

Deletion of Secretory Group V Phospholipase A₂ Attenuates Cell Migration and Airway Hyperresponsiveness in Immunosensitized Mice¹

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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. *The Journal of Immunology*, 2007, 179: 4800–4807.

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 (PLA₂)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

V 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 arachidonoyl 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, gIIaPLA₂, and gXPLA₂, 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.

GIIaPLA₂ is a close homolog of gVPLA₂; however, unlike gVPLA₂, gIIaPLA₂ has low affinity for zwitterionic phosphatidylcholine-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 PLA₂ 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^{null} mice; WT, wild type; *pla2g5*^{+/+}, gVPLA₂ WT mice; *pla2g4*^{-/-}, cytosolic gIVaPLA₂^{null} mice; *pla2g4*^{+/+}, cytosolic gIVaPLA₂ WT mice; Rrs, airway lung resistance; MCh, methacholine; BALF, bronchoalveolar lavage fluid.

of airway hyperresponsiveness (AHR) in allergic guinea pigs (21). Other studies have demonstrated substantially greater AHR in immunosensitized gIVaPLA₂ wild-type (WT) mice than in gIVaPLA₂ knockout mice (23, 34, 37). Although much attention has been focused on gIVaPLA₂ 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 gVPLA₂ can trigger the biosynthesis and release of LTB₄ from human neutrophils (32, 33) in a gIVaPLA₂-dependent manner and LTC₄ from human eosinophils in a gIVaPLA₂-independent manner (34, 35). We and others have also reported recently that epithelial cells are a natural source of gVPLA₂ (35, 36, 38) 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 gIVaPLA₂ (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 nonspecific AHR elicited by methacholine (MCh) challenge was caused by endogenous gVPLA₂ secreted from airways of immunosensitized mice. In additional studies, we used allergic gVPLA₂^{null} 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 gIVaPLA₂ activation. Our data are the first demonstration that 1) gVPLA₂ regulates AHR in immunosensitized mice and 2) induction of AHR by gVPLA₂ is independent of cytosolic gIVaPLA₂.

Materials and Methods

Isolation of secretory PLA₂s

The mutagenesis of gVPLA₂ to the W31A mutant was performed as described previously (32–35). gIIaPLA₂, 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*^{−/−} and WT control *pla2g5*^{+/+} mice. Homozygous BALB/c gIVaPLA₂^{null} mice (*pla2g4*^{−/−}) and littermate controls (*pla2g4*^{+/+}) were derived as described (20, 29, 37). Control C57BL/6 mice, 6–8 wk old, were purchased from The Jackson Laboratory. These mice were housed in specific pathogen-free conditions at the University of Chicago animal facility. Experimental protocols conform to the principles outlined by the Animal Welfare and the National Health services guidelines for the care and use of animals in biomedical research.

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 DeVilbiss 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 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/lane) 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'-GATGCA CGACCGTTGTATG-3'; reverse, 5'-TAATCTAATGGAAGAGCCTC AGGT-3'. Primers for NEO are: forward, 5'-GCTGTGCTCGACGTTG TCACT-3'. Primers used for gIVaPLA₂ genotyping are: number 1, 5'-CG ACTCATACAGTCCTTCATCAC; number 2, 5'-GGGAACCTCTCTGA CTAGGGG; number 3, 5'-TGTTGTACAATCTTTGTGTGTTTCA.

Preparation of animals

Mice were anesthetized with 30 mg/ml xylazine plus 80 mg/ml ketamine i.p. and were ventilated quasibiosinusoidally at a frequency of 150 breaths/min (tidal volume, 10 ml/kg) as described previously (42). Increasing concentrations of sPLA₂s 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 through a computer-controlled small-animal ventilator as described previously (42). MCh (0–10 mg/ml) or 14–1400 ng/ml gVPLA₂, gIIaPLA₂, or W31A were given via Aeroneb to mice. Rrs was measured during continuous nebulization (15 s) of MCh, gVPLA₂, gIIaPLA₂, 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 gIVaPLA₂, 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 isotype-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 infusion 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 using 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.

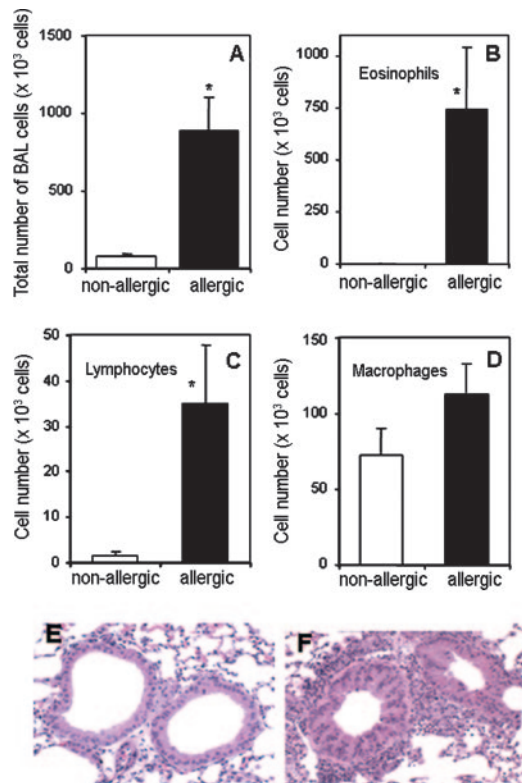


FIGURE 1. Bronchoalveolar lavage (BAL) cellular infiltrates in non-allergic and allergic mice. *A*, The total number of cells in BALF in allergic mice ($n = 5$) compared with nonallergic mice ($n = 5$); *B*, eosinophil count; *C*, lymphocyte count; *D*, macrophage count. Histologic assessment of tissue eosinophilia and airway remodeling ($\times 20$) 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 microsections 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 histosides 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 microsections 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. 2*F*). Immunoblotting analysis showed that gVPLA₂ was highly expressed in lungs of allergic mice (Fig. 2*G*); 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. 3*A*). 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. 3*B*). 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 μ g of MCL-3G1 (after phase I) was administered to mice for 3 consecutive days before OVA challenge (Fig. 4*A*, treatment 1). The change in Rrs caused by gVPLA₂ was measured 24 h after the last OVA challenge (after phase II). Maximal airway narrowing was 2.9 ± 0.011 cm H₂O/ml/s in response to 14 ng/ml gVPLA₂ for control mice receiving isotype-matched control Ab, IgG1. Higher concentrations of gVPLA₂ had no further effect on Rrs (Fig. 4*B*, $\square$). The contractile response to increasing concentrations of gVPLA₂ was attenuated by MCL-3G1 to baseline level (Fig. 4*B*, $\blacksquare$). In separate studies, MCL-3G1 was injected 24 h after phase II (Fig. 4*A*, treatment 2), and the mice were challenged with gVPLA₂ (Fig. 4*B*, $\blacksquare$). 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 $1240 \pm 98.6 \times 10^3$ cells in mice receiving 20 μ g of MCL-3G1 after phase II compared with $153 \pm 14.6 \times 10^3$ cells for mice receiving 20 μ g of MCL-3G1 after phase I and before each of three inhalation challenges with OVA in phase

FIGURE 2. Expression of gVPLA₂ in saline-treated (nonallergic) and OVA-sensitized/-challenged airway (allergic). Values are representative of five mice per group. *A*, nonallergic airway microsection stained with isotype-matched control, IgG1; *B*, allergic airway microsection stained with isotype-matched control, IgG1; *C*, nonallergic airway microsection stained with MCL-3G1 mAb; *D*, allergic airway microsection stained with MCL-3G1 mAb; *E*, inset, nonallergic airway stained with MCL-3G1, IgG1; *F*, inset, allergic airway stained with MCL-3G1 mAb, red arrows indicate the expression of gVPLA₂ in the airway epithelium (EPI), airway smooth muscle (ASM) and macrophages (mΦ); *G*, lung and heart homogenates from nonallergic and allergic mice probed with MCL-3G1 mAb and analyzed by Western blot. The expression of gVPLA₂ in airway microsection was visualized by light microscopy.

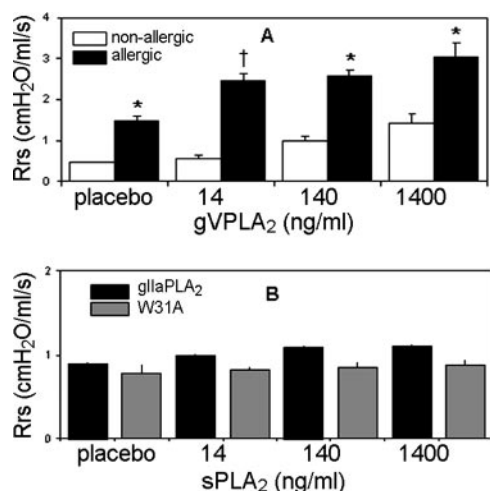
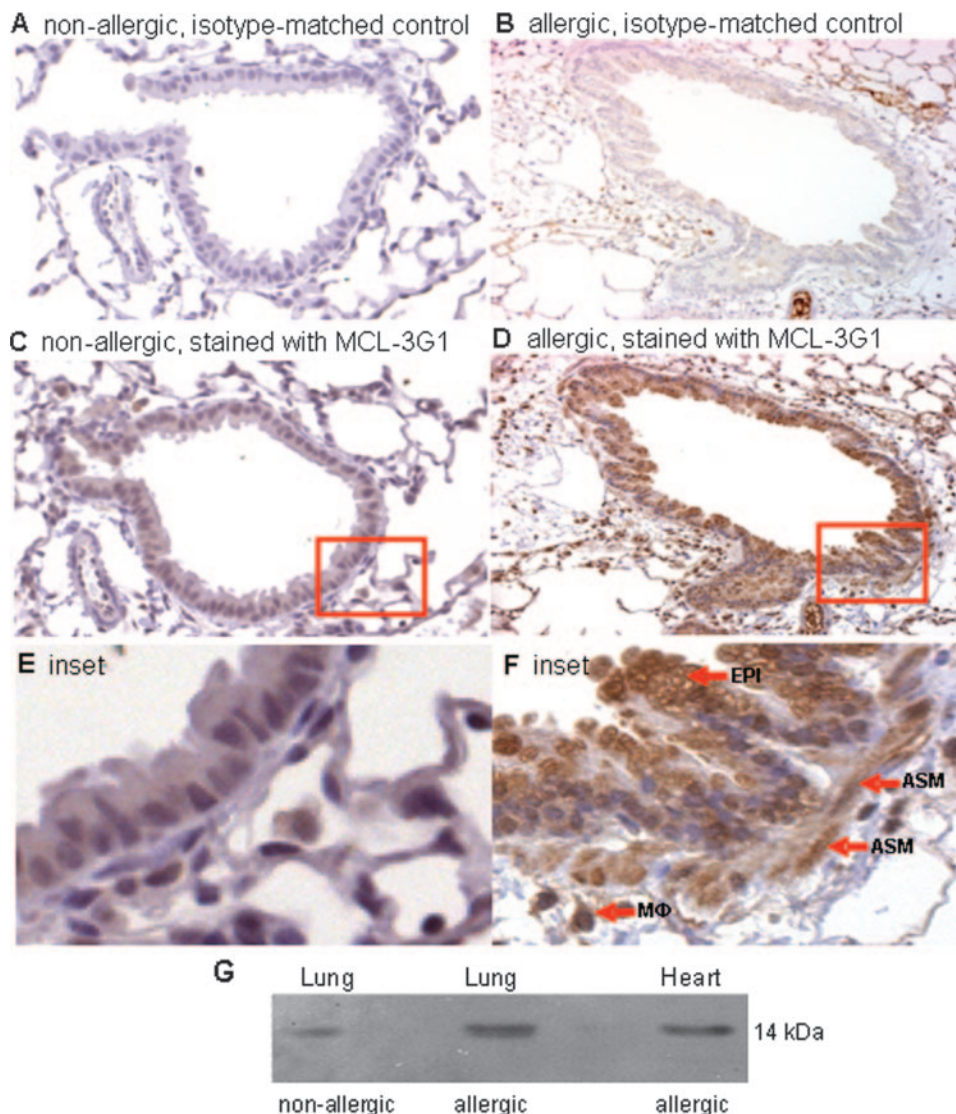


FIGURE 3. Nonresponsiveness to gIIaPLA₂ and the inactive W31A gVPLA₂ mutant. *A*, Increase in airway resistance (Rrs; cm H₂O/ml/s) elicited by gVPLA₂ in nonallergic mice (□, *n* = 5 mice) and allergic mice (■, *n* = 5 mice); *B*, absence of change in airway lung resistance (Rrs; cm H₂O/ml/s) elicited by gIIaPLA₂, a close homolog of gVPLA₂ (■, *n* = 4 mice) and W31A, an inactive mutant of gVPLA₂ (□, *n* = 4 mice) in allergic mice. *, *p* < 0.05 and †, *p* < 0.01 vs control value of nonallergic mice for each group compared with allergic mice. Data are expressed as mean ± SEM.

II (Fig. 4C, *p* < 0.001). The total inflammatory cell count decreased from $1100 \pm 87 \times 10^3$ cells to $120 \pm 0.635 \times 10^3$ cells for eosinophils (*p* < 0.001), from $160 \pm 52.5 \times 10^3$ cells to $23 \pm 13.5 \times 10^3$ cells for macrophages (*p* < 0.05) and from $115 \pm 29 \times 10^3$ cells to $10.3 \pm 3.5 \times 10^3$ cells for lymphocytes (*p* < 0.05). These data demonstrate that blockade of endogenous gVPLA₂ is sufficient to block both AHR (Fig. 4B) and cell migration before phase II (Fig. 4C) 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 (Fig. 4A).

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 ± 0.02 cm H₂O/ml/s and increased progressively to 5.55 ± 0.43 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 ≤ 10 mg/ml 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 1). 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).

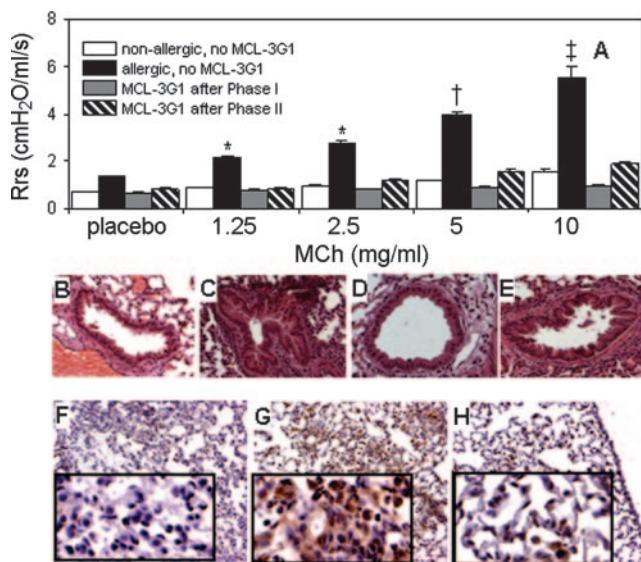
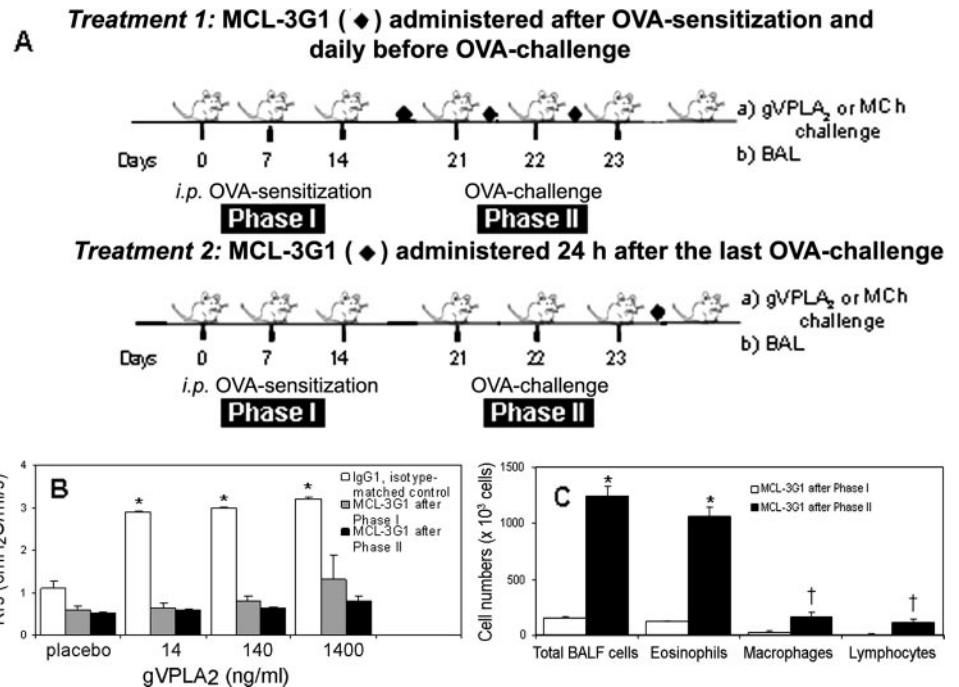


FIGURE 5. Cumulative concentration-response curve to methacholine (MCh) in the presence or absence of MCL-3G1 in nonallergic and allergic mice. Change in airway resistance (Rrs; cm H₂O/ml/s) caused by MCh. **A**, □, nonallergic, no MCL-3G1 (*n* = 7 mice); ■, allergic mice, no MCL-3G1 (*n* = 7 mice); ▒, 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 airway, not receiving MCL-3G1; **C**, allergic airway, 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.



blocked the airway narrowing caused by MCh inhalation (see Fig. 4A, treatment 2). Administration of IgG1, an irrelevant Ab, had no effect on AHR (Fig. 5A). Accordingly, these data suggest that gVPLA₂, which is up-regulated in immunosensitized airways (Fig. 2), is required for the nonspecific bronchial airway responsiveness characteristic of immunosensitization as reflected by increased responsiveness to MCh.

We also assessed the effect of MCL-3G1 on histological and morphological alterations in bronchial airways before and after OVA challenge (see Fig. 4A). All lungs were inflated to 20–25 cm H₂O before infusion of 10% formalin solution into the lung. There were no morphological differences between the airway microsections of nonallergic mice (Fig. 5B) and mice treated with MCL-3G1 administered daily before OVA challenge (Fig. 5D; see Fig. 4A, treatment 1). By contrast, airway narrowing was evident in allergic mice not receiving MCL-3G1 during immunosensitization (Fig. 5C). MCL-3G1 administered 24 h after the last OVA challenge (see Fig. 4A, treatment 2) caused a marked reduction of bronchoconstriction caused by MCh-inhalation. (Fig. 5E). These airways were less constricted than in the allergic mice receiving no MCL-3G1 (Fig. 5C).

Histological examination of peripheral airways obtained from allergic mice demonstrated substantial gVPLA₂ expression (Fig. 5, F–H). Fig. 5F, which demonstrates lack of gVPLA₂, was stained by isotype IgG1 control. Synthesis of gVPLA₂ was apparent by brown staining in allergic airways (Fig. 5G); by contrast, only very modest staining for gVPLA₂ was shown in mice receiving MCL-3G1 (after phase I) daily before OVA challenge (Fig. 5H).

Deletion of gVPLA₂ gene inhibits AHR and cell migration in allergic mice: role of gVPLA₂

We further assessed the specificity of our findings by examining the effect of gVPLA₂ deletion on AHR and cell migration in mice genetically deficient in gVPLA₂ (*pla2g5*^{−/−}) and WT littermate controls (*pla2g5*^{+/+}). The immunosensitized littermate controls *pla2g5*^{+/+} had greater airway responsiveness to MCh (Fig. 6A) than did the allergic *pla2g5*^{−/−} mice even after exposure to the same concentration of OVA. In allergic *pla2g5*^{+/+} littermate control mice, airway narrowing (absolute number minus baseline

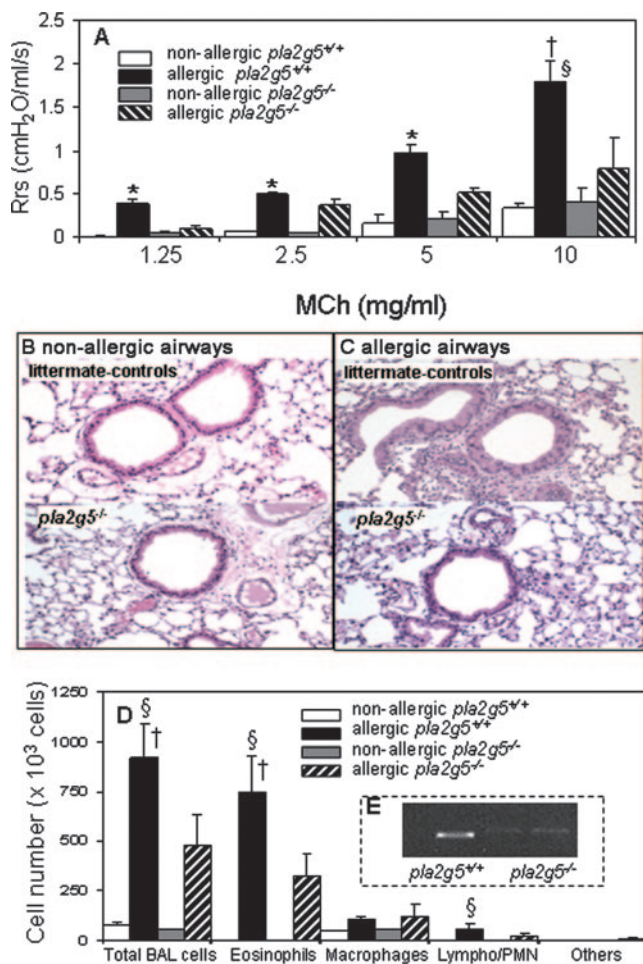


FIGURE 6. Comparison of MCh-induced bronchoconstriction and cell accumulation in BAL 24 h after the saline and OVA challenge with gVPLA₂ littermate control (*pla2g5*^{+/+}) and gVPLA₂-deficient (*pla2g5*^{-/-}) mice. **A**, Airway narrowing assessed as change in airway resistance (Rrs; cm H₂O/ml/s) caused by MCh (*n* = 5 mice for each group); **B**, Photomicrographs of representative lung tissues obtained from *pla2g5*^{+/+} and *pla2g5*^{-/-} nonallergic mice and **C**, *pla2g5*^{+/+} littermate control mice and allergic *pla2g5*^{-/-} mice, stained with H&E; **D**, The BALF total and differential cell count, which indicates the total number of individual cells (*n* = 7 mice for each group). **Inset**, gVPLA₂ expression in lung homogenates as assessed by PCR. *, *p* < 0.05, and †, *p* < 0.01 compared with all nonallergic mice; §, *p* < 0.05 vs allergic *pla2g5*^{-/-} mice. Data are expressed as mean ± SEM.

value) progressively increased by 1) 0.385 ± 0.05 cm H₂O/ml/s after 1.25 mg/ml MCh, 2) 0.5 ± 0.02 cm H₂O/ml/s after 2.5 mg/ml MCh, 3) 0.98 ± 0.11 cm H₂O/ml/s after 5 mg/ml MCh, and 4) 1.79 ± 0.41 cm H₂O/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 *R_L* 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 $76.6 \pm 18.4 \times 10^3$ cells to $918 \pm 168 \times 10^3$ cells for *pla2g5*^{+/+}

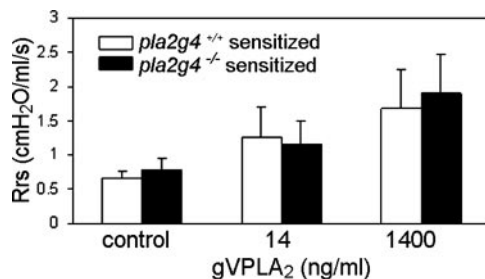


FIGURE 7. Cumulative concentration-response curve to gVPLA₂ in allergic BALB/c *pla2g4*^{+/+} and *pla2g4*^{-/-} mice. Increase in airway resistance (Rrs; cm H₂O/ml/s) caused by administration of gVPLA₂ into *pla2g4*^{+/+} is comparable with that elicited in *pla2g4*^{-/-} mice for all concentrations tested (*n* = 3 animals/group). Data are expressed as mean ± SEM.

mice after OVA challenge (Fig. 6D; *p* < 0.01), which was attenuated by >45% in allergic *pla2g5*^{-/-} mice ($481 \pm 154 \times 10^3$ cells; *p* < 0.05 vs allergic *pla2g5*^{+/+} mice). The BALF eosinophil count was $748 \pm 181 \times 10^3$ cells in allergic *pla2g5*^{+/+} and $320.8 \pm 115 \times 10^3$ 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 $0.12 \pm 0.07 \times 10^3$ cells (saline control) to $60 \pm 30 \times 10^3$ cells (*p* < 0.001), which was attenuated >50% to $24 \pm 16 \times 10^3$ 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^{null} 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 H₂O/ml/s, 1.28 ± 0.43 cm H₂O/ml/s after 14 ng/ml gVPLA₂ (*p* < 0.002 vs control) and 1.67 ± 0.58 cm H₂O/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 cytological 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, naive 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 pathobiological 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 gVLA₂ to be extremely potent and efficacious in causing bronchoconstriction (Fig. 3). Maximal bronchoconstriction was elicited in allergic mice with 14 ng/ml gVPLA₂ ($=1 \times 10^{-9}$ M; Fig. 3A); comparable bronchoconstriction required 2.5 mg/ml MCh ($=1 \times 10^{-3}$ M; see Fig. 5A) (47). Because secreted gVPLA₂ binds rapidly to the phosphatidylcholine-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 gIVaPLA₂ 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 gIVaPLA₂ 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

The authors have no financial conflict of interest.

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