cells for macrophages (p < 0.05) and from 115 to 10.3 cells for lymphocytes (p < 0.05). These data demonstrate that blockade of endogenous gVPLA2 is sufficient to block both AHR and cell migration before phase II caused by immunosensitization. By contrast, after full sensitization with OVA (after phase II), MCL-3G1 did not affect the migration of inflammatory cells; however, the airway narrowing still was blocked significantly.

MCL-3G1, a gVPLA2-blocking Ab, attenuated MCh-induced bronchoconstriction in allergic mice

Initial studies established the increase in AHR measured as change in Rrs in nonallergic and allergic mice (Fig. 5A). Baseline Rrs in allergic mice was 1.36 cm H2O/ml/s and increased progressively to 5.55 cm H2O/ml/s with inhalation of 10 mg/ml MCh (p < 0.01 vs baseline value). There was no significant increase in Rrs at any concentration of MCh in the nonallergic group. Pretreatment with i.p. MCL-3G1 after phase I and before each challenge with OVA blocked substantially the increase in Rrs caused by MCh-inhalation (see Fig. 4A, treatment I). Similarly, i.p. MCL-3G1 administered after phase II still
FIGURE 4. Effect of MCL-3G1, a gVPLA2 blocking Ab, on gVPLA2-induced bronchoconstriction and cell migration before and after OVA challenge. A, Protocol design: administration of MCL-3G1 before gVPLA2 or MCh inhalation. B, Change in airway resistance (Rrs; cm H2O/ml/s) caused by gVPLA2. Isotype-matched control (IgG1; □, no MCL-3G1; n = 5 mice). Blockade of AHR by 20 μg of MCL-3G1 alone administered (after phase I) daily before OVA challenge performed on days 21, 22, and 23 (■, treatment 1; n = 5 mice) and 20 μg of MCL-3G1 administered (after phase II) 24 h after the last OVA challenge (■, treatment 2; n = 5 mice). C, The total BALF and differential cell count. *, p < 0.001 and †, p < 0.01 vs MCL-3G1 before OVA challenge (after phase I). Data are expressed as mean ± SEM. IgG1 isotype-matched control was used as negative control in allergic mice (n = 5 mice per group).

FIGURE 5. Cumulative concentration-response curve to methacholine (MCh) in the presence or absence of MCL-3G1 in nonallergic and allergic mice. Change in airway resistance (Rrs; cm H2O/ml/s) caused by MCh. A, □, nonallergic, no MCL-3G1 (n = 7 mice); ■, allergic mice, no MCL-3G1 (n = 7 mice); ○, MCL-3G1 administered (after phase I) before OVA challenge (n = 5 mice); ●, MCL-3G1 administered 24 h after the last OVA challenge (n = 5 mice). B–E, Representative airway microsections stained with H&E from the same experimental mice in A. B, Nonallergic mice, not receiving MCL-3G1; C, allergic mice, not receiving MCL-3G1; D, MCL-3G1 administered after OVA-sensitization (phase I) and daily before OVA challenge (phase II); E, MCL-3G1 administered 24 h after the last OVA challenge (after phase II). F–H, Histological identification of gVPLA2 expression in airway of immune-sensitized mice; F, allergic airway, stained with isotype-matched control. IgG1; G, allergic mice stained with MCL-3G1; H, MCL-3G1 administered after OVA sensitization (phase I) and daily before OVA challenge (before phase II), stained with MCL-3G1. *, p < 0.05, †, p < 0.01, and ‡, p < 0.001 compared with nonallergic mice, to MCL-3G1 treated mice before OVA challenge, and to 24 h after the last OVA challenge.
value) progressively increased by 1) 0.385 ± 0.05 cm H_2O/ml/s after 1.25 mg/ml MCh, 2) 0.5 ± 0.02 cm H_2O/ml/s after 2.5 mg/ml MCh, 3) 0.98 ± 0.11 cm H_2O/ml/s after 5 mg/ml MCh, and 4) 1.79 ± 0.41 cm H_2O/ml/s after 10 mg/ml MCh. An increase in Rrs caused by MCh also was blocked substantially in gVPLA2-deficient mice. There was no difference in change in Rrs for pla2g5+/− and pla2g5−/− saline-treated mice (p = NS).

Histological examination of Airways revealed the absence of inflammatory cells and typical appearance of normal bronchial airways in nonallergic pla2g5+/− (Fig. 6B, upper left), nonallergic pla2g5−/− mice (Fig. 6B, lower left) as well as in allergic pla2g5−/− mice (Fig. 6C, lower right). By contrast, Airways from allergic pla2g5+/− mice had extensive eosinophilic infiltrates within the submucosa that extended through the epithelial lining of the bronchial Airways (Fig. 6C, upper right).

The total number of BALF cells increased significantly from 76.6 ± 18.4 × 10^3 cells to 918 ± 168 × 10^3 cells for pla2g5+/− mice after OVA challenge (Fig. 6D, p < 0.01), which was attenuated by >45% in allergic pla2g5−/− mice (481 ± 154 × 10^3 cells; p < 0.05 vs allergic pla2g5+/− mice). The BALF eosinophil count was 748 ± 181 × 10^3 cells in allergic pla2g5+/− and 320.8 ± 115 × 10^3 cells for allergic mice lacking gVPLA2, gene (p < 0.05). The number of macrophages did not change significantly in any treatment group. The total number of lymphocytes in pla2g5+/− was less than the eosinophil count; however, allergic pla2g5+/− mice had a 500-fold increase from 0.12 ± 0.07 × 10^3 cells (saline control) to 60 ± 30 × 10^3 cells (p < 0.001), which was attenuated >50% to 24 ± 16 × 10^3 cells in pla2g5−/− mice exposed to the same concentration of OVA (p < 0.05). Unlike in pla2g5+/− mice, gVPLA2 expression was not identified by PCR in lung homogenate obtained from pla2g5−/− mice (Fig. 6E). These data further suggest that gVPLA2 is a critical enzyme in the regulation of cell migration and AHR in this murine model of allergic airway inflammation.

To examine the possible mechanism of gVPLA2-induced AHR in allergic mice, we administered gVPLA2 to immunosensitized gIVaPLA2−/− BALB/c mice (pla2g4−/−) and measure the change in Rrs (Fig. 7). In allergic littermate control pla2g4+/−, basal Rrs was 0.68 ± 0.09 cm H_2O/ml/s, 1.28 ± 0.43 cm H_2O/ml/s after 14 ng/ml gVPLA2 (p < 0.002 vs control) and 1.67 ± 0.58 cm H_2O/ml/s in response to 1400 ng/ml gVPLA2 (p < 0.01 vs 14 ng/ml gVPLA2; p < 0.002 vs control). These data are identical with those from allergic pla2g4−/− mice (p = NS for all comparisons) and indicate that gVPLA2-induced airway narrowing is effected by mechanisms distinct from gIVaPLA2 activation.

**Discussion**

The objective of this study was to characterize the role of gVPLA2 in cytopathological and morphological alterations that accompany the induction of AHR in allergic lungs of C57BL/6 mice. Our data show that immunosensitization caused 1) airway inflammation and remodeling (Fig. 1), 2) biosynthesis of gVPLA2 in the Airways (Fig. 2), and 3) AHR to gVPLA2 (Fig. 3A) and MCh (Fig. 5A) compared with sham-sensitized mice. Injection i.p. of MCL-3G1, a gVPLA2 blocking Ab, before OVA challenge (after phase I) blocked cell migration and AHR (Figs. 4 and 5). An important finding in this study is that MCL-3G1 still blocked the bronchoconstriction caused by MCh in highly inflamed Airways (Fig. 5A). We also found that neither gIIaPLA2, a close homolog of gVPLA2, nor W31A, a mutant of gVPLA2, that does not bind to the outer plasma membrane to release arachidonic acid (32–34) caused development of AHR in allergic mice (Fig. 3B). Hence, these effects are specific to gVPLA2 and do not result from activation of the cytosolic gIVaPLA2 enzyme (Fig. 7). Blockade of cell migration...
and AHR was replicated by deletion of gVPLA2 in allergic pla2g5−/− mice (Fig. 6). Our study is the first to describe the involvement of gVPLA2 in AHR and airway inflammation in a murine model of allergic pulmonary inflammation. In this study, naïve C57BL/6 mice were utilized as a proper control for pla2g5−/− mice which were bred to a C57BL/6 background; pla2g4−/− mice were derived from BALB/c strain. Accordingly, both strains were responsive to Ag challenge, and there was a clear and significant increase in airway lung resistance.

Asthma is a chronic disease characterized by AHR, cellular infiltrates, and reversible narrowing of airways (1–6). The airway inflammation is associated with infiltration of leukocytes, including T lymphocytes, macrophages, and eosinophils (1–6). However, the primary signals for the infiltration of the lung tissue with leukocytes have yet to be identified. Although cytokines enhance the differentiation, migration, and pathological efficiency of eosinophils, neither GM-CSF, nor IL-5, nor IL-3 activates eosinophils to cause bronchoactive mediator release (46). Prior studies have reported that cells that normally reside in bronchial airways can generate bronchoactive mediators, which can directly activate the neighboring inflammatory cells (35–36); yet the endogenous trigger for activation of inflammatory cell functions inside the lung has not been identified.

In this study, we found gVPLA2 to be extremely potent and efficacious in causing bronchoconstriction (Fig. 3). Maximal bronchoconstriction was elicited in allergic mice with 14 ng/ml gVPLA2 (∼1 × 10−3 M; Fig. 3A); comparable bronchoconstriction required 2.5 mg/ml MCh (∼1 × 10−3 M; see Fig. 5A) (47). Because secreted gVPLA2 binds rapidly to the phosphotidylycholine-rich cell membrane of circulating inflammatory cells, the quantitation of this enzyme in BALF is not possible. Administration of MCL-3G1 attenuated both eosinophilic inflammation and AHR in allergic mice. Our data also demonstrate that blockade of gVPLA2 alone is sufficient to inhibit the AHR and eosinophilic migration elicited by MCh or gVPLA2 inhalation of immunosensitized mice.

Specificity of gVPLA2 in the induction of cell migration and AHR was confirmed by replicating our findings in gVPLA2-deficient mice. We found that in allergic pla2g5−/− mice, airway inflammation (Fig. 6C) and AHR to MCh challenge (Fig. 6A) were substantially reduced compared with immunosensitized pla2g5−/− mice. These data are identical with those from allergic mice treated with MCL-3G1 before OVA challenge (Fig. 5A). By contrast, there are no statistical differences in airway narrowing caused by gVPLA2 inhalation in allergic pla2g4+/− and pla2g4−/− mice (Fig. 7). These data suggest 1) a functional role for gVPLA2 in induction of bronchoconstriction that does not utilize glVAla2 activation (Fig. 7) and 2) a potential role of anti-gVPLA2 mAb in the future prevention or management of allergic inflammation and AHR.

It is important to note some limitations of our findings. Although our results define a unique mechanism by which AHR and cell migration into the airway lumen requires endogenously secreted gVPLA2, our data are based on studies in immunosensitized mice. The morphological changes of the airway in this process resemble those of human asthma; however, our data cannot necessarily be extrapolated to the human state given that mice do not spontaneously develop asthma (42). Nonetheless, our data suggest a potential role of human gVPLA2 as a messenger protein in allergic pulmonary inflammation and thus suggest the potential value of evaluating the efficacy of gVPLA2 inhibitors in humans.

In summary, we demonstrate that gVPLA2 inhibition by MCL-3G1, a specific blocking Ab against gVPLA2, causes blockade of inflammatory cell trafficking into the airway lumen and blocks bronchoconstriction to allergen challenge and MCh, a generalized measure of AHR, in immunosensitized airways. Furthermore, MCL-3G1 blocked AHR to both gVPLA2 and MCh when administered after OVA challenge of sensitized mice even in the presence of OVA-induced inflammation. Our data also show that gVPLA2 induces AHR by a mechanism that does not utilize a glVAla2 pathway. Deletion of the gVPLA2 gene reduced allergic pulmonary inflammation and completely blocks the AHR to MCh challenge even in the immunosensitized state. Accordingly, blockade of endogenous secreted gVPLA2 could provide a potential new therapeutic approach for treating diverse phenotypes of human asthma.

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Disclosures
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