Published in final edited form as:

J Immunol. 2020 June 15; 204(12): 3097–3107. doi:10.4049/jimmunol.2000102.

Secreted phospholipase A_2 group X acts as an adjuvant for type-2 inflammation leading to an allergen-specific immune response in the lung

Herbert Luke Ogden[*,‖], **Ying Lai**[*,‖], **James D. Nolin**[*,‖], **Dowon An**[*], **Charles W. Frevert**[*,†], **Michael H. Gelb**[‡,§], **William A. Altemeier**[*], **Teal S. Hallstrand**[*,#]

[†] Department of Comparative Medicine, University of Washington, Seattle, WA

[‡] Department of Chemistry, University of Washington, Seattle, WA

[§] Department of Biochemistry, University of Washington, Seattle, WA

[*] Department of Medicine, Division of Pulmonary, Critical Care and Sleep, University of Washington, Seattle, WA

Abstract

Secreted phospholipase A_2 ($sPLA_2$) enzymes release free fatty acids, including arachidonic acid and generate lysophosphospholipds from phospholipids, including membrane phospholipids from cells and bacteria and surfactant phospholipids. We have shown that an endogenous enzyme $sPLA_2$ group X ($sPLA_2$-X) is elevated in the airways of asthmatics and that mice lacking the $sPLA_2$-X gene (*Pla2g10)* display attenuated airway hyperresponsiveness (AHR), innate and adaptive immune responses, and type-2 cytokine production in a model of airway sensitization and challenge using a complete allergen that induces endogenous adjuvant activity. This complete allergen also induces the expression of $sPLA_2$-X/*Pla2g10*. In the periphery, a $sPLA_2$ found in bee venom (bee venom PLA_2, $bvPLA_2$) administered with the incomplete antigen ovalbumin (OVA) leads to an antigen specific immune response. In this study, we demonstrate that both $bvPLA_2$ and murine $sPLA_2$-X have adjuvant activity, leading to a type-2 immune response in the lung with features of airway hyperresponsiveness (AHR) and antigen-specific type-2 airway inflammation following peripheral sensitization and subsequent airway challenge with OVA. Further, the adjuvant effects of $sPLA_2$-X that result in the type-2 biased OVA-specific adaptive immune response in the lung was dependent upon the catalytic activity of the enzyme, as a catalytically inactive mutant form of $sPLA_2$-X does not elicit the adaptive component of the immune response, although other components of the immune response were induced by the inactive enzyme suggesting receptor mediated effects. Our results demonstrate that exogenous and endogenous $sPLA_2$s play an important role in peripheral sensitization resulting in airway responses to inhaled antigens.

[#] To whom correspondence should be addressed: Teal S. Hallstrand, MD, MPH, Division of Pulmonary, Critical Care and Sleep Medicine, Department of Medicine, University of Washington, Seattle, WA, USA., Center for Lung Biology, Box 358052, 850 Republican Street, Seattle, WA 98109-4714, Phone: (206) 221-6506, Fax: (206) 221-0739, tealh@uw.edu.

[‖]These authors contributed equally to this manuscript.

Introduction

Secreted phospholipase A_2 ($sPLA_2$) are multi-functional enzymes that act to regulate phospholipid metabolism and bind to specific C-type lectin receptors that are involved in innate immunity (1, 2). Both cytosolic PLA_2s and $sPLA_2$s mediate the rate-limiting step in the eicosanoid pathway by releasing free fatty acids (FFA)s including arachidonic acid (AA) from the *sn2* position of phospholipids, which also leads to the generation of lysophospholipids (LysoPL)s (3–5). The *sn2* position of membrane phospholipids is preferentially substituted with poly unsaturated fatty acids (PUFA)s such as AA that initiates the eicosanoid cascade, but FFAs and LysoPLs also play important independent roles as regulators of inflammation. Because $sPLA_2$s act within the cell prior to secretion and also act in the extracellular environment following secretion (6), the activities attributed to this group of enzymes includes effects mediated on the outer cell membrane of mammalian cells, the cell wall of bacteria and on phospholipids in the airways such as surfactant-associated phospholipids (7).

After the initial discovery that $sPLA_2$ activity is increased in asthma and allergic rhinitis following allergen challenge (8–10), we found that one specific $sPLA_2$, $sPLA_2$ group X ($sPLA_2$-X), is expressed at high levels in the airways, particularly the airway epithelium and bronchial macrophages (11) and that the levels of this protein are elevated in human asthma in association with severity of asthma and the severity of airway hyperresponsiveness (AHR) (12, 13). Murine models of asthma with mice lacking the $sPLA_2$-X gene (*Pla2g10)* have demonstrated an important role for $sPLA_2$-X in the development of type-2 inflammation and AHR and point to a central role for this enzyme in both the innate and adaptive immune response to inhaled allergen. Deletion of this single enzyme is associated with a marked reduction in several critical pathways including in the formation of eicosanoids, particularly cysteinyl leukotrienes (CysLTs, LTs C_4, D_4, E_4), the type-2 dominated cellular influx into the airways, and IL-33 protein levels in the airways (14, 15). Here we examine the possibility that $sPLA_2$-X acts during the sensitization phase, leading to features of allergen-induced airways dysfunction upon subsequent antigen exposure in the airways and lung.

Prior work examining bee venom PLA_2 ($bvPLA_2$), a small PLA_2 that has similar catalytic properties to endogenous mammalian $sPLA_2$s, demonstrated that $bvPLA_2$ administered peripherally with an antigen led to an antigen-specific immune response that was type-2 biased and involved type-2 innate lymphoid cells (ILC2)s (16). Thus, we hypothesized that the $sPLA_2$-X enzyme functions as an endogenous adjuvant to “complete” allergens that can cause sensitization in the absence of an exogenous adjuvant, and that subsequent exposure to the antigen in the lung would lead to a type-2 biased immune response and features of AHR. We base this hypothesis in part on the evidence that the complete allergen house dust mite (HDM) induces $sPLA_2$-X/*Pla2g10* in the lung (15). We conducted initial studies with $bvPLA_2$ because of the known adjuvant effect, and then examined the function of endogenous $sPLA_2$-X. We tested our hypothesis using the incomplete antigen ovalbumin (OVA) along with catalytically active and inactive $sPLA_2$ proteins during the sensitization phase, followed by airway exposure to the antigen alone. This model allowed us to break down the components of the sensitization phase. Our results demonstrate that $sPLA_2$-X acts as an efficient adjuvant, acting through both the innate and adaptive arms of the immune

system, and leading to features of type-2 inflammation and airway dysfunction following exposure to inhaled antigen. Furthermore, our results show that sPLA$_2$-X adjuvant activity promotes an antigen-specific type-2 inflammatory response upon exposure to antigen *in vivo* and *ex vivo* in lung leukocytes. The development of the adaptive immune response, antigen-specific type-2 cytokine response, and bronchoalveolar lavage eosinophilia was dependent upon the active sPLA$_2$-X enzyme, while an enzymatically inactive mutant sPLA$_2$-X protein induced AHR and some alterations in lung tissue leukocyte infiltration suggesting that both receptor mediated and enzymatic functions of sPLA$_2$-X are involved in the development of the type-2 biased immune response to antigen that is triggered upon exposure in the lung following peripheral sensitization.

Materials and Methods

Mice

Wild type (WT) BALB/c mice were used in all experiments and were obtained from Jackson Laboratories. Animals were maintained under specific pathogen-free conditions at the University of Washington and had *ad libitum* access to food and water. Female mice between 6–8 weeks old were used in experiments in order to reduce heterogeneity. The University of Washington Institutional Animal Care and Use Committee approved all animal studies.

OVA with PLA$_2$ Adjuvant Model of Experimental Asthma

During the peripheral sensitization phase, WT BALB/c mice received intraperitoneal sensitization with 100 μg low-endotoxin OVA (EndoFit™ OVA, Invivogen, San Diego, CA) with either 50 μg bvPLA$_2$ (Sigma, St.Louis, MO), recombinant murine sPLA$_2$-X or the enzymatically inactive H46Q mutant form of sPLA$_2$-X on days 0 and 14 (17)(Supplemental Figures S1 **and** S2). The levels of endotoxin were measured in key reagents using a Limulus Assay as reported in Supplemental Table 1 (Genscript, Piscataway, NJ). On days 26–28, mice received oropharyngeal doses of 50 μg OVA in 50 μl saline. An additional control group received an oropharyngeal dose of saline following the administration of bvPLA$_2$ in the peritoneum. On day 29, mice were intubated and ventilated using a flexiVent small animal ventilator (SCIREQ Inc., Montreal, Quebec, CA). Lung function was measured following increasing doses of methacholine (0, 6.25, 12.5, 25, and 50 mg) and dynamic resistance (R) and elastance (E) were calculated using the single frequency forced oscillation technique.

Assessment of Airway Inflammation

The lungs were lavaged post-mortem via tracheal cannula with ice-cold PBS and total bronchoalveolar lavage (BAL) cell counts were determined using a Cellometer Auto 2000 (Nexcelom Bioscience, Lawrence, MA). The BAL fluid was spun down and cells were aliquoted for cell differential analysis via multi-color flow cytometry and the supernatant stored at −80°C with 1× protease inhibitor cocktail.

Lung Leukocyte Isolation

The left lung was minced in warmed digestion solution (RPMI 1640 with 5% FCS, 0.5 mg/ml Liberase TL and 40 U/ml DNase I). Digestion was stopped with cold HBSS containing 10 mM EDTA and the digested tissue was pushed through a 40 μm cell strainer and washed several times with wash buffer (RPMI 1640 with 5% FCS). Red blood cells were removed using ACK red blood cell lysis buffer (eBioscience, San Diego, CA). Lung digest was resuspended in wash buffer, and an aliquot was removed for cell count and viability using the Cellometer Auto 2000. Cells were centrifuged and resuspended in MACS/FACS buffer (HBSS w/o Ca and Mg, 2 mM EDTA and 0.5% BSA) containing CD45 MACS microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD45+ lung leukocytes were isolated using a Miltenyi LS column and magnetic separator.

Multi-Color Flow Cytometry for Cell Differentials

Single cell suspensions from lung leukocytes and BAL cells were stained for cell differentials using a cocktail of the following antibodies: CD11c (clone N418, eBioscience), Siglec-F (clone E50–2440, BD Bioscience), Ly-6G (clone RB6–8C5, eBioscience), CD45 (clone 30-F11, Biolegend, San Diego, CA) CD3e (clone 145–2C11, eBioscience) and MHCII (M5/114.15.2, BD Biosciences, San Jose, CA). Cells were blocked using Fc block (BD Biosciences) and incubated with the primary antibody cocktail for 30 min at 4°C in the dark. Cells were washed and resuspended in PBS containing eFluor 450 fixable viability dye (eBioscience) and incubated for 15 min at 4°C in the dark. Cells were then fixed using 4% paraformaldehyde and stored at 4°C in FACS buffer until analyzed via flow cytometry. Cells were analyzed using a FACS LSR II flow cytometer (Becton Dickinson Instrument Systems, Franklin Lakes, NJ) and gating was performed using FlowJo software (TreeStar, Ashland, OR). Single stained controls of lung leukocytes were used to set voltages and for color compensation, and leukocyte populations were determined as previously reported (18, 19). Briefly, our gating strategy consisted of first selecting singlets using forward scatter width and area, followed by selection of viable cells using the fixable viability dye. Leukocytes were then identified using side scatter and CD45. $CD45^+$ cells were gated for $CD3^+$ T cells and the remaining $CD3^-CD45^+$ cells were further subdivided into $MHCII^{high}$ and $MHCII^{low}$ populations. In the $MHCII^{low}$ population, eosinophils were identified as $CD11c^-SiglecF^+$ leukocytes, resident macrophages as $CD11c^{high}SiglecF^{high}$ leukocytes and neutrophils were identified as $CD11c^-SiglecF^-$ leukocytes that were also $Ly\text{-}6G^+$. In the $MHCII^{high}$ population, $CD11c^+SiglecF^-$ dendritic cells (DC) and $CD11c^-SiglecF^-$ monocyte-derived macrophages (recruited macrophages) were identified. A similar gating strategy was used to identify leukocyte subsets in the BAL fluid.

Multi-Color Flow Cytometry for ILC2s

Single cell suspensions from peritoneal cells were stained for detection of ILC2s using the following antibodies: Mouse Hematopoietic Lineage eFluor 450 Cocktail (eBioscience), containing CD3 (17A2), CD45R/B220 (RA3–6B2), CD11b (M1/70), Ter119 (TER-119) and Gr-1/Ly6G (RB6–8C5) with the addition of eFluor 450-conjugated antibodies for CD4 (RM4–5, eBioscience), CD8a (53–6.7, eBioscience), CD11c (N418, eBioscience), CD19 (1D3, eBioscience), NK1.1 (PK136, eBioscience) and Fc epsilon Receptor I alpha (FcεR1)

(MAR-1, eBioscience). Further selection was performed on the lineage negative (Lin^-) population using the following antibodies: CD45 (30-F11, eBioscience), CD90.2/Thy-1.2 (53–2.1, eBioscience), CD25 (PC61.5, eBioscience), T1/ST2 (DJ8, MD Biosciences, Oakdale, MN).

Cells were washed and resuspended in PBS, blocked using Fc block (BD Biosciences), washed and subsequently incubated with the primary antibodies for 30 min at 4°C in the dark. The cells were then washed and resuspended in PBS containing fixable UV blue dead cell viability stain (eBioscience) and incubated for 15 min at 4°C in the dark. The cells were fixed in 4% paraformaldehyde for 5 min at room temperature and stored at 4°C in FACS buffer until analyzed via flow cytometry. Cells were analyzed using a FACS LSR II flow cytometer and gating was performed using FlowJo software. Our gating strategy for ILC2s consisted of selecting singlets using forward scatter width and area, followed by selection of viable leucocytes using the fixable viability dye and CD45. We then identified Lin- cells expressing CD90.2 as ILC2s and further enumerated CD25+ and ST2+ cells among this population. Single stained controls and color compensation was conducted using anti-CD3 antibodies containing the different fluorophores. Fluorescence minus one (FMO) controls were prepared by omitting CD90.2, CD25, and T1/ST2 antibodies to set the appropriate thresholds for these gates.

Antigen Re-stimulation of Cultured Lung Leukocytes

For OVA re-stimulation of isolated lung leukocytes, 2×10^5 cells were plated in each well of a 96-well tissue culture plate. Cells were stimulated with 100 μg/ml OVA or with saline as a control. Following stimulation for 24h, the cell culture supernatant was isolated and stored at -80°C with 1× protease inhibitor cocktail (Pierce). Cell culture supernatant was later analyzed for cytokine levels via ELISA.

Cytokine and Immunoglobin Analysis by ELISA

Murine IL-33 protein content from BAL fluid, lung tissue lysates and peritoneal fluid were determined by ELISA per manufacturer's instructions (R&D, Minneapolis, MN) and analyzed on a Biotek EL800 platereader (Winooski, VT). Detection of other murine cytokines (IL-4, IL-5, IL-13, IL-10, IL-6, IL-2, TNF, KC) from antigen restimulated leukocytes was performed using the Mouse V-PLEX Pro-inflammatory Panel 1 assay and the Mouse IL-13 Ultrasensitive assay per manufacturer's instructions (Meso Scale Diagnostics, Rockville, MD) and plates were analyzed using a Sector S 600 plate reader. The number of samples assayed was based on the number of wells available on the plate and all data were reported. OVA-specific IgE and IgG1 from mouse plasma samples were assayed by ELISA (Cayman, Ann Arbor, MI).

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism software (version 8.3.1; GraphPad, La Jolla, CA). Differences in AHR to MCh were evaluated with a 2-way ANOVA test with contrasts between MCh dose and sensitization condition. *Post hoc* analysis was performed at each dose after correction for multiple comparisons. Differences in features of inflammation including cell differentials in BAL fluid and lung tissue, and immunoglobulin levels were

assessed with a 1-way ANOVA with contrasts based on the type of sensitization and challenge. The production of cytokines from restimulated leukocytes for the bvPLA$_2$ studies were assessed by an unpaired 2-tailed Welch's t test to account for the lower variance of the unstimulated leukocytes. Cytokine production from restimulated leukocytes for the sPLA$_2$-X studies were assessed with a 2-way ANOVA test with contrasts between sensitization condition and OVA restimulation. Correction for multiple comparisons was made with the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (20). For the analysis of ILC2s, the data were assessed with an unpaired t test or 1-way ANOVA with contrasts based on the type of sensitization and challenge without further correction for multiple comparisons. For all tests, a *P value* less than 0.05 was considered significant and in the case of correction for multiple comparisons, a *q value* of less than 0.05 was considered significant.

Results

Bee venom PLA$_2$ acts as an adjuvant that facilitates an antigen-specific type-2 immune response in the lung following peripheral sensitization.

Prior work demonstrated that in the periphery bvPLA$_2$ delivered along with an antigen leads to the development of a type-2 biased antigen-specific immune response (16). Thus, we hypothesized that both exogenous and endogenous secreted PLA$_2$s could serve as effective adjuvants leading to features of type-2 allergic inflammation upon re-exposure of the airways to antigen. There is strong evidence that such peripheral sensitization is important for the development of allergic asthma in humans (21). To examine this question, we established a model using bvPLA$_2$ as an adjuvant in combination with the antigen OVA, as OVA alone does not elicit a strong type-2 immune response. In our model, we administered sensitization doses 2 weeks apart in the peritoneum, followed by airway challenge with antigen on days 26–28 (Supplemental Fig. 1A). One day after the last airway challenge, AHR was assessed with increasing concentrations of inhaled methacholine. Mice sensitized with bvPLA$_2$ plus OVA (bvPLA$_2$/OVA) and subsequently challenged with OVA had increased AHR compared to mice given bvPLA$_2$ alone (bvPLA$_2$/Sal) and challenged with saline (Fig. 1A). Mice sensitized with saline plus OVA (Sal/OVA) and challenged with OVA also displayed elevated AHR, similar to previous observations (22, 23); however, these mice did not develop AHR to the same extent as mice in the bvPLA$_2$/OVA group (Fig. 1A). Sensitization in the presence of bvPLA$_2$ led to a significant increase in the bronchoalveolar lavage (BAL) leukocytes relative to OVA alone or bvPLA$_2$ without antigen (Fig 1B), but the number of lung leukocytes was not altered in this model (Fig. 1C).

Evaluation of the cellular influx into the BAL fluid by multicolor flow cytometry revealed marked increases in the concentration of eosinophils, T cells, recruited (CD3-CD45+MHCII^{high}CD11c^{-}SiglecF^{-}) macrophages and dendritic cells (DC)s facilitated by sensitization to OVA in the presence of bvPLA$_2$ (Figs. 1D-G), while OVA without an adjuvant also increased the concentration of eosinophils (*P=0.04*), T cells (*P=0.007*), and DCs (*P=0.0007*), but not recruited macrophages (*P=0.14*) relative to the bvPLA$_2$/Sal control. In the BAL fluid there was also an increase in the concentration of neutrophils (PMN) with either OVA alone (P<0.0001) or with bvPLA$_2$/OVA (P<0.0001) relative to the bvPLA$_2$/Sal

control, but there is no difference between these conditions (*P=0.09*) and the magnitude of the PMN influx was small relative to the influx of eosinophils. We also found that there was an increase in the number of resident (CD3-CD45+MHCII^{low}CD11c^{+}SiglecF^{+}) macrophages when bvPLA$_2$/OVA was administered relative to bvPLA$_2$/Sal (*P=0.0003*) or relative to OVA without an adjuvant (*P=0.005*); while ova without an adjuvant was modestly different than the bvPLA$_2$/Sal control (*P=0.06*).

Lung leukocyte populations were altered to less of a degree by the adjuvant properties of bvPLA$_2$ in this model, suggesting that antigen-challenge following peripheral sensitization mediated by bvPLA$_2$ led predominantly to an immune response that was largely confined to the airways following antigen administration. Although the number of eosinophils in the lung was higher in both groups that were challenged with OVA (*P=0.04* OVA alone relative to bvPLA$_2$/Sal), the concentration of eosinophils was highest when bvPLA$_2$ was administered during sensitization relative to either group (Supplemental Fig. 1B). In contrast, lung T cells, recruited macrophages and resident macrophages were not significantly altered by the administration of bvPLA$_2$ during the sensitization phase (Supplemental Figs. 1C-E); however, it is also notable that when OVA was administered alone, there was a significant decrease in resident macrophages (*P=0.02*). We also noted that the number of DCs was increased either by the administration of OVA alone (*P<0.0001*) or with bvPLA$_2$ as an adjuvant, while there were no notable differences in the concentration of PMNs (Supplemental Figs. 1F-G). These findings demonstrate that bvPLA$_2$ acts as an adjuvant, and subsequent airway challenge with antigen drives a type-2 skewed immune response in the lung characterized by AHR and leukocyte recruitment predominantly to the airways.

Peripheral sensitization to OVA with bvPLA$_2$ facilitates antigen-specific cytokine production by lung leukocytes following antigen challenge.

To gain a better understanding of the events that are established following peripheral sensitization in the presence of bvPLA$_2$ followed by antigen challenge in the lung, we isolated leukocytes from the lungs of sensitized and challenged mice and stimulated these cells *ex vivo* with OVA for 24 hours. Cells from bvPLA$_2$/OVA sensitized mice produced more of the type-2 cytokines IL-4 and IL-5 than cells from saline/OVA sensitized mice; however, we did not observe a difference in IL-13, although the levels of IL-13 were surprisingly low in this model, while the levels of IL-5 were markedly elevated (Figs. 2A-C). We also detected significantly higher levels of IL-10, IL-6, IL-2 (Figs. 2D-H), as well as KC (*P=0.001*) and TNF (*P=0.003*) in lung leukocytes isolated from bvPLA$_2$/OVA mice. Leukocytes that were sensitized with bvPLA$_2$ alone in the absence of the antigen challenge with OVA did not produce measurable cytokines following *in vitro* OVA stimulation (data not shown). These data suggest that peripheral sensitization mediated through bvPLA$_2$ enhances activation of leukocytes in the lung following antigen exposure in the lung and specifically leads to the antigen-specific generation of both type-1 and type-2 cytokines.

Sensitization with bvPLA$_2$ promotes an adaptive immune response to antigen in the lung.

To evaluate the effects of bvPLA$_2$ on the adaptive immune response to OVA, we measured antigen-specific IgE and IgG. Although the levels of OVA-IgE were increased relative to the

bvPLA$_2$/Sal control, the levels of OVA-IgE were significantly higher in the bvPLA$_2$/OVA group compared to both controls (Fig. 3A) In contrast, OVA-IgG was modestly elevated in the bvPLA$_2$/OVA group, but this increase was not seen in the group that received OVA in the absence of an adjuvant and there was also a modest trend towards an increase in OVA-IgG compared to the Sal/OVA group (Fig. 3B). These results indicate that bvPLA$_2$ adjuvant activity enhances the adaptive immune response in an antigen-specific manner and that this response is more prominent for antigen-specific IgE, although the levels of OVA-specific IgE are lower than OVA-specific IgG.

Adjuvant activity of sPLA$_2$-X facilitates the development of allergic airway disease following sensitization in the periphery.

Next we sought to expand upon these findings by investigating the potential adjuvant properties of a recombinant version of endogenous sPLA$_2$-X that we have implicated in human asthma (12, 14, 15, 24). Both sPLA$_2$-X and bvPLA$_2$ are small PLA$_2$s that act on the outer cell membrane of mammalian cells with similar enzymatic activity, but these sPLA$_2$s are structurally dissimilar, a critical distinction since they both act through receptors but are divergent in this regard (25, 26). In prior studies, we demonstrated that mice lacking sPLA$_2$-X/*Pla2g10* are protected from developing allergic airway disease and have impaired innate and adaptive immune responses to the complete allergen HDM (15) and in a model with the exogenous adjuvant alum (14). Here we sought to determine the ability of sPLA$_2$-X to act as an adjuvant and to investigate whether peripheral sensitization in the presence of this endogenous enzyme facilitated a type-2 response in the lung. Further, we examined the dependence on the enzymatic activity of sPLA$_2$-X through the use of a mutant version of this enzyme in which a single amino acid substitution from histidine to glutamine in the active site (H46Q mutant sPLA$_2$-X) renders it enzymatically inactive but maintains the secondary protein structure preserving receptor binding (17). We exposed mice to OVA plus recombinant WT sPLA$_2$-X or to H46Q mutant sPLA$_2$-X according to our model (Supplemental Fig. 2A). Following sensitization in the periphery with each protein followed by airway challenge with antigen, both the WT version of sPLA$_2$-X and the H46Q mutant sPLA$_2$-X tended to induce AHR above the saline controls (*P=0.07* H46Q vs. Sal), but the WT sPLA$_2$-X increased AHR over both the saline control and the H46Q mutant administered during the sensitization phase resulting in significant difference overall based on the type of protein administered during sensitization and methacholine dose (*P=0.03*, Fig. 4A). In this model, we excluded the 50 mg/ml concentration of methacholine because several of the WT mice died at this concentration due to enhanced AHR. The cellular influx into the airways was notable for a significant increase of leukocytes in the BAL fluid of mice sensitized with WT sPLA$_2$-X plus OVA that was not seen in mice sensitized with the H46Q mutant form of sPLA$_2$-X (Fig. 4B). The concentration of lung tissue leukocytes tended to increase in the mice that received WT sPLA$_2$-X plus OVA, but this was not significantly different from the group that received the inactive form of sPLA$_2$-X (Fig. 4C).

Multi-color flow cytometry to decipher leukocyte populations in the BAL fluid revealed marked increases in eosinophils, T cells, recruited macrophages and DCs in mice sensitized with WT sPLA$_2$-X compared to saline controls (Figs. 4D-G). Sensitization with the inactive H46Q mutant did not result in any notable change in leukocyte populations (Figs. 4D-G).

We also noted an increase in the concentration of PMNs in the BAL fluid mediated by WT $sPLA_2$-X compared to either control group (*P=0.0004*), but the concentration of PMNs was very low in this model. We also did not observe any differences in the concentrations of resident macrophages.

Further characterization of leukocyte subsets in the lung tissue revealed that there were significant increases in lung tissue eosinophils with WT $sPLA_2$-X that did not occur with the mutant H46Q protein, while there were no significant differences in T cells or recruited macrophages (Supplemental Figs. 2B-D). We also noted that the concentration of resident macrophages was reduced when WT $sPLA_2$-X was administered during sensitization, while the concentrations of DCs and PMNs in lung tissue were both increased following sensitization in the presence of WT $sPLA_2$-X followed by subsequent challenge of the lung with OVA (Supplemental Figs. 2E-G). Together, these data indicate that $sPLA_2$-X activity is required for the activation and recruitment of leukocyte populations to the airways in response to inhaled antigen, and that these responses are accompanied by similar changes of lesser magnitude in lung tissue leukocytes following OVA rechallenge of the airways.

Enzymatic activity of $sPLA_2$-X is required for the adaptive type-2 immune response and airway inflammation.

Lung leukocytes isolated from control mice sensitized with saline plus OVA (Sal) displayed modest changes in type-2 cytokines following *ex vivo* stimulation with OVA (Figs. 5A-C). In contrast, leukocytes from mice sensitized with WT $sPLA_2$-X protein plus OVA (WT) produced robust levels of IL-4, IL-5 and IL-13, whereas leukocytes from mice sensitized with the inactive $sPLA_2$-X mutant (H46Q) plus OVA produced levels of IL-4, IL-5 and IL-13 following OVA rechallenge that were similar to leukocytes from saline control mice (Figs. 5A-C). In addition to type 2 cytokines, leukocytes from mice sensitized with WT $sPLA_2$-X produced significantly higher levels of IL-10, IL-6 and IL-2 compared to leukocytes from mice sensitized with inactive H46Q $sPLA_2$-X or leukocytes isolated from saline control mice in response to OVA restimulation (Figs. 5D-F). An exception was that the levels of IL-6 increased significantly following OVA restimulation when inactive H46Q $sPLA_2$-X was administered during the sensitization phase (Fig 5E, *P=0.04*). In contrast to the results with bvPLA2/OVA, we did not observe any differences in the levels of TNF or KC with either WT $sPLA_2$-X or H46Q $sPLA_2$-X at baseline or after antigen restimulation.

To further evaluate *ex vivo* cytokine production in immune cell populations, we stimulated splenocytes from these mice with OVA and analyzed cytokine production as with lung leukocytes above. Compared to lung leukocytes, production of cytokines by splenocytes in response to restimulation with OVA was attenuated, with no differences observed in IL-13, IL-10, IL-6 or IL-2 based on the type of sensitization received (Supplemental Fig. 3A-F); However, OVA restimulated splenocytes from mice sensitized with OVA plus $sPLA_2$-X produced significantly more IL-5 than splenocytes from mice sensitized with OVA plus the inactive H46Q $sPLA_2$-X or with saline control and there was a trend towards more IL-4 from splenocytes from mice sensitized with WT $sPLA_2$-X relative to inactive H46Q $sPLA_2$-X (Supplemental Figs. 3A**&**B). There were no differences in the levels of TNF or KC with either WT $sPLA_2$-X or H46Q $sPLA_2$-X. These findings suggest that $sPLA_2$-X activity is

essential for the type-2 skewed adaptive immune response to antigen and that these responses are markedly accentuated in the lung following 3 days of antigen rechallenge in the airways.

To assess the potential for $sPLA_2$-X adjuvant activity to elicit an adaptive immune response, we measured OVA-specific IgE and IgG using the same method as for the $bvPLA_2$ experiments. Both OVA-IgE and OVA-IgG were significantly elevated in the serum of mice sensitized with WT $sPLA_2$-X, a response that was completely abrogated in mice sensitized with the H46Q mutant form of $sPLA_2$-X (Figs. 6A&B). The OVA-specific IgE and IgG did not differ between the saline controls and those sensitized in the presence of H46Q mutant $sPLA_2$-X. It should also be noted that the absolute levels of the antigen-specific immunoglobins were substantially higher than in the studies with $bvPLA_2$. These data show that the adjuvant properties of $sPLA_2$-X that drive activation of both the innate and adaptive immune responses are dependent on the enzymatic activity of $sPLA_2$-X.

Enzymatic activity of $sPLA_2$-X is not necessary for the leukocyte influx into the peritoneum following intraperitoneal administration.

Prior work using $bvPLA_2$ demonstrated dependence upon ILC2s during the sensitization process and also identified release of IL-33 in response to this enzyme both *in vivo* and *ex vivo* (16). Previously, we found that mice lacking $sPLA_2$-X/*Pla2g10* have lower levels of IL-33 in the airways following sensitization and challenge with HDM (15). To further characterize this response and examine the dependence upon enzymatic activity, we performed intraperitoneal (i.p.) injections of WT $sPLA_2$-X or saline on three consecutive days and evaluated the number and activation state of ILC2s in the peritoneum (**Model**, Fig. 7A). From the peritoneal lavage, we found a significant leukocyte influx induced by $sPLA_2$-X (Fig. 7B) that included a modest increase in ILC2s (Fig. 7C), but a more notable increase in the number of ILC2s that expressed both CD25 and the IL-33 transmembrane receptor ST2 (Fig. 7D) as well as the total number of ILC2s that expressed ST2 (Fig. 7E). We repeated this experiment with both the WT $sPLA_2$-X and the inactive H46Q $sPLA_2$-X and confirmed that the active enzyme induced a cellular influx into the peritoneum ($P=0.03$), but this cellular influx did not differ from that of the inactive enzyme ($P = 0.74$). Similarly, the number of viable CD45+ leukocytes did not differ between the active and inactive enzymes by flow cytometry (Fig. 7F) and there were similar increases in ILC2s expressing CD25 and ST2 (Fig. 7G) as well as the total number of ILC2s that expressed ST2 (Fig. 7H) as we had seen with the experiments using saline or the active enzyme and the enzymatic activity did not appear essential for this response. We also measured the level of IL-33 in the peritoneum following sequential injections of both the WT and H46Q $sPLA_2$-X and found that the levels of IL-33 were below the level of detection of the assay. We also assess the numbers of cells in the BAL fluid and the lung following multiple i.p. injections of the WT $sPLA_2$-X enzyme without challenging the airways with antigen and found no evidence of a cellular influx in either the lung or BAL fluid, including no notable differences in the cellular differential by flow cytometry (data not shown). These results suggest that enzymatic activity of $sPLA_2$-X is not necessary for the generation of a cellular influx in the peritoneum or for activation of ILC2s, suggesting that receptor mediated effects may be involved.

Discussion

In the present study we used two different small extracellular PLA_2s to examine the potential adjuvant effects of PLA_2 enzymes focusing on the possibility that these enzymes serve to facilitate the development of airway inflammation and AHR following the subsequent inhalation of an allergen that itself does not have significant intrinsic adjuvant activity. This work is particularly relevant to human asthma as our lab has identified a specific endogenous $sPLA_2$ ($sPLA_2$-X) that is increased in human asthma (11–13, 15) and plays a central role in both the innate and adaptive immune response in mouse models of asthma (14, 15, 24). We used $bvPLA_2$ initially because a recent study demonstrated that in the periphery, $bvPLA_2$ serves as an effective adjuvant leading to an antigen specific immune response in the hind leg (16). We extend these findings here to examine whether such peripheral sensitization leads to an immune response relevant to asthma in the lung when the antigen is subsequently inhaled. We found that both $bvPLA_2$ as well as a recombinant version of endogenous $sPLA_2$-X serve as effective adjuvants in the periphery leading to a type-2 skewed immune response in the lung after re-exposure to antigen in the lung, although the severity of the immune response and AHR appeared to be greater when $sPLA_2$-X was used as the adjuvant. Through the use of a recombinant mutant $sPLA_2$-X enzyme with a targeted substitution in the active site that renders it catalytically inactive (17), we demonstrated that the adaptive immune response to the antigen in the lung, including the antigen-specific generation of cytokines by lung leukocytes and allergen-specific IgE were dependent upon the enzymatic activity of the $sPLA_2$, while features of AHR as well as some aspects of leukocyte trafficking to the lung were not dependent upon the enzymatic activity and are more likely a consequence of receptor-mediated actions. Further, the generation of ILC2s expressing markers of activation in the peritoneum could be induced by either the active or inactive enzyme. Another possibility is that residual endotoxin in the reagents led to the non-enzymatic features of inflammation, but this is unlikely given the low levels of endotoxin in the reagents.

Both $bvPLA_2$ and $sPLA_2$-X cleave the *sn2* position of phospholipids derived from cellular membranes and other sources such as surfactant phospholipids leading to the release of FFAs and LysoPLs. The *sn2* position is particularly important because it is preferentially substituted with PUFAs including AA that lead to the generation of eicosanoids. Through the use of an inactive mutant $sPLA_2$-X that maintains the secondary enzymatic structure of the enzyme, but is devoid of PLA_2 activity, we demonstrated with certainty that the adaptive immune responses facilitated by the $sPLA_2$-X enzyme was dependent upon the catalytic activity of the enzyme. We found that although AHR was not entirely dependent upon the enzymatic activity, the generation of an adaptive immune response to inhaled antigen was dependent upon enzymatic activity during the sensitization phase, leading to the recruitment of leukocytes to the lung that responded to antigen in an allergen-specific manner with the generation of key cytokines by lung leukocytes. Additionally, sensitization in the presence of either $bvPLA_2$ or WT $sPLA_2$-X caused the same features of the adaptive immune response including the allergen-specific generation of cytokines by lung leukocytes and allergen-specific IgE, although it is notable that the intensity of the immune response was greater for $sPLA_2$-X, particularly the higher levels of antigen-induced IL-13 in lung leukocytes and

allergen-specific immunoglobins. It is also notable that while there was a clear signal that the immune response was skewed towards a type-2 response, there were also allergen-induced generation of cytokines that are not classically type-2 including IL-2, IL-10, and IL-6 for both $sPLA_2$s, as well as an increase in both TNF and KC following the administration of $bvPLA_2$ during the sensitization phase. It should also be noted that the antigen-induced levels of IL-5 were particularly elevated in both models and antigen-induced increases in splenocytes could be seen following sensitization in the presence of $sPLA_2$-X. Lung leukocytes were thus primed in this context to generate many different cytokines in an allergen-specific manner since OVA elicits very little immune response in the absence of adjuvant-driven sensitization. Our data also demonstrates that while sensitization leads to antigen-induced production of IL-5 in the lung and spleen and was dependent upon active $sPLA_2$-X, the antigen-specific generation of IL-13 only occurred in the lung and was dependent upon the enzymatic activity of $sPLA_2$-X.

Our results demonstrate a significant dissociation between tissue leukocyte infiltration and leukocytes in the BAL fluid that tend to track closely with peri-airway inflammation in our prior studies (15, 19). The changes in BAL leukocyte trafficking were also closely associated with the adaptive immune response that is dependent upon enzymatic activity of the $sPLA_2$. These results indicate that the response to a simple antigen such as OVA in the lung requires the generation of allergen-specific IgE that was facilitated by the enzymatic activity of either $bvPLA_2$ or $sPLA_2$-X leading to an airway centered immune response that is very similar to the findings in human asthma, with less of a difference in tissue infiltration with leukocytes. Because enzymatic activity was necessary for OVA-specific IgE, these findings are consistent with recent work revealing that IgE binding to the low affinity IgE receptor CD23 on the epithelium is necessary for efficient epithelial transcytosis of antigen (27). Another possibility is that efficient generation of high-affinity IgE mediated through specialized T cell subsets (28) is facilitated by a product of $sPLA_2$ activity in the periphery, altering the subsequent response in the airways through cells bearing the high-affinity IgE receptor (FcεRI).

These results are in contrast to the inhalation of complex antigens such as HDM that can induce features of airways disease in the absence of IgE (29), and specifically that C-type lectin binding of HDM proteins to dectin-2 during the effector phase is independent of IgE (30). We found here that the generation of antigen-specific IgG is dependent on the enzymatic activity of $sPLA_2$-X. Since we demonstrated previously that in the lung $sPLA_2$-X/*Pla2g10* expression and protein levels are elevated in response to inhaled HDM allergen, and that the generation of antigen-specific IgG is dependent on $sPLA_2$-X/*Pla2g10*, these results suggest that the induction of $sPLA_2$-X provides a mechanism for sensitization endogenous antigens such as HDM that may be dependent upon CysLTs (31, 32) and also the ability of complete antigens to assist in the sensitization to “incomplete” allergens such as OVA that require the presence of an adjuvant for effective allergenicity.

Although prior work with $bvPLA_2$ indicates that sensitization in the periphery is dependent upon IL-33 signaling through the ST2 receptor, our results indicate that the allergen-specific generation of IL-13 that occurs in the lung, but not in the spleen is dependent upon the enzymatic activity of $sPLA_2$-X, although the induction of activated ILC2s expressing the

ST2 receptor does not require this enzymatic activity. These results suggest that there are several PLA_2-mediated processes that lead to the subsequent response to inhaled allergen and that multiple different epithelial/structural cell-derived cytokines fully regulate type-2 immunity (33). Prior studies have identified PLA_2 products as important elements of the sensitization process relevant to the lung, particularly the sensitization to HDM that occurs in the absence of adjuvant, but is dependent upon the endogenous generation of CysLTs by C-type lectin binding of HDM proteins to dectin-2 in dendritic cells (32) and that sensitization is dependent upon the CysLT1 receptor (CysLT1R) in this context (31). For human $sPLA_2$-X, we have demonstrated that this enzyme serves as regulator of the generation of CysLTs by human eosinophils (34, 35), and in mice the deletion of $sPLA_2$-X/*Pla2g10* causes a marked reduction in the elevated levels of CysLTs found in murine models of asthma (14, 15). In addition to the generation of eicosanoids, PLA_2s also release other FFAs and LysoPLs. As inhaled proteolytic allergens leads to the generation of IL-33 (15, 36), and the deletion of *Pla2g10* in this context leads to a marked reduction in IL-33 (15), these results suggest that signals in addition to CysLTs are involved. In fact, recent work demonstrates that FFAs act along with IL-33 to regulate the activation of ILC2s through FFA-receptor-1 (37). Thus, while sensitization in the periphery was dependent on signaling through ST2, leading to a subsequent and allergen specific T-cell response (16), the subsequent response in the lung appears more complex and requires additional PLA_2 related signals, particularly catalytic activity of the enzyme for the adaptive immune response and other factors that are not dependent upon the catalytic activity that serve to regulate the development of AHR.

Although both enzymes used in this study have PLA_2 enzymatic activity, $bvPLA_2$ enzymatically is most like the mammalian group III $sPLA_2$ ($sPLA_2$-III) enzyme; however, the secondary structures of $bvPLA_2$ and $sPLA_2$-III are divergent (26). In our study, we found that the adaptive immune response to inhaled allergen could be facilitated by either enzyme, but there were differences as either active or inactive $sPLA_2$-X caused a degree of lung leukocyte infiltration, while $bvPLA_2$ caused much less of an alteration in lung leukocytes. With regard to $sPLA_2$-X, the murine enzyme binds with high affinity to the “M-type” PLA_2 receptor Pla2r1, which is a paralog of the mannose receptor (CD206) and has reported signaling functions as well as serving as a soluble receptor that serves to decrease the levels of several different $sPLA_2$ (19, 25, 38). Functions attributed to Pla2r1 include cell proliferation (39), vascular smooth muscle contraction (40) and activation of the mitogen activated kinase (MAPK) cascade (41). We found that deletion of the *Pla2r1* gene leads to higher basal levels of $sPLA_2$-X, heightened AHR and increased levels of cytokines produced by lung leukocytes both with and without antigen stimulation, suggesting that Pla2r1 is functioning in the context of the OVA/alum model primarily to reduce the levels of specific $sPLA_2$s (19). In contrast, $bvPLA_2$ does not bind to Pla2r1 but binds to the “N-type” PLA_2 receptor which may be responsible for neurotoxicity (25, 42, 43) and also binds to the mannose receptor CD206 (44). The possibility that $bvPLA_2$ binds directly to the IL-33 receptor ST2 has been raised (26) based on the effects of $bvPLA_2$ in the periphery that are mediated through ST2 (16), but there has been no direct evidence of binding to this receptor.

In regard to the potential non-enzymatic functions of $sPLA_2$-X, we demonstrated that either active $sPLA_2$-X or the inactive H46Q mutant $sPLA_2$-X lead to both a cellular influx as well

as the activation of ILC2s in the peritoneum. The generation of ILC2s and release of IL-33 in the peritoneum was previously demonstrated for bvPLA$_2$, but the dependence on enzymatic activity was not ascertained (16). As the global deletion of sPLA$_2$-X/*Pla2g10* causes a reduction in both IL-33 and ILC2s in the lung (15), we examined the administration of sPLA$_2$-X on 3 subsequent days in the peritoneum. In contrast to the direct administration of IL-33 in the peritoneum (45), we did not observe any increase in lung leukocytes early after the administration of sPLA$_2$-X in the peritoneum. Thus, the majority of our results indicate that an enzymatic product is necessary for both the increase in antigen specific generation of IL-5 in the spleen and antigen specific generation of IL-4, IL-5, and IL-13 by lung leukocytes; however, sensitization with the inactive enzyme leads to the local generation of ILC2s in the peritoneum and subsequently a modest increase in lung eosinophil infiltration and features of AHR through a mechanism that is not dependent upon the catalytic activity of the enzyme.

Although bvPLA$_2$ was sufficient to act as an effective adjuvant leading to sensitization and an allergen specific immune response in the lung, it is known that bvPLA$_2$ also has suppressive effects when administered on a regular basis around the time of sensitization in the OVA/alum model, and that this effect is mediated through the mannose receptor CD206, leading to regulatory T-cell dependent suppression of OVA-induced airway dysfunction (46, 47). Remarkably, features of airway inflammation and AHR in the OVA/alum model were suppressed by the intratracheal administration of bvPLA$_2$ given 3 days subsequent to the sensitization doses of OVA/alum (48). Thus, bvPLA$_2$ is sufficient to induce an allergen specific immune response in the periphery (16), which leads to a subsequent airway-centered adaptive immune response in the lung, as we have shown here, while at the same time bvPLA$_2$ has a suppressive effect on inflammation acting directly through receptor binding to the mannose receptor CD206 (47). These considerations could explain the relatively attenuated immune response with bvPLA$_2$ relative to sPLA$_2$-X, or simply other differences in the adjuvant properties of the enzymes. The suppressive effects of bvPLA$_2$ are most well-established for the anaphylactic response to bvPLA$_2$, where the generation of IgE antibodies leads to suppression of the anaphylactic response to bee venom (16, 49). Our results would suggest that this direct antibody-mediated protection is most likely due to the enzymatic function of bvPLA$_2$, while more general suppressive functions may be receptor-mediated. Transgenic mice expressing the murine sPLA$_2$-III/*Pla2g3* which closely mirrors the enzymatic but not receptor binding function of bvPLA$_2$ spontaneously develop multiple features of inflammation including skin inflammation and lymphoid aggregates in the airway as well as mucus hypersecretion in the airway (50), suggesting as we have found here that the catalytic activity of bvPLA$_2$ facilitates an antigen-specific response in the lung. Earlier work demonstrated that bvPLA$_2$ causes the generation of leukotrienes from peripheral blood basophils (51) as we have demonstrated for human sPLA$_2$-X acting on eosinophils (34, 35). To our knowledge, no suppressive functions have been directly attributed to sPLA$_2$-X through receptor-mediated functions; however, we have provided evidence that the receptor that it binds (Pla2r1) has suppressive functions primarily through binding and removing sPLA$_2$-X (19).

Our results indicate that the endogenous murine sPLA$_2$-X enzyme plays a role in the sensitization phase of allergen-induced airway immunopathology. Sensitization with either

this enzyme or bvPLA$_2$ was sufficient to induce an adaptive immune response in the lung upon the subsequent inhalation of allergen. These results indicate that both sPLA$_2$s have the capacity to act as an adjuvant relevant to the development of immunopathology in the lung. The adaptive immune response and the antigen-induced generation of IL-13 in the lung was dependent on the catalytic activity of the enzyme, but the results also suggest receptor-mediated effects can alter some aspects of the innate immune response and AHR. As endogenous sPLA$_2$-X is increased in human asthma, can be upregulated at mucosal surfaces and is upregulated in the skin during inflammatory responses (52), these results have important implications for the underlying etiology of allergen-induced asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We are grateful for assistance of Farideh Ghomashchi for the preparation of the recombinant proteins and the thoughtful advice of Dr. Gerard Lambeau during the development of this work.

**Funding: NIH Grants R01HL089215, K24AI130263, U19AI125378, and F32HL134217

References

1. Murakami M, Nakatani Y, Atsumi GI, Inoue K, and Kudo I 2017 Regulatory Functions of Phospholipase A2. Crit Rev Immunol 37: 121–179. [PubMed: 29773019]
2. Murakami M 2019 Novel functions of phospholipase A2s: Overview. Biochim Biophys Acta Mol Cell Biol Lipids 1864: 763–765. [PubMed: 30769093]
3. Dennis EA, Cao J, Hsu YH, Magrioti V, and Kokotos G 2011 Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. Chemical reviews 111: 6130–6185. [PubMed: 21910409]
4. Aloulou A, Ali YB, Bezzine S, Gargouri Y, and Gelb MH 2012 Phospholipases: an overview. Methods in molecular biology (Clifton, N.J.) 861: 63–85.
5. Murakami M, Taketomi Y, Miki Y, Sato H, Yamamoto K, and Lambeau G 2014 Emerging roles of secreted phospholipase A2 enzymes: the 3rd edition. Biochimie 107 Pt A: 105–113. [PubMed: 25230085]
6. Jemel I, Ii H, Oslund RC, Payre C, Dabert-Gay AS, Douguet D, Chargui K, Scarzello S, Gelb MH, and Lambeau G 2011 Group X secreted phospholipase A2 proenzyme is matured by a furin-like proprotein convertase and releases arachidonic acid inside of human HEK293 cells. The Journal of biological chemistry 286: 36509–36521. [PubMed: 21878635]
7. Nolin JD, Murphy RC, Gelb MH, Altemeier WA, Henderson WR Jr., and Hallstrand TS 2019 Function of secreted phospholipase A2 group-X in asthma and allergic disease. Biochim Biophys Acta Mol Cell Biol Lipids 1864: 827–837. [PubMed: 30529275]
8. Bowton DL, Seeds MC, Fasano MB, Goldsmith B, and Bass DA 1997 Phospholipase A2 and arachidonate increase in bronchoalveolar lavage fluid after inhaled antigen challenge in asthmatics. American journal of respiratory and critical care medicine 155: 421–425. [PubMed: 9032172]
9. Chilton FH, Averill FJ, Hubbard WC, Fonteh AN, Triggiani M, and Liu MC 1996 Antigen-induced generation of lyso-phospholipids in human airways. The Journal of experimental medicine 183: 2235–2245. [PubMed: 8642333]
10. Stadel JM, Hoyle K, Naclerio RM, Roshak A, and Chilton FH 1994 Characterization of phospholipase A2 from human nasal lavage. American journal of respiratory cell and molecular biology 11: 108–113. [PubMed: 8018333]

11. Hallstrand TS, Chi EY, Singer AG, Gelb MH, and Henderson WR Jr. 2007 Secreted phospholipase A2 group X overexpression in asthma and bronchial hyperresponsiveness. American journal of respiratory and critical care medicine 176: 1072–1078. [PubMed: 17901411]
12. Hallstrand TS, Lai Y, Ni Z, Oslund RC, Henderson WR Jr., Gelb MH, and Wenzel SE 2011 Relationship between levels of secreted phospholipase A(2) groups IIA and X in the airways and asthma severity. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology 41: 801–810. [PubMed: 21255140]
13. Hallstrand TS, Lai Y, Altemeier WA, Appel CL, Johnson B, Frevert CW, Hudkins KL, Bollinger JG, Woodruff PG, Hyde DM, Henderson WR Jr., and Gelb MH 2013 Regulation and function of epithelial secreted phospholipase A2 group X in asthma. American journal of respiratory and critical care medicine 188: 42–50. [PubMed: 23614662]
14. Henderson WR Jr., Chi EY, Bollinger JG, Tien YT, Ye X, Castelli L, Rubtsov YP, Singer AG, Chiang GK, Nevalainen T, Rudensky AY, and Gelb MH 2007 Importance of group X-secreted phospholipase A2 in allergen-induced airway inflammation and remodeling in a mouse asthma model. The Journal of experimental medicine 204: 865–877. [PubMed: 17403936]
15. Nolin JD, Lai Y, Ogden HL, Manicone AM, Murphy RC, An D, Frevert CW, Ghomashchi F, Naika GS, Gelb MH, Gauvreau GM, Piliponsky AM, Altemeier WA, and Hallstrand TS 2017 Secreted PLA2 group X orchestrates innate and adaptive immune responses to inhaled allergen. JCI insight 2.
16. Palm NW, Rosenstein RK, Yu S, Schenten DD, Florsheim E, and Medzhitov R 2013 Bee venom phospholipase A2 induces a primary type 2 response that is dependent on the receptor ST2 and confers protective immunity. Immunity 39: 976–985. [PubMed: 24210353]
17. Shridas P, Bailey WM, Gizard F, Oslund RC, Gelb MH, Bruemmer D, and Webb NR 2010 Group X secretory phospholipase A2 negatively regulates ABCA1 and ABCG1 expression and cholesterol efflux in macrophages. Arterioscler Thromb Vasc Biol 30: 2014–2021. [PubMed: 20844270]
18. Scoville DK, Nolin JD, Ogden HL, An D, Afsharinejad Z, Johnson BW, Bammler TK, Gao X, Frevert CW, Altemeier WA, Hallstrand TS, and Kavanagh TJ 2019 Quantum dots and mouse strain influence house dust mite-induced allergic airway disease. Toxicol Appl Pharmacol 368: 55–62. [PubMed: 30682383]
19. Nolin JD, Ogden HL, Lai Y, Altemeier WA, Frevert CW, Bollinger JG, Naika GS, Kicic A, Stick SM, Lambeau G, Henderson WR Jr., Gelb MH, and Hallstrand TS 2016 Identification of Epithelial Phospholipase A2 Receptor 1 as a Potential Target in Asthma. American journal of respiratory cell and molecular biology 55: 825–836. [PubMed: 27448109]
20. Benjamini Y, Krieger A, and Yekutieli D 2006 Adaptive linear step-up false discovery rate controlling procedures. Biometrika 93: 491–507.
21. Lluis A, Depner M, Gaugler B, Saas P, Casaca VI, Raedler D, Michel S, Tost J, Liu J, Genuneit J, Pfefferle P, Roponen M, Weber J, Braun-Fahrlander C, Riedler J, Lauener R, Vuitton DA, Dalphin JC, Pekkanen J, von Mutius E, Schaub B, and Protection Against Allergy G: Study in Rural Environments Study. 2014 Increased regulatory T-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood. The Journal of allergy and clinical immunology 133: 551–559. [PubMed: 23993223]
22. Hessel EM, Zwart A, Oostveen E, Van Oosterhout AJ, Blyth DI, and Nijkamp FP 1995 Repeated measurement of respiratory function and bronchoconstriction in unanesthetized mice. Journal of applied physiology (Bethesda, Md. : 1985) 79: 1711–1716.
23. Janssen EM, van Oosterhout AJ, Nijkamp FP, van Eden W, and Wauben MH 2000. The efficacy of immunotherapy in an experimental murine model of allergic asthma is related to the strength and site of T cell activation during immunotherapy. Journal of immunology (Baltimore, Md. : 1950) 165: 7207–7214.
24. Henderson WR Jr., Oslund RC, Bollinger JG, Ye X, Tien YT, Xue J, and Gelb MH 2011 Blockade of human group X secreted phospholipase A2 (GX-sPLA2)-induced airway inflammation and hyperresponsiveness in a mouse asthma model by a selective GX-sPLA2 inhibitor. The Journal of biological chemistry 286: 28049–28055. [PubMed: 21652694]
25. Lambeau G, and Lazdunski M 1999 Receptors for a growing family of secreted phospholipases A2. Trends Pharmacol Sci 20: 162–170. [PubMed: 10322502]

26. Lee G, and Bae H 2016 Bee Venom Phospholipase A2: Yesterday's Enemy Becomes Today's Friend. Toxins (Basel) 8: 48. [PubMed: 26907347]
27. Palaniyandi S, Liu X, Periasamy S, Ma A, Tang J, Jenkins M, Tuo W, Song W, Keegan AD, Conrad DH, and Zhu X 2015 Inhibition of CD23-mediated IgE transcytosis suppresses the initiation and development of allergic airway inflammation. Mucosal Immunol 8: 1262–1274. [PubMed: 25783969]
28. Gowthaman U, Chen JS, Zhang B, Flynn WF, Lu Y, Song W, Joseph J, Gertie JA, Xu L, Collet MA, Grassmann JDS, Simoneau T, Chiang D, Berin MC, Craft JE, Weinstein JS, Williams A, and Eisenbarth SC 2019 Identification of a T follicular helper cell subset that drives anaphylactic IgE. Science 365.
29. McKnight CG, Jude JA, Zhu Z, Panettieri RA Jr., and Finkelman FD 2017 House Dust Mite-Induced Allergic Airway Disease Is Independent of IgE and FcepsilonRIalpha. American journal of respiratory cell and molecular biology 57: 674–682. [PubMed: 28700253]
30. Parsons MW, Li L, Wallace AM, Lee MJ, Katz HR, Fernandez JM, Saijo S, Iwakura Y, Austen KF, Kanaoka Y, and Barrett NA 2014 Dectin-2 regulates the effector phase of house dust mite-elicited pulmonary inflammation independently from its role in sensitization. Journal of immunology (Baltimore, Md. : 1950) 192: 1361–1371.
31. Barrett NA, Rahman OM, Fernandez JM, Parsons MW, Xing W, Austen KF, and Kanaoka Y 2011 Dectin-2 mediates Th2 immunity through the generation of cysteinyl leukotrienes. The Journal of experimental medicine 208: 593–604. [PubMed: 21357742]
32. Barrett NA, Maekawa A, Rahman OM, Austen KF, and Kanaoka Y 2009 Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells. Journal of immunology (Baltimore, Md. : 1950) 182: 1119–1128.
33. Van Dyken SJ, Nussbaum JC, Lee J, Molofsky AB, Liang HE, Pollack JL, Gate RE, Haliburton GE, Ye CJ, Marson A, Erle DJ, and Locksley RM 2016 A tissue checkpoint regulates type 2 immunity. Nature immunology 17: 1381–1387. [PubMed: 27749840]
34. Hallstrand TS, Lai Y, Hooper KA, Oslund RC, Altemeier WA, Matute-Bello G, and Gelb MH 2016 Endogenous secreted phospholipase A2 group X regulates cysteinyl leukotrienes synthesis by human eosinophils. The Journal of allergy and clinical immunology 137: 268–277 e268. [PubMed: 26139511]
35. Lai Y, Oslund RC, Bollinger JG, Henderson WR Jr., Santana LF, Altemeier WA, Gelb MH, and Hallstrand TS 2010 Eosinophil cysteinyl leukotriene synthesis mediated by exogenous secreted phospholipase A2 group X. The Journal of biological chemistry 285: 41491–41500. [PubMed: 20974857]
36. Hara K, Iijima K, Elias MK, Seno S, Tojima I, Kobayashi T, Kephart GM, Kurabayashi M, and Kita H 2014 Airway uric acid is a sensor of inhaled protease allergens and initiates type 2 immune responses in respiratory mucosa. Journal of immunology (Baltimore, Md. : 1950) 192: 4032–4042.
37. Yamaguchi M, Zacharia J, Laidlaw TM, and Balestrieri B 2016 PLA2G5 regulates transglutaminase activity of human IL-4-activated M2 macrophages through PGE2 generation. J Leukoc Biol 100: 131–141. [PubMed: 26936936]
38. Lambeau G, Schmid-Alliana A, Lazdunski M, and Barhanin J 1990 Identification and purification of a very high affinity binding protein for toxic phospholipases A2 in skeletal muscle. The Journal of biological chemistry 265: 9526–9532. [PubMed: 2160984]
39. Arita H, Hanasaki K, Nakano T, Oka S, Teraoka H, and Matsumoto K 1991 Novel proliferative effect of phospholipase A2 in Swiss 3T3 cells via specific binding site. The Journal of biological chemistry 266: 19139–19141. [PubMed: 1918029]
40. Kanemasa T, Arimura A, Kishino J, Ohtani M, and Arita H 1992 Contraction of guinea pig lung parenchyma by pancreatic type phospholipase A2 via its specific binding site. FEBS Lett 303: 217–220. [PubMed: 1607021]
41. Kinoshita E, Handa N, Hanada K, Kajiyama G, and Sugiyama M 1997 Activation of MAP kinase cascade induced by human pancreatic phospholipase A2 in a human pancreatic cancer cell line. FEBS Lett 407: 343–346. [PubMed: 9175881]

42. Lambeau G, Barhanin J, Schweitz H, Qar J, and Lazdunski M 1989 Identification and properties of very high affinity brain membrane-binding sites for a neurotoxic phospholipase from the taipan venom. The Journal of biological chemistry 264: 11503–11510. [PubMed: 2544597]

43. Nicolas JP, Lin Y, Lambeau G, Ghomashchi F, Lazdunski M, and Gelb MH 1997 Localization of structural elements of bee venom phospholipase A2 involved in N-type receptor binding and neurotoxicity. The Journal of biological chemistry 272: 7173–7181. [PubMed: 9054413]

44. Mukhopadhyay A, and Stahl P 1995 Bee venom phospholipase A2 is recognized by the macrophage mannose receptor. Arch Biochem Biophys 324: 78–84. [PubMed: 7503563]

45. Kondo Y, Yoshimoto T, Yasuda K, Futatsugi-Yumikura S, Morimoto M, Hayashi N, Hoshino T, Fujimoto J, and Nakanishi K 2008 Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. Int Immunol 20: 791–800. [PubMed: 18448455]

46. Choi MS, Park S, Choi T, Lee G, Haam KK, Hong MC, Min BI, and Bae H 2013 Bee venom ameliorates ovalbumin induced allergic asthma via modulating CD4+CD25+ regulatory T cells in mice. Cytokine 61: 256–265. [PubMed: 23121887]

47. Park S, Baek H, Jung KH, Lee G, Lee H, Kang GH, Lee G, and Bae H 2015 Bee venom phospholipase A2 suppresses allergic airway inflammation in an ovalbumin-induced asthma model through the induction of regulatory T cells. Immun Inflamm Dis 3: 386–397. [PubMed: 26734460]

48. Jung KH, Baek H, Shin D, Lee G, Park S, Lee S, Choi D, Kim W, and Bae H 2016 Protective Effects of Intratracheally-Administered Bee Venom Phospholipase A2 on Ovalbumin-Induced Allergic Asthma in Mice. Toxins (Basel) 8.

49. Marichal T, Starkl P, Reber LL, Kalesnikoff J, Oettgen HC, Tsai M, Metz M, and Galli SJ 2013 A beneficial role for immunoglobulin E in host defense against honeybee venom. Immunity 39: 963–975. [PubMed: 24210352]

50. Sato H, Taketomi Y, Isogai Y, Masuda S, Kobayashi T, Yamamoto K, and Murakami M 2009 Group III secreted phospholipase A2 transgenic mice spontaneously develop inflammation. Biochem J 421: 17–27. [PubMed: 19371233]

51. Mustafa FB, Ng FS, Nguyen TH, and Lim LH 2008 Honeybee venom secretory phospholipase A2 induces leukotriene production but not histamine release from human basophils. Clin Exp Immunol 151: 94–100. [PubMed: 18005261]

52. Haas U, Podda M, Behne M, Gurrieri S, Alonso A, Furstenberger G, Pfeilschifter J, Lambeau G, Gelb MH, and Kaszkin M 2005 Characterization and differentiation-dependent regulation of secreted phospholipases A in human keratinocytes and in healthy and psoriatic human skin. J Invest Dermatol 124: 204–211. [PubMed: 15654975]

Key Points

1. $sPLA_2$-X serves as an effective adjuvant for a T2 immune response in the lungs.
2. The adaptive immune response requires the enzymatic activity of $sPLA_2$-X.
3. The innate immune response is not fully dependent upon $sPLA_2$-X enzymatic activity.

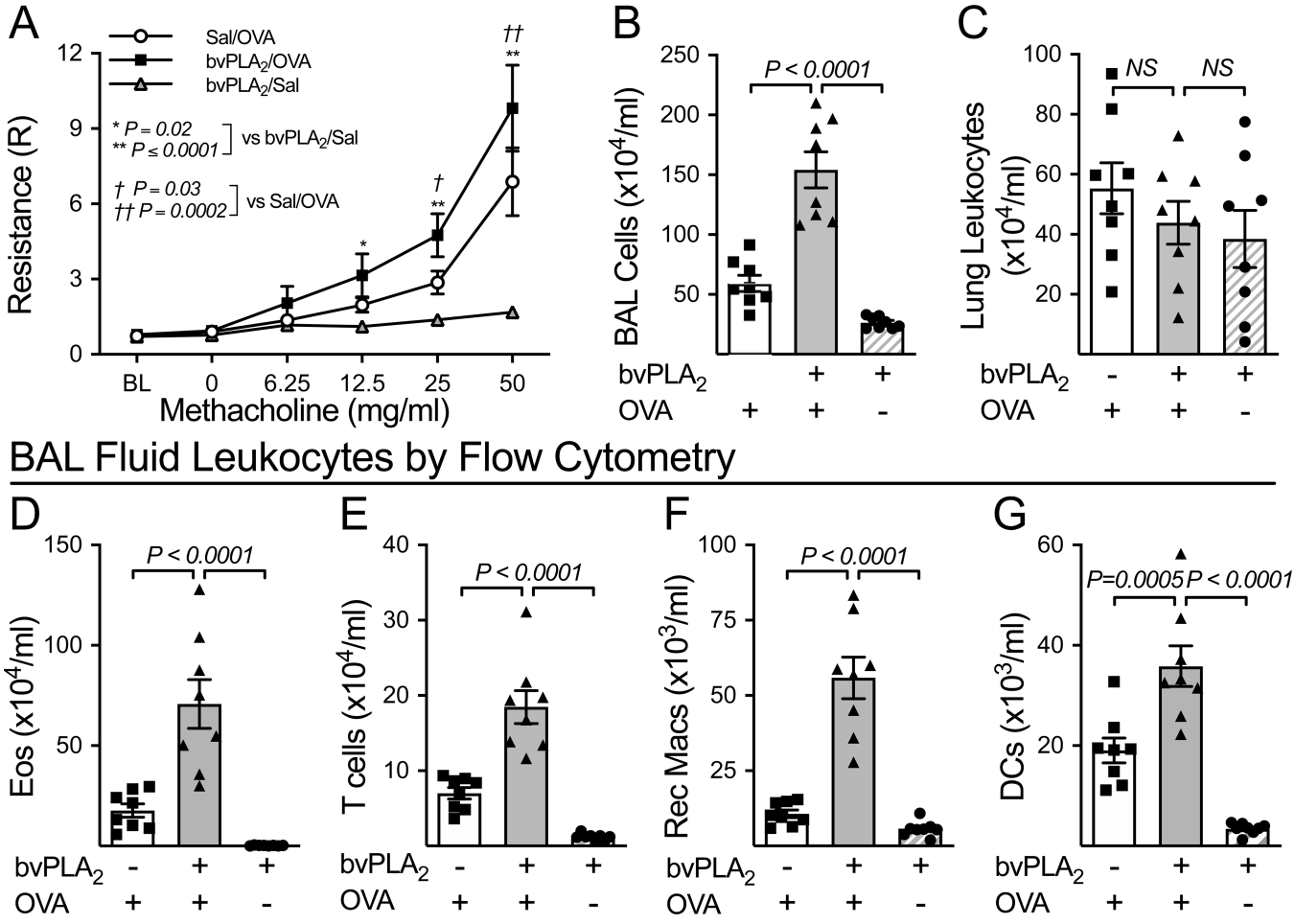

FIGURE 1.

Bee venom PLA_2 ($bvPLA_2$) serves as an effective adjuvant for a type-2 inflammation and airway hyperresponsiveness. (**A**) Sensitization to OVA in the presence of $bvPLA_2$ ($bvPLA_2$/OVA) followed by OVA challenge caused increased airway hyperresponsiveness (AHR) in response to progressive concentrations of methacholine compared to the administration of OVA in the absence of an adjuvant during the sensitization phase (Sal/OVA) and relative to $bvPLA_2$ alone with no antigen ($bvPLA_2$/Sal). (**B**) The concentration of BAL cells was increased when $bvPLA_2$ was administered during the sensitization phase followed by re-exposure to OVA in the airways. (**C**) In contrast, the concentration of CD45+ leukocytes in the lung did not differ between the groups. (**D-G**) The concentrations of eosinophils (**D**), T cells (**E**), recruited macrophages (**F**, Rec Macs), and dendritic cells (**G**, DCs) assessed by multicolor flow cytometry were all increased in the BAL fluid when $bvPLA_2$ was administered during the sensitization phase along with the antigen OVA. The data are represented as the mean +/− SEM. There were 2 experimental replicates with n=4 mice per group for a total of 8 mice in each group. The reported *P values* were corrected for multiple comparisons as described in the statistical methods.

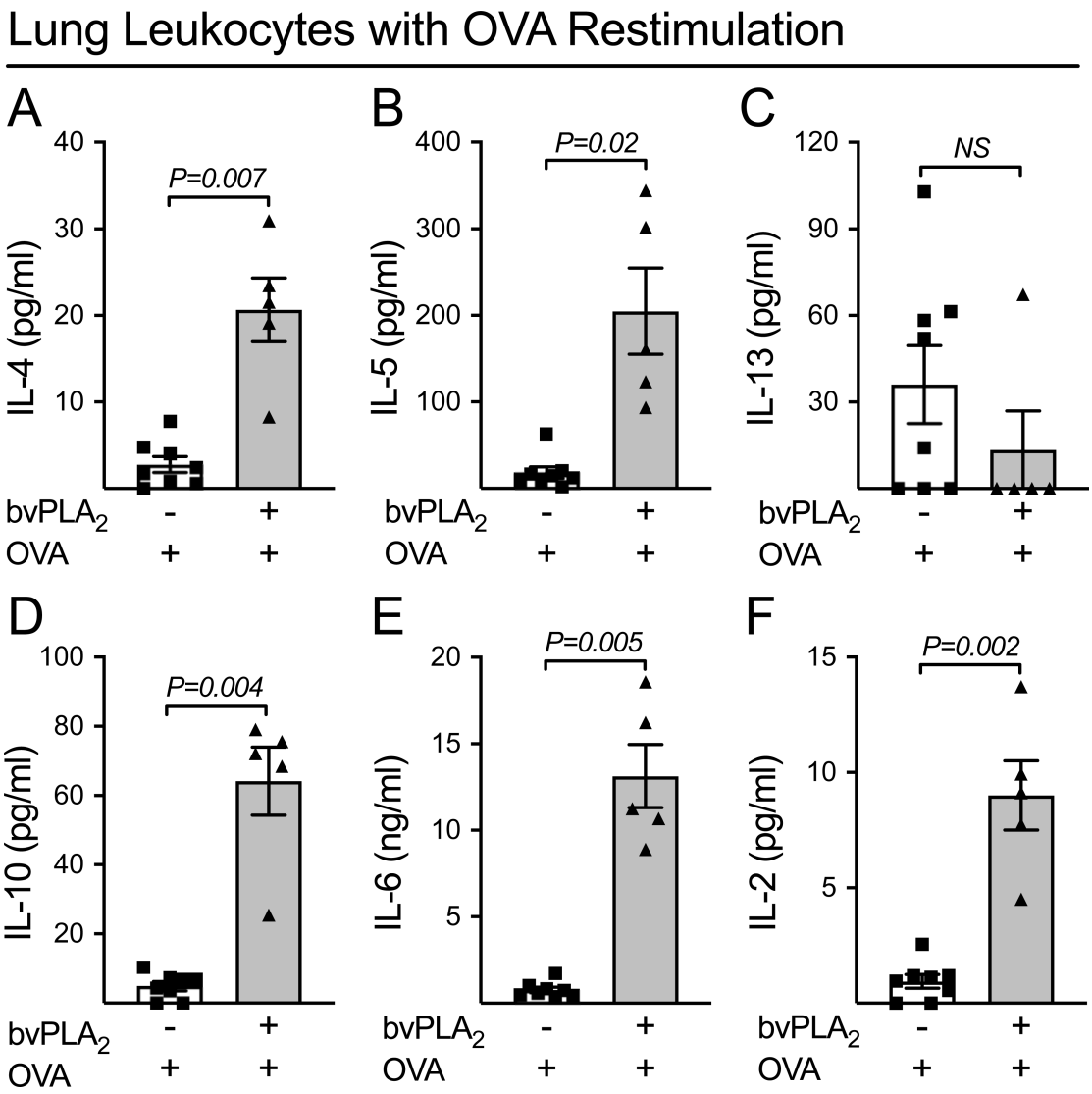

FIGURE 2.

Cytokine production by lung leukocytes in response to OVA restimulation is increased when bvPLA$_2$ was administered during the sensitization phase. (**A-C**) Cytokine production following *in vitro* OVA restimulation of CD45+ lung leukocytes following sensitization and challenge had increased production of the type-2 cytokines IL-4 (**A**), IL-5 (**B**), but not IL-13 (**C**). (**D-F**) The production of cytokines that are not classically type-2 including IL-10 (**D**), IL-6 (**E**) and IL-2 (**F**) were also increased in mice that received bvPLA$_2$ during sensitization. The data are represented as the mean +/− SEM. There were 8 lung samples in the control group that received OVA and 5 in the group that received bvPLA$_2$/OVA; *P values* represent the Welch's t test.

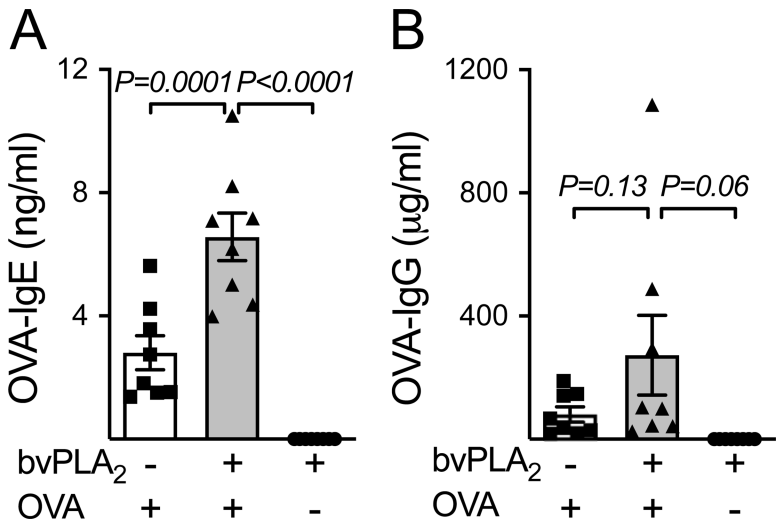

FIGURE 3.

The antigen-specific immune response is enhanced by bvPLA$_2$ during the sensitization phase. (A) OVA-specific IgE in serum following sensitization and challenge was significantly increased when bvPLA$_2$ was administered during the sensitization phase. (B) A similar trend was identified for the levels of OVA-specific IgG in this model. The data are represented as the mean +/− SEM. There were 8 samples in each group and the reported *P values* were corrected for multiple comparisons.

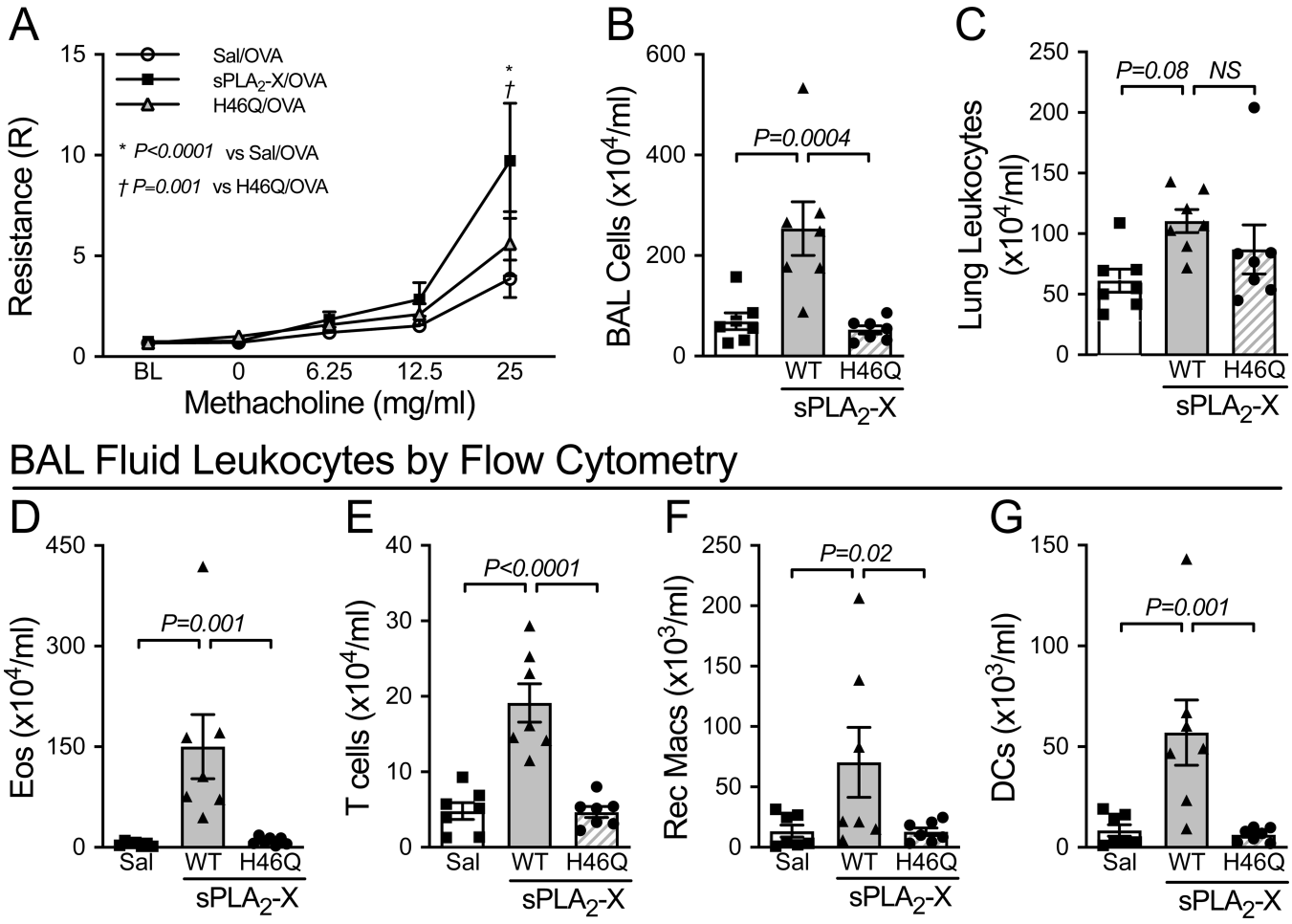

FIGURE 4.

The endogenous protein sPLA$_2$-X acts as an effective adjuvant for type-2 inflammation in the lung and the adaptive component of this responses is dependent upon the PLA$_2$ activity of this enzyme. (**A**) Sensitization to OVA in the presence of sPLA$_2$-X (sPLA$_2$-X/OVA) followed by OVA challenge caused increased airway hyperresponsiveness (AHR) in response to progressive concentrations of methacholine compared to the administration of OVA in the absence of an adjuvant during the sensitization phase (Sal/OVA). The administration of a catalytically inactive mutant sPLA$_2$-X (H46Q sPLA$_2$-X) in place of the wild type (WT) enzyme followed by OVA challenge (H46Q/OVA) also caused an increase in AHR relative to Sal/OVA. (**B**) In contrast, the concentration of BAL cells was increased only when catalytically active sPLA$_2$-X was administered during the sensitization phase followed by re-exposure to OVA in the airways. (**C**) The concentration of CD45+ leukocytes in the lung tended to increase in the lung when catalytically active sPLA$_2$-X was administered during the sensitization phase. (**D-G**) The concentrations of eosinophils (**D**), T cells (**E**), recruited macrophages (**F**, Rec Macs), and dendritic cells (**G**, DCs) assessed by multicolor flow cytometry were all increased in the BAL fluid when catalytically active sPLA$_2$-X was administered during the sensitization phase along with the antigen OVA. The data are represented as the mean +/− SEM. There were 2 experimental replicates with n=4 and n=3

mice per group for a total of 7 mice in each group and the reported *P values* were corrected for multiple comparisons as described in the statistical methods.

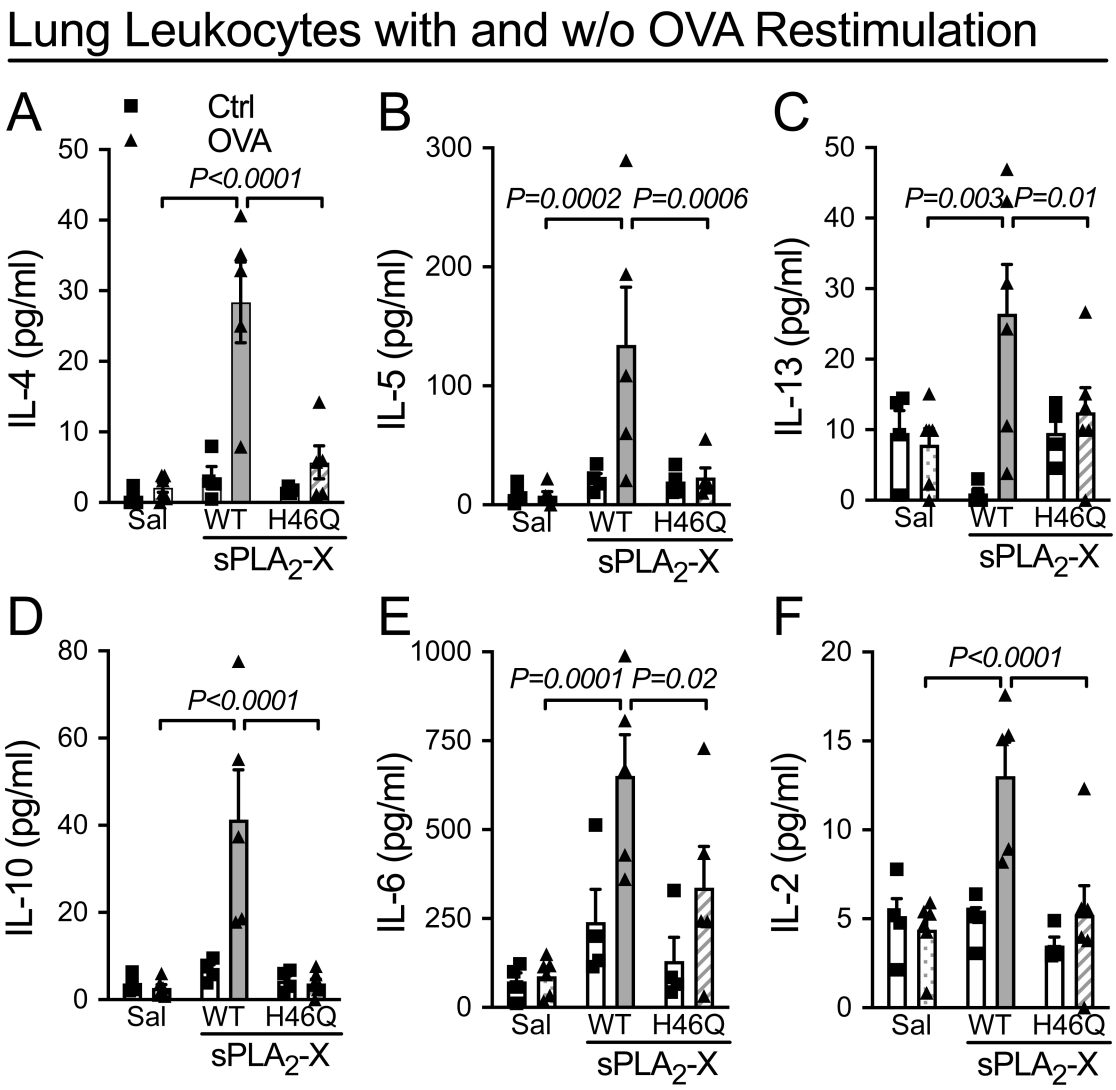

FIGURE 5.

The antigen-specific cytokine production by lung leukocytes following restimulation with OVA restimulation is increased primarily when catalytically active sPLA$_2$-X was administered during the sensitization phase. (**A-C**) Cytokine production following *in vitro* OVA restimulation of CD45+ lung leukocytes following sensitization and challenge revealed marked increases in the production of multiple different cytokines. Specifically, the administration of catalytically active sPLA$_2$-X during the sensitization phase caused the marked increase in the production of the type-2 cytokines IL-4 (**A**), IL-5 (**B**), and IL-13 (**C**) in the lung. (**D-F**) The production of cytokines that are not classically type-2 including IL-10 (**D**), IL-6 (**E**) and IL-2 (**F**) were also increased in mice that received catalytically active sPLA$_2$-X during the sensitization phase. The catalytically inactive mutant sPLA$_2$-X (H46Q sPLA$_2$-X) administered during the sensitization phase, also led to the subsequent increase in IL-6 following OVA restimulation (E, P =0.04). The data are represented as the mean +/− SEM. There were 4 lung samples that were incubated with saline from each of the groups

and 5–6 lung samples from each of the groups that were incubated with OVA; P values represent a 2-way ANOVA with correction for multiple comparisons.

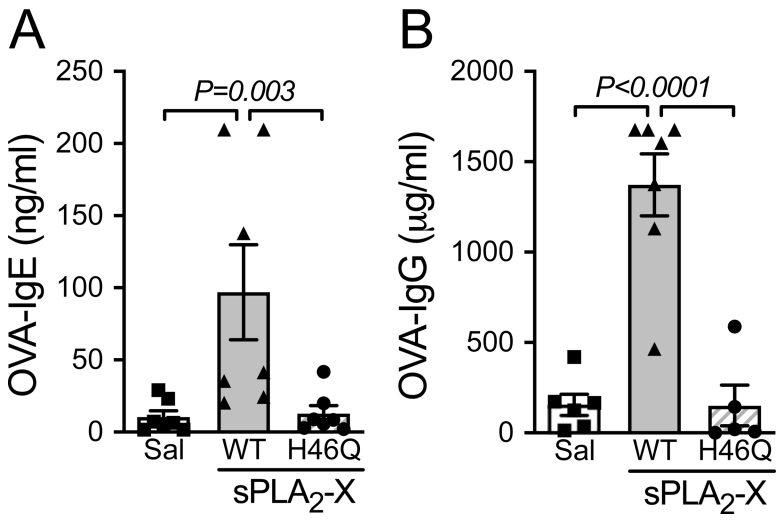

FIGURE 6.

The production of antigen-specific antibodies is increased when catalytically active $sPLA_2$-X was administered during the sensitization phase and is dependent upon the catalytic activity of the enzyme. OVA-specific IgE in serum following sensitization and challenge was significantly increased when catalytically active $sPLA_2$-X was administered during the sensitization phase, but not when catalytically inactive H46Q mutant $sPLA_2$-X was administered during the sensitization phase. (B) Catalytically active $sPLA_2$-X during the sensitization phase increased the levels of OVA-specific IgG in this model. The data are represented as the mean +/− SEM. There were 7 samples in each group for the OVA-specific IgE and 5–7 samples in each group for the OVA-specific IgG based on the availability of serum; the reported *P values* were corrected for multiple comparisons.

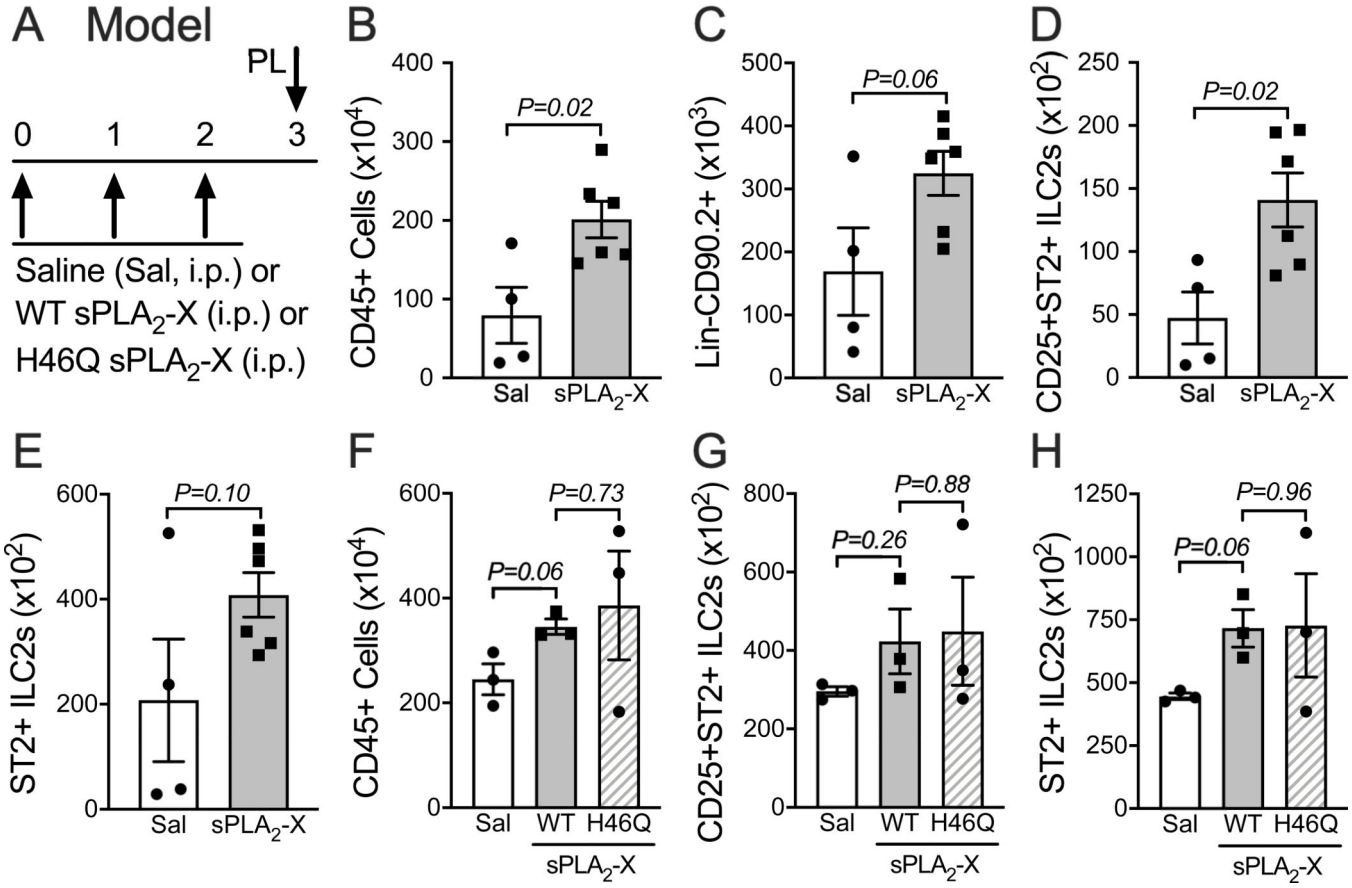

FIGURE 7.

Catalytically active sPLA$_2$-X and catalytically inactive H46Q mutant sPLA$_2$-X caused a cellular influx including activated ILC2s in the peritoneum. (**A**) Saline, active sPLA$_2$-X or catalytically inactive H46Q mutant sPLA$_2$-X were administered in the peritoneum (i.p.) on 3 subsequent days follow by peritoneal lavage (PL). (**B-E**) Flow cytometry comparing saline to wild type (WT) sPLA$_2$-X revealed an increase in the total number of CD45+ leukocytes (**B**), including an increase in the number of Lin-CD90.2+ type-2 innate lymphoid cells (ILC2)s (**C**), particularly ILC2s expressing both CD25 and ST2 (**D**), and total ILC2s expressing ST2 (**E**). (**F-H**) Further experimentation including the inactive H46Q mutant sPLA$_2$-X identified an increase in total CD45+ leukocytes that occurred in the absence of enzymatic activity (**F**), and similar trends for the number of ILC2s expressing both CD25 and ST2 (**G**), and total ILC2s expressing ST2 (**H**). There were 2 experimental replicates with n=2 saline and n=3 WT sPLA$_2$-X treated mice per group in the first experiment (**B-E**) and one experimental group with n=3 treated mice per group comparing saline, WT sPLA$_2$-X and H46Q mutant sPLA$_2$-X (**F-H**). The data are represented as the mean +/− SEM; *P values* represent either an unpaired t test (**B-E**) or a 2-way ANOVA(**F-H**).