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Function of Secreted Phospholipase A₂ Group-X in asthma and allergic disease

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Abstract

Elevated secreted phospholipase A₂ (sPLA₂) activity in the airways has been implicated in the pathogenesis of asthma and allergic disease for some time. The identity and function of these enzymes in asthma is becoming clear from work in our lab and others. We focused on sPLA₂ group X (sPLA₂-X) after identifying increased levels of this enzyme in asthma, and that it is responsible for a large portion of sPLA₂ activity in the airways and that the levels are strongly associated with features of airway hyperresponsiveness (AHR). In this review, we discuss studies that implicated sPLA₂-X in human asthma, and murine models that demonstrate a critical role of this enzyme as a regulator of type-2 inflammation, AHR and production of eicosanoids. We discuss the mechanism by which sPLA₂-X acts to regulate eicosanoids in leukocytes, as well as effects that are mediated through the generation of lysophospholipids and through receptor-mediated functions.

Introduction

Asthma is a disease with components of variable airflow obstruction, airway hyperresponsiveness (AHR) and structural remodeling of the airways that are linked to inflammation of the airways. Work to define the underlying basis of airway inflammation has identified components of both the innate and adaptive immune systems that are accentuated in asthma and biased towards a type-2 immune response, typified by production

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of the key type-2 cytokines, IL-4, IL-5, and IL-13. The precise underlying basis for this altered immune response in asthma is not fully understood; however, there is significant evidence that there are alterations in the biosynthesis of eicosanoids in asthma. These mediators are responsible for many of the clinical features of asthma, and can serve as central regulators of the immune response in asthma. A central discovery was the overproduction of a group of mediators collectively known as the cysteinyl leukotrienes (CysLT) that are over-produced in asthma, replicate many of the features of asthma, and can be targeted therapeutically. Work from our laboratories have revealed that CysLTs play a key role in the regulation of inflammation and structural remodeling in murine models of asthma [1-3], and that the increased production of CysLTs in asthma is linked to features of “indirect” AHR [4]. The identification of patient populations with increased production of CysLTs and other eicosanoids spurred further work in this area to identify upstream regulators of eicosanoid biosynthesis in asthma. This led to the identification of secreted PLA₂ (sPLA₂) group-X (sPLA₂-X) as a potential regulator of airway inflammation in asthma. In particular, our labs have shown that this enzyme is expressed in epithelial cells, macrophages and eosinophils in the airways [5, 6], is clearly elevated in the airway fluid of patients with asthma [2] and is linked to a number of features of asthma including AHR and asthma severity. Murine models with the deletion of this enzyme have revealed that sPLA₂-X serves as a central regulator of many of the features of asthma that are replicated in this model system [1, 2]. Using a small molecule inhibitor of the human sPLA₂-X, our work has revealed that sPLA₂-X serves as a key regulator of CysLT formation and eosinophil activation, and that such an inhibitor can be effective in a transgenic murine model of asthma where the human *PLA2G10* gene was substituted for the murine *Pla2g10*, identifying the therapeutic potential of the inhibition of this pathway.

Functions of secreted PLA₂s relevant to asthma

A central factor that contributes to the pathogenesis of asthma is the biosynthesis of eicosanoids in the airways [7], including cysteinyl leukotrienes (CysLT) and prostaglandins (PG). The limiting factor in eicosanoid biosynthesis is the release of free fatty acids, namely arachidonic acid (AA), from the *sn*2 position of membrane phospholipids through the action of phospholipase A₂ (PLA₂) enzymes [8]. In addition to the well-studied group IVA cytosolic PLA₂-alpha (cPLA₂α), there are 10 known secreted PLA₂s (sPLA₂) that have been described in humans and 11 in mice, and studies suggest that eicosanoid biosynthesis may involve the coordinated action of both cPLA₂α and sPLA₂s [9-11]. Individual sPLA₂s display unique specificity towards certain phospholipids, dictating the cellular or molecular target for each enzyme. Compared to other sPLA₂s, sPLA₂-X was shown to be the most efficient at releasing free fatty acids from mammalian cells due to its high affinity for phosphatidylcholine (PC) rich cell membranes [12-14]. This specificity also implicates sPLA₂-X in the degradation of pulmonary surfactant, as a large portion of endogenous surfactants contain PC (e.g. dipalmitoylphosphatidylcholine, DPPC) and can be a target of sPLA₂ activity [15]. Degradation of surfactants has a well-established role in lung injury [16, 17], and surfactant dysfunction can also contribute to loss of lung function in asthma, specifically through disruption of surface tension and resultant closure of peripheral airways [18-20]. Interestingly, *in vivo* overexpression of constitutively active sPLA₂-X in

macrophages leads to neonatal death due to severe pneumonia and surfactant dysfunction [21]. The alveolar macrophages in these animals contain copious amounts of surfactant, indicating that unrestrained sPLA₂-X activity has deleterious effects on lung function. In another study, transgenic expression of sPLA₂-V, but not sPLA₂-X, resulted in neonatal lethality due to degradation of lung surfactant and resultant severe lung dysfunction [22]. The lack of a phenotype following transgenic sPLA₂-X expression was attributed to the presence of a propeptide that requires enzymatic cleavage to generate the active enzyme, as active sPLA₂-X was only observed after the initiation of tissue inflammation where enzymatic cleavage and activation of sPLA₂-X occurred.

Further evidence supporting the likely role of sPLA₂-X in asthma was revealed following allergen challenge in both animal models and human subjects. We found that allergen challenge induced expression of the sPLA₂-X gene (mouse, *Pla2g10*; human, *PLA2G10*) and resulted in increased release of sPLA₂-X protein in the airways of both mice and humans [2, 5, 23, 24]. Moreover, our group has demonstrated the importance of sPLA₂-X in initiating CysLT synthesis in human eosinophils [6, 25], providing evidence that products from elevated sPLA₂-X activity in asthma feed into the eosinophil LTC₄ synthase pathway, increasing the CysLT pool in the lung. We have also shown that mice lacking the *Pla2g10* gene display blunted CysLT production in the airways following exposure to house dust mite (HDM) [2]. In asthma, CysLTs and PGD₂ are the primary eicosanoids implicated in disease pathogenesis and are also involved in promoting type-2 inflammation and immune cell activation [26-28]. Taken together, sPLA₂-X is a central regulator of eicosanoid biosynthesis in the lung and has emerged as a strong candidate for therapeutic intervention.

CysLTs were thought to play a major role in asthma early on, evidenced by their abundance in the airways of asthmatics and potent induction of smooth muscle contraction [29, 30]. Early studies showed that direct inhalational administration of LTC₄ or LTD₄ resulted in bronchoconstriction in both asthmatics and non-asthmatics [29, 31, 32]. While LTE₄ inhalation resulted in substantially less severe bronchoconstriction overall, it was more potent in asthmatics than healthy controls [33]. A study by Gauvreau and colleagues further demonstrated that airway challenge with LTE₄ but not LTD₄ led to cellular influx of eosinophils in the airways [34]. This persistent eosinophilia could be prevented using a CysLT1 receptor (CysLTR1) antagonist, zafirlukast, following inhalation of LTE₄ in mild asthma [35]. Despite these studies, the exact role of LTE₄ in asthma is still not well understood, as LTE₄ is a weak activator of both canonical CysLT receptors, CysLTR1 and CysLTR2. In fact, in mice lacking both CysLTR1 and CysLTR2, LTE₄ administration promotes airway inflammation and eosinophil influx via activation of the purinergic receptor, P2Y₁₂ [36]. Recent evidence also suggests that there is a third type of CysLT receptor (i.e. GPR99) that preferentially binds LTE₄ [37] and stimulates mast cell-dependent mucous release from airway epithelial cells [38]. The presence of additional receptors that are not well-studied might explain some of the heterogeneous results from leukotriene receptor antagonist therapy, which primarily targets CysLTR1, and offer an alternative avenue for intervention. PGD₂ signaling is dependent on two known receptors: D prostanoid receptor 1 (DP1) and DP2, also called chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) [39]. The major contribution of PGD₂ to asthma pathogenesis has been linked to enhanced eosinophil activation in transgenic mice

overexpressing PGD₂ synthase [26]. PGD₂-induced pro-inflammatory effects are largely governed by CRTH2, which is highly expressed in eosinophils, as well as Th2 cells and ILC2s [40-42]. Following PGD₂-dependent activation via CRTH2, Th2 cells and ILC2s produce abundant type 2 cytokines, a hallmark feature of allergic asthma [43-45]. The implication of CRTH2 in asthma made this a key target for therapeutic development; however, initial studies using CRTH2 antagonists to treat asthma have shown only modest effect [46]. While the involvement of PGD₂ in driving inflammation through CRTH2 is clear, the role of DP1 appears to be more distinct in activating sensory nerves. Evidence suggests that DP1 activation may be a key signal for bronchoconstriction and cough via activation of sensory neurons in the airways, and targeting this receptor may be effective in the setting of asthma [47]. These data highlight the central role of CysLTs and PGD₂ in the pathogenesis and exacerbation of asthma, and reveal the potential impact of targeting specific eicosanoids and their receptors, and the potential to target upstream regulators of eicosanoid production such as sPLA₂-X in asthma.

A consequence of phospholipid hydrolysis by sPLA₂s, including sPLA₂-X, is the release of lysophospholipids (LysoPL; e.g. lysophosphatidylcholine, LysoPC; lysophosphatidic acid, LysoPA), which are readily detected in human BAL fluid, and are elevated after allergen challenge [48]. Subsequent studies focused on understanding the specific function of LysoPL species have implicated LysoPLs in asthma and inflammation. For instance, LysoPLs can possess adjuvant-like properties to drive antigen sensitization [49] and the LysoPC-derived platelet activating factor (PAF) plays a critical role in inflammation and anaphylaxis [50, 51]. In asthmatics with impaired lung function, increased levels of bioactive forms of LysoPC (18:0, 16:0) were detected in bronchoalveolar lavage (BAL) fluid [52]. In vitro studies have revealed that the airway epithelium is a major source of LysoPC, as stimulation of cultured bronchial epithelial cells with growth factors led to increased release of bioactive forms of LysoPC [53]. Bansal and colleagues investigated the importance of LysoPC in a mouse model following exposure to cockroach extract and found that LysoPC promoted the maturation and recruitment of a subset of NKT cells to the airways, offering insights into the potential function of LysoPC in human asthma [54]. Another LysoPL byproduct from sPLA₂ activity, LysoPA, has both pro- and anti-inflammatory roles in the airways, acting to regulate cytokines and chemokines in the airway epithelium [55-57].

Of the 6 known LysoPA receptors (LPA_{i1-6}) in mice, LPA₂ has emerged as an important mediator of inflammation in the airways via activation of cell-protective signals. In LPA₂-deficient mice exposed to ovalbumin (OVA), airway inflammation and AHR were elevated compared to WT animals [58]. In addition, activation of LPA₂ signaling using a LPA₂ agonist protected against HDM-induced airway inflammation [59]. In contrast to these findings, two studies suggest that lack of LPA₂ confers resistance to the development of lung inflammation, including a study using a *Schistosoma* egg antigen model in LPA₂ heterozygous mice [60] and another study using the HDM, ragweed and *Aspergillus* model of allergic lung inflammation in LPA₂-deficient mice [61]. The etiology of these discrepant findings are uncertain, but may reflect differences in the model antigen used and the genetic background of animals in experiments, and dominance of pro- versus anti-inflammatory effects. As with other LysoPL species [48], LysoPA is detectable in the BAL fluid at

baseline and is elevated following segmental airway allergen challenge [62]. Relevant to asthma, LysoPA also induces expression and release of IL-13R alpha 2 (IL-13R α 2) in bronchial epithelial cells [63]. This receptor is shared by both IL-4 and IL-13 and is thought to act in part as a decoy receptor to mediate the extent of type-2 inflammation. The exact function of IL-13R α 2, however, may depend on its membrane-bound or soluble form. Overexpression of membrane-bound IL-13R α 2 in airway epithelial cells leads to increased eosinophil influx and lung inflammation following allergen challenge [64]. Interestingly, both soluble and membrane-bound forms of IL-13R α 2 are found in mice, but only the membrane-bound form can be found in humans [65, 66], highlighting the translational relevance of studies focusing on membrane-bound IL-13R α 2. While targeting downstream components of the type-2 immune response has proven to be successful in certain subsets of patients with asthma, it is intriguing to consider whether inhibiting the inflammatory cascade further upstream of eicosanoid formation (i.e. sPLA₂-X activity) may represent a more effective option for the treatment of asthma.

The expression of the sPLA₂-X gene is inducible in the airways, but production of functional enzyme requires additional modification at the post-translational level. Secretion of sPLA₂-X can occur via either the classical secretory pathway or through degranulation [67]. Similar to sPLA₂-IB, sPLA₂-X is translated as a zymogen and requires enzymatic cleavage of an N-terminal pro-peptide to generate an active, mature form of the enzyme [68]. The 11 amino acid N-terminal pro-peptide ends with a dibasic doublet [69], indicating that it is the target of a pro-protein convertase. Unlike sPLA₂-X, the highly active sPLA₂-V, which is found at high levels in airway macrophages, does not require proteolytic cleavage for activation [70]. Subsequent investigation discovered that maturation of the pro-sPLA₂-X can occur via the intracellular activity of a furin-like pro-protein convertase, and activation is likely differentially regulated depending on the disease state and the cellular source of the enzyme [71, 72]. Interestingly, Jemel and colleagues also note that secretion of the zymogen and subsequent extracellular activation of pro-sPLA₂-X by trypsin-like proteases cannot be ruled out [72]. This raises important questions about extracellular regulation of sPLA₂-X in settings of asthma, where proteolytic allergen exposure or elevated mast cell proteases in the airways of asthmatics may act to augment sPLA₂-X activity [73].

In addition to their enzymatic functions, sPLA₂s can also bind to receptors, including the C-type lectin receptor, PLA₂R1 [74]. PLA₂R1 is closely related to receptors in the C-type lectin superfamily, and is a paralog of the macrophage mannose receptor [75] which can bind sPLA₂s and potentially other carbohydrates [76]. PLA₂R1 plays a multifaceted role, as ligation by sPLA₂s has been implicated in both activation of cellular signaling events [77-79] and clearance of systemic sPLA₂s [74, 80, 81], depending on the presence of its membrane-bound or soluble form. Investigations into the binding specificity of mouse sPLA₂s to PLA₂R1 revealed a subset of sPLA₂s, including sPLA₂-IIA and sPLA₂-X, which are high affinity ligands [76]. This receptor-ligand interaction results in a decrease in enzymatic activity of the bound sPLA₂, further indicating the possible regulatory role of PLA₂R1 [76]. This is of particular importance, as sPLA₂-X appears to have a pathogenic role in asthma; thus, regulation of enzyme levels by PLA₂R1 may represent a critical means of controlling sPLA₂-directed inflammation. In support of this, our group has shown that the expression of this receptor is elevated in asthmatics, particularly in the airway epithelium

[82], and findings in animal models indicate a likely role for PLA₂R1 in regulating effects of sPLA₂ activity in asthma [82, 83]. Taken together, sPLA₂-X has several functions relevant to the immunopathogenesis of asthma and may represent a potential opportunity for therapeutic intervention.

Implication of sPLA₂-X in Human Asthma

Asthma is a complex disorder that has significant heterogeneity in the phenotypic manifestations of the disease as well as the underlying immunopathogenesis. The common phenotypic manifestations of asthma include variable airflow obstruction and symptoms of cough and shortness of breath that often occurs in response to specific triggers including allergens, respiratory viruses and exercise. In general, airway inflammation, secretion of gel forming mucins and structural remodeling of the airways contributes to the clinical features of asthma, but also occurs in other airway diseases. A highly specific feature of asthma is called indirect AHR, where a stimulus such as exercise or allergen exposure causes airway narrowing by virtue of release of endogenous mediators from the airways. Work from our lab and others demonstrates that patients with indirect AHR have increased levels of CysLTs [4]. In patients with indirect AHR, dry air exercise challenge induces airway narrowing called exercise-induced bronchoconstriction (EIB). This is accompanied by the release of eicosanoids, including CysLTs and PGD₂, that are mechanistically linked to the development of airway narrowing induced by exercise challenge [84, 85]. This phenotypic variation in the manifestations of asthma provides an excellent platform to further unravel the basis of dysregulated eicosanoid synthesis in asthma.

As we have discussed, dysregulated eicosanoid synthesis has been strongly implicated in the pathogenesis of asthma for many years, but the precise alterations leading to the accentuated production of eicosanoids in asthma is not fully understood. The rate-limiting step in eicosanoid generation is the release of AA by PLA₂-dependent hydrolysis of membrane phospholipids, feeding into both the cyclooxygenase (COX) and 5-lipoxygenase (5-LO) pathways. Although intracellular PLA₂s serve as key regulators of eicosanoids, these enzymes are not released into the extracellular space. It was noted early on that the airway lining fluid contains an enzyme that has activity consistent with a sPLA₂, but the identity of the enzymes responsible for this sPLA₂ activity were not ascertained. In addition to the release of AA, sPLA₂s also lead to the generation of LysoPL and can degrade surfactant phospholipids, contributing to airway dysfunction [15]. Initial studies noted that total sPLA₂ activity is elevated in the airways of asthmatics compared to healthy controls and that allergen challenge in both the upper and lower airways leads to an increase in the sPLA₂ activity accompanied by AA release and eicosanoids biosynthesis [48, 86]. These results suggested that a sPLA₂ can serve as a key checkpoint leading to the overproduction of eicosanoids (such as CysLTs) in asthma and can be upregulated following allergen or exercise challenge.

To further ascertain the identity of the specific sPLA₂ responsible for increased activity in asthma, we conducted studies on the expression level of both the secreted and cytosolic PLA₂s. We also obtained samples of airway cells from patients with asthma who displayed notable indirect AHR, as well as a healthy control group [5]. We further examined the

effects of exercise challenge in both of these groups and showed a marked alteration in the balance of eicosanoids favoring the production of CysLTs in asthmatics relative to PGE₂, which generally plays a protective role in the airways [87]. The results of screening based on gene expression and immunostaining of airway cells showed that several of the sPLA₂ enzymes, including sPLA₂-IB, -IIA, -IID, -IIF, -X and -XIIA, were expressed in airway cells although the expression of sPLA₂-IIE, -III, -V and -XIIB could not be detected at the level of PCR. A striking finding was that sPLA₂-X and sPLA₂-XIIA were expressed at high levels in the airways. Further comparisons between asthmatics and healthy controls using immunostaining of airway cells revealed that subjects with asthma have greater immunostaining for both sPLA₂-X and sPLA₂-XIIA (Fig 1). We also identified some immunostaining for sPLA₂-V, however this was in a smaller portion of airway cells and was not statistically different from the control group. Following analysis of immunostaining for sPLA₂-X, we found that airway macrophages, epithelial cells and eosinophils were significant sources of this enzyme and that after exercise there was an increase in the immunostaining in macrophages and epithelial cells in the group of subjects with asthma with evidence of indirect AHR (Fig 2). As the protein is secreted we examined the levels of the protein by Western blot and found that there was generally an increase in the sPLA₂-X immunostaining in induced sputum supernatant in the group with asthma compared to the control group after exercise challenge. These results suggested that several of the sPLA₂s are present in the airways, but that sPLA₂-X was the enzyme that exhibited the most profound difference in asthma and post exercise challenge, suggesting it is a key target. Strengthening the link between sPLA₂-X and asthma, the *PLA2G10* gene is located on chromosome 16 (16p13.1-p.12), near the gene for the IL-4 receptor (*IL4R*), a region that is associated with asthma [88, 89]. It is also notable that sPLA₂-XIIA appears to be significantly dysregulated in asthma; however, this enzyme is less implicated as a regulator of eicosanoid synthesis as it does not contribute significantly to the AA pool [12, 90], further pointing to the importance of sPLA₂-X in asthma.

Based on our results in patients with indirect AHR, we further examined the relationship between sPLA₂s and asthma severity using samples from the severe asthma research program where differences in lung function were more prominent [23]. We further used this opportunity to ascertain differences in the subtype responsible for the PLA₂ activity in the airways as well as the cellular localization of sPLA₂ enzymes. Using small molecule inhibitors that target specific sPLA₂s, we found that the majority of PLA₂ activity in the BAL fluid could be attributed to sPLA₂-IIA and -X. Additionally, we found that sPLA₂-X expression was markedly higher in the epithelium compared to the other sPLA₂s, and sPLA₂-IIA and -X were expressed at high levels in cells from BAL fluid (Fig 3). Using the time resolved immunofluorescent assay (TRIFA), we found that the level of sPLA₂-X protein was increased in the BAL fluid of severe asthmatics, and this was related to the severity of lung function abnormalities, the extent of neutrophilic inflammation and the levels of PGE₂ in the airways. In this study, we were not able to detect other eicosanoids that had been assayed at the time of sample collection. It is also notable in this patient population that the level of sPLA₂-IIA was increased in severe asthma while the levels of sPLA₂-V were below the detection limit in BAL fluid (Fig 4).

Because of the studies implicating the involvement of sPLA₂-X in asthma, we conducted a definitive study using detailed characterization of AHR by both direct and indirect stimuli and collected endobronchial biopsies at the time of research bronchoscopy [24]. This allowed us to further characterize the level of sPLA₂-X in the airway epithelium and confirm that in asthma the expression of sPLA₂-X gene (*PLA2G10*) is much higher in the epithelium than in cells that are in the airway lumen. At the expression level, we did not observe any difference between subjects with asthma and healthy controls in either epithelial brushings or sputum cells; however, we did note that the protein level of sPLA₂-X was markedly elevated in asthma relative to the healthy controls, suggesting an association between sPLA₂-X and asthma, and particularly associated with enhanced AHR and decreased lung function (Figs 4 & 5). We used quantitative morphometry to examine the volume of sPLA₂-X staining in the epithelium relative to the surface area of the basal lamina revealing a trend towards greater protein levels in the epithelium associated with greater indirect AHR. The immunostaining also revealed that basal cells, which are increased in the airway epithelium in asthma [91], also prominently stain for sPLA₂-X [24]. These results revealed that the expression level, which is high in both healthy controls as well as subjects with asthma, was not different, but sPLA₂-X protein in airway epithelial cells and the airway lumen are increased in asthma. This may suggest that the key regulatory point is at the level of protein production or secretion into the airways, a process that involves cleavage of a pro-peptide from the enzyme, resulting in its active form. We used primary airway epithelial organotypic cell culture to further examine the regulation of sPLA₂-X and found that the enzyme increases markedly during cellular differentiation, possibly while basal cells are differentiating into ciliated cells and that the enzyme is constitutively expressed and secreted into the apical fluid. Retinoic acid, a key epithelial differentiation factor, also induces the expression of the enzyme, although there was no difference between cells from asthmatics and healthy controls [24]. In the epithelium the combination of TNF plus IL-1 β , or IL-13 or IL-17 alone induce the expression *PLA2G10* [24]. These results suggest that in human asthma sPLA₂-X may serve as a key source of sPLA₂ activity and is a key regulator of eicosanoid synthesis, LysoPL generation and surfactant dysfunction which can all contribute to the development of airflow obstruction, asthma severity and AHR.

Function of sPLA₂-X in the Biosynthesis of Cysteinyl Leukotrienes by Leukocytes

The biosynthesis of CysLTs is dependent on the presence of 5-LO and LTC₄ synthase (LTC₄S), the latter of which conjugates glutathione (GSH) to LTA₄, generating LTC₄, the first of the CysLTs [92]. LTC₄ can then be rapidly metabolized to LTD₄, and ultimately the more stable LTE₄, by gamma glutamyl transpeptidases and dipeptidases, respectively. Expression of the LTC₄S gene is primarily limited to immune cells, including mast cells, basophils, monocytes/macrophages and eosinophils [93]. Structural cells in the airways, including epithelial cells, are not known to express high levels of LTC₄S, although a prior study suggested primary human bronchial epithelial cells can express LTC₄S *in vitro* [94].

Several studies have shown that structural cell-derived sPLA₂s can indirectly contribute to the CysLT pool by providing AA which can be readily taken up by immune cells containing

5-LO and LTC₄S [95]. Epithelial cells in asthmatics do, however, express high levels of sPLA₂-X [5], which likely contributes to the CysLT pool indirectly by releasing abundant AA into the airway lumen. AA is cell permeable and can readily be used by immune cells as a substrate for 5-LO to generate LTA₄, which is metabolized by LTC₄S to LTC₄ or by LTA₄ hydrolase (LTA₄H) to LTB₄. Studies have demonstrated the importance of transcellular biosynthesis of CysLTs and LTB₄ in *in vivo* models of inflammation, which likely proceeds via the uptake of LTA₄ [96, 97].

The high levels of sPLA₂-X identified in the airways prompted us to examine the potential role of this enzyme as a regulator of CysLT formation in target cells such as eosinophils in the airways. Because of the ability of free fatty acids to cross the plasma membrane, as well as the ability of sPLA₂-X to act on the outer cell membrane of mammalian cells, this enzyme has the potential to initiate significant eicosanoid synthesis. We found that when sPLA₂-X was added exogenously to eosinophils there was the rapid and dose-dependent increase in both the AA release and CysLT formation which reached a maximum at approximately 20 minutes [25]. This ability to initiate CysLT synthesis was dependent upon sPLA₂ enzymatic activity as demonstrated through both the use of an active site-directed small molecule inhibitor and by heat deactivation of the enzyme. Addition of sPLA₂-X also led to the release of LysoPL species that are enriched in the outer cell membrane of mammalian cells. Because sPLA₂-X has been shown in transfected cells to release AA before and after secretion and to be secreted via the classical secretory pathway, we further investigated the mechanism of CysLT formation.

It is known that in mammalian cells, cPLA_{2α} plays a dominant role in the formation of eicosanoids including CysLTs [98-100]. It has also been shown that in some instances sPLA₂s act in concert with cPLA_{2α} to coordinate eicosanoid synthesis [9]. In our studies, the addition of sPLA₂-X to eosinophils led to a rapid release of AA that was not dependent on cPLA_{2α}; however, maximal CysLT formation mediated through sPLA₂-X was dependent on the activation of cPLA_{2α} [25]. This coordinated function may be important for the pathogenesis of asthma since it may lead to augmented release of CysLT in the local environment of the airways. In particular, we found that sPLA₂-X both led to phosphorylation of cPLA_{2α} as well as intracellular calcium flux indicating that sPLA₂-X could activate cPLA_{2α}. Further examination of the potential mechanism revealed that sPLA₂-X mediated CysLT formation through the p38 MAPK and c-Jun N-terminal kinase (JNK) pathways. This could potentially be through receptor-mediated signaling via PLA₂R1, or through activation of eosinophils by LysoPC, as LysoPC addition also induced CysLT formation [25] (Fig 6). Finally, we used pre-activation of eosinophils with fMLP, which is known to strongly activate cPLA_{2α}, in order to demonstrate that the addition of sPLA₂-X led to further augmentation of CysLT formation which may be a key finding in understanding the augmented levels of these eicosanoids that are identified in the airways of patients with asthma.

In our initial study in which we examined the cellular location of sPLA₂-X in the airways of subjects with asthma, we noted immunostaining in eosinophils [6] along with bronchial macrophages and epithelial cells [5]. Prior studies have shown that sPLA₂-V is present in eosinophils and can mediate CysLT formation in the absence of cPLA_{2α} activation [101].

We further confirmed the presence of sPLA₂-X at the protein level in lysates from eosinophils from allergic donors as well as at the gene expression level [6]. The eosinophil lysates show a slight gel shift to a larger molecular weight suggesting that the protein has been modified, but we did not ascertain the etiology of the protein modification. We studied endogenous PLA₂ activation in eosinophils using fMLP as a model stimulus revealing that sPLA₂-X localized to the endoplasmic reticulum, eosinophil granules and lipid bodies following stimulation. More importantly, an active site-directed small molecule inhibitor of sPLA₂-X attenuated both the release of AA, and fMLP-mediated CysLT release in a dose-dependent manner. Further, a structural analog of this inhibitor containing a methyl group preventing it from docking in the active site of sPLA₂-X did not inhibit the formation of CysLTs. We further examined the relationship between cPLA_{2α} and sPLA₂-X and showed that sPLA₂-X appeared to serve as a signal for further activation through the MAPK cascade, particularly ERK1/2 and p38. As we had seen before, cPLA_{2α} activation was essential for CysLT formation and 5-LO translocation to the perinuclear space indicating that cPLA_{2α} is necessary for sustained CysLT formation and that sPLA₂-X serves as a key signal leading to the amplification of CysLT formation. Because of our findings that sPLA₂-X is inducible and may serve as a pro-inflammatory mediator in asthma, we examined the ability of pro-inflammatory cytokines to induce *PLA2G10* gene expression in eosinophils. We found that *PLA2G10* expression was induced by a combination of TNF and IL-1β or by IL-13 alone but was not induced by common activating signals for eosinophils, such as IL-5 and GM-CSF, nor by IL-33 or TSLP [6]. To further demonstrate the relevance of the ability of pro-inflammatory cytokines to prime the synthesis of CysLTs through sPLA₂-X, we showed that TNF in combination with IL-1β enhanced the release of CysLTs following stimulation with fMLP. In summary, these results suggest that sPLA₂-X plays a key role in the heightened synthesis of CysLTs by eosinophils in asthma, and further clarify the respective roles of sPLA₂-X and cPLA_{2α} in the generation of eicosanoids such as CysLTs that are secreted by eosinophils.

Function of sPLA₂-X in Animal Models of Asthma

A central role for sPLA₂-X in Th2 cytokine-driven airway inflammation has been demonstrated in mouse asthma models utilizing animals that are globally deficient in the *Pla2g10* gene [1, 2]. Since sPLA₂-X exhibits the greatest phospholipid hydrolysis activity of the sPLA₂ enzymes, it is a potential target for interrupting eicosanoid formation by inflammatory cells in the immunopathogenesis of asthma. In mice, sPLA₂-X co-localizes to airway epithelial cells expressing MUC5AC protein and is also present in alveolar macrophages and splenic mononuclear cells [1]. In initial studies, *Pla2g10*^{-/-} mice were generated by retroviral-mediated insertion of an exon-trapping cassette [1]. In acute and chronic mouse asthma models using OVA as allergen that reflects key features of Th2 cytokine-driven asthma with airway remodeling, *Pla2g10*^{-/-} mice had significantly reduced airway inflammation and decreased AHR compared to WT controls [1]. Characteristic features of airway remodeling such as goblet cell metaplasia and mucus hypersecretion, smooth muscle hyperplasia, and subepithelial fibrosis were significantly decreased in *Pla2g10*^{-/-} mice in the chronic asthma model. Mice deficient in the sPLA₂-X protein exhibit marked impairment of the Th2 cytokine response to OVA with reduction in OVA-specific

IgE, CD3⁺/CD4⁺/CD8⁺ T cell trafficking to the airway lumen, and type-2 cytokine (i.e., IL-4, IL-5 and IL-13) levels in lung tissue and BAL fluid. Generation of both 5-LO (i.e., CysLTs and LTB₄) and COX (i.e., PGE₂ and PGD₂) products were markedly reduced in the OVA-treated *Pla2g10*^{-/-} mice compared to controls [1]. The 5-LO products of AA metabolism have many key effects on the biologic actions of the cells associated with asthma. In mice, the CysLTs increase dendritic cell activation and recruitment, goblet cell mucus release and hyperplasia, endothelial cell vascular permeability, smooth muscle cell contractility and proliferation, and fibroblast/myofibroblast contractility and proliferation [102]. LTB₄ and CysLTs recruit eosinophils, mast cells, and T cells to the lungs and stimulate type-2 inflammatory responses [102]. These data indicate that sPLA₂-X plays a critical role in AA metabolism to leukotrienes and prostaglandins and impairment of eicosanoid generation by deficiency of sPLA₂-X prevents key features of type-2 cytokine-driven airway inflammation, remodeling and hyperresponsiveness.

In mouse asthma models using a proteolytic allergen relevant to induction of human asthma [e.g., house dust mite (HDM) or cockroach], there is less dependence on T cells and greater involvement of epithelial and innate immune mechanisms, especially IL-33-driven responses in comparison to the OVA model. Studies using mice lacking sPLA₂-X demonstrated similar protection in HDM-driven AHR and infiltration by T cells and eosinophils, and type-2 cytokine production by antigen-triggered leukocytes [2]. In particular, the *Pla2g10*^{-/-} mice had decreased levels of IL-33 in the airway lumen and fewer type-2 innate lymphoid cells (ILC2) in the lungs after HDM challenge compared to wild-type controls [2]. Other significant immunologic defects in the sPLA₂-X-deficient mice stimulated by HDM are reduction in IL-33-stimulated IL-13 production by mast cells and abrogation of new recruitment of macrophages and subsequent M2 polarization in the airways. These data indicate that sPLA₂-X is a pivotal regulator of both adaptive and innate immune responses induced by proteolytic allergen relevant in the pathogenesis of human asthma [2]. This protection of allergen-induced airway inflammation and airway dysfunction is abolished following knock-in of the human *PLA2G10* gene into the *Pla2g10*^{-/-} mice, showing that functional enzyme drives many of the features associated with asthma [103]. These mice were generated with knock-in of human *PLA2G10* to assess the effect of pharmacologic blockade of sPLA₂-X-mediated responses in the airways in a mouse model. Knock-in of human *PLA2G10* in the *Pla2g10*^{-/-} mice restored allergen-driven airway eosinophilic inflammatory cell response, mucus hypersecretion, and AHR to methacholine in the *Pla2g10*^{-/-} mice. *In vivo* systemic administration of a specific human sPLA₂-X inhibitor [104] to a plasma concentration substantially greater than the IC₅₀ for *in vitro* inhibition of the human enzyme significantly reduced the allergen-driven asthma phenotype with impairment of airway inflammation, mucus hypersecretion, and hyperresponsiveness to methacholine after OVA treatment.

These studies together indicate that sPLA₂-X plays a pathogenic role in different models of experimental asthma, likely through its enzymatic activity, and contributes to airway inflammation and dysfunction. Furthermore, inhibition of human sPLA₂-X activity in a knock-in mouse model provides strong evidence that sPLA₂-X inhibition may be a novel direction for further drug development in asthma. One of the most important findings from the studies of *Pla2g10*^{-/-} animals is the dependence on functional sPLA₂-X for propagation

of type-2-driven inflammation. In these studies, lack of the *Pla2g10* gene significantly attenuated the type-2 immune response in both OVA- and HDM-exposed animals. This provides further evidence that targeting the eicosanoid pathways further upstream instead of targeting the resultant mediators (CysLTs, PGD₂) may be a more effective and promising treatment option.

Interaction of sPLA₂-X through receptor-mediated functions

As we noted before, several sPLA₂s, including sPLA₂-X act as high affinity ligands for a receptor designated as PLA₂R1. This receptor is a 180-kD integral transmembrane receptor that has a large extracellular domain but a short cytoplasmic domain. The receptor belongs to the superfamily of C-type lectin receptors and is a paralog of the macrophage mannose receptor and is closely related to other receptors within this family [75]. PLA₂R1 is strongly implicated as a key auto-antigen in membranous nephropathy, where the glomerular basement membrane becomes leaky [105]. Although several studies have revealed that ligands of this receptor can induce cellular signaling, a major function has been attributed to the release of the soluble form of the enzyme either through alternative transcription or cleavage of the large extracellular domain. Thus, the enzyme may serve to bind and remove high affinity sPLA₂ ligands, including groups IB, IIA, IIE, IIF, and X, limiting the overall sPLA₂ activity [76]. It is also likely that PLA₂R1 acts as a pleiotropic receptor that binds to non-sPLA₂ ligands.

We became interested in the potential function of this receptor in asthma following a genome wide expression study of airway epithelial cells collected from children with atopic asthma as compared to a healthy nonatopic control group showed that this receptor was significantly overexpressed in asthma [82] and (Fig 7A). Our results were further confirmed in a second group of epithelial brushings from a distinct cohort of children with and without atopic asthma that also revealed an increase in the expression of this receptor in asthma using qPCR [82]. In epithelial brushings, the expression of this receptor was strongly correlated with the expression of *PLA2G1B*, *PLA2G2F*, *PLA2G5* and *PLA2G12A*, but tended to be inversely correlated with the expression of *PLA2G10*. Immunostaining for PLA₂R1 in endobronchial biopsies from subjects with asthma revealed immunostaining for this receptor that is prominent in submucosal glandular epithelium as well as columnar airway epithelial cells [82] and (Fig 7B-E).

To further characterize the function of PLA₂R1 and its relation to sPLA₂-X we conducted an asthma model in mice lacking PLA₂R1. Our model involved sensitization to ovalbumin (OVA) in the presence of an exogenous adjuvant Alum followed by OVA challenge via the airways. A prior study had shown reduced clearance of sPLA₂-IB in mice lacking PLA₂R1 [83]. We found that the basal levels of sPLA₂-X were markedly higher in mice lacking PLA₂R1 [82], suggesting that in mice this receptor is primarily acting to enhance the clearance of sPLA₂s. Our results revealed that mice lacking PLA₂R1 had a marked increase in AHR to methacholine challenge and that there was a modest but significant increase in airway inflammation associated with hyperresponsiveness. In particular, the number of eosinophils and T cells was increased in the BAL fluid and the number of CD11c⁺ dendritic cells was increased in the lung. In concordance with these findings, the levels of antigen

specific IgG were also elevated in the blood of the receptor deficient mice. As the accentuation of AHR was more prominent than might have been expected based on the differences in inflammation, we further characterized airway permeability and mucus release, two mechanisms that are known to contribute to AHR [106]. We found that airway permeability measured by the transit of the large protein IgM to the airways following methacholine challenge was increased in the receptor deficient mice and that the mucin content of the airway epithelium was significantly reduced following methacholine challenge in the receptor deficient mice suggesting enhanced mucin release predominantly from goblet cells. The basis for these findings are not entirely certain, but could be related to changes in the permeability of the basement membrane as has been described in membranous nephropathy.

An area that needs more attention regarding the function of PLA₂R1 in relation to asthma is the potential to regulate inflammation. The accentuated inflammation in knockout mice described in our study and the study by Tamaru and colleagues [82, 83] could simply be the consequence of the increased levels of sPLA₂-X and other sPLA₂s, or due to other mechanisms entirely. To further understand this alteration in inflammation we examined the release of cytokines from lung leukocytes that were restimulated with the antigen OVA. While we found that the antigen-induced increases in IL-2, IL-4, and IL-17 were attenuated in the PLA₂R1-deficient animals, the production of a broad array of pro-inflammatory cytokines were increased in lung leukocytes from the receptor knockouts. Relating these findings back to human asthma, these findings might suggest that the increased expression of the receptor in the airway epithelium may be a protective mechanism that occurs in response to airway inflammation.

Summary

Since the discovery of the sPLA₂-X enzyme, our work has revealed that it may be a key therapeutic target in asthma (Fig 8). In particular, the enzyme has a number of attributes that implicate it as a regulator of immune processes through the release of free fatty acids and LysoPLs with high activity from mammalian cell membranes. Further, our work has revealed that although there are no differences in the expression of the enzyme in the epithelium in asthma, there are clear differences in the amount of the secreted protein in the airways that are related to features of asthma, specifically AHR. In humans, we have found that the airway epithelium, bronchial macrophages and eosinophils are key sources of the enzyme, and have further identified the expression in mast cells in murine models. The increased levels of this enzyme are also implicated in the dysregulated production of eicosanoids in asthma. Murine models further revealed that sPLA₂-X serves as a central regulator of allergen-induced inflammation and AHR. In particular, lack of sPLA₂-X in a T-cell dependent model of asthma reveals that the lack of this enzyme disrupts T-cell signaling as well as the production of key eicosanoids including CysLTs. The lack of this enzyme in a complete allergen model of airway dysfunction mediated through HDM, reveals that the lack of this enzyme alters both the innate and adaptive immune response to allergen and alters type-2 inflammation as well as the levels of IL-33 in the airways suggesting it may have an upstream effect on a key pathway leading to type 2 inflammation. Cellular studies have further revealed that sPLA₂-X serves as a key regulator of CysLT synthesis both as an

extracellular activator of immune cells such as eosinophils, as well as in endogenous regulator of CysLT formation acting in concert with cPLA_{2α}. Collectively, these studies suggest that sPLA₂-X serves as an important regulator of airway dysfunction in asthma and may serve as a critical target for therapeutically managing airway inflammation in individuals with asthma.

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Figure 1:

Comparison of immunostaining for sPLA₂s in induced sputum cells from healthy subjects compared to individuals with asthma and exercise-induced bronchoconstriction. The percentage of sputum cells staining positive for (A) sPLA₂-V was not different between groups. Staining for (B) sPLA₂-X or (C) sPLA₂-XIIIA was significantly elevated in asthma (N = 5/group, mean $\pm$ SEM; paired *t* test). Adapted from Hallstrand et al, Am J Resp Crit Care Med, 2007 [5].

Figure 2:

Effects of exercise challenge on sPLA₂-X levels in the airways of healthy and asthmatic subjects. (A) Immunostaining for sPLA₂-X was increased in bronchial macrophages (Mac) and epithelial cells (Epi), but not in eosinophils (EOS) in induced sputum cells from asthmatic subjects following exercise challenge (N = 9/group, mean $\pm$ SEM; paired *t* test). (B) Analysis of induced sputum supernatant by Western blot revealed an increase in sPLA₂-X protein in subjects with asthma post exercise challenge (N = 5/group, mean $\pm$ SEM; paired *t* test). Adapted from Hallstrand et al, Am J Resp Crit Care Med, 2007 [5].

Figure 3:

Expression of sPLA₂ enzymes in epithelial cells and BAL cells from humans. (A) Expression of sPLA₂-X was significantly elevated in airway epithelial cells compared to sPLA₂-IIA or sPLA₂-V. In BAL cells, sPLA₂-IIA and sPLA₂-X are elevated, but not sPLA₂-V. (B) There is a significant difference in sPLA₂-X compared to sPLA₂-V, but not sPLA₂-IIA in BAL cells (N = 15/gene, mean ± SEM; Kruskal-Wallis test with Dunn's post-hoc test). Adapted from Hallstrand et al, Clin Exp Allergy, 2011 [23].

Figure 4:

Differences in the levels of sPLA₂-X in BAL fluid relative to lung function and asthma severity. (A) The levels of sPLA₂-X protein differed by asthma severity, and were specifically increased in the group of individuals with severe asthma relative to nonasthmatic controls (N = 18-20/group, medians and interquartile ranges shown in graph). Kruskal-Wallis test used to compare three groups (*) and comparisons between subgroups done using Dunn's post-hoc test (†). (B) The level of sPLA₂-X protein in BAL fluid was related to the percentage of predicted FEV₁ across the study population by linear regression analysis. Adapted from Hallstrand et al, Clin Exp Allergy, 2011 [23].

Figure 5:

Quantification of sPLA₂-X in induced sputum and airway tissue relative to features of airway hyperresponsiveness. (A) The concentration of sPLA₂-X protein in induced sputum was elevated in asthmatics relative to control subjects. These differences are more accentuated in subjects who have exercise-induced bronchoconstriction (EIB+) compared to individuals without EIB (EIB-) and controls (N = 10-19/group; Kruskal-Wallis test with Dunn's post-hoc test). (B) In asthmatics, the amount of positive immunostaining for sPLA₂-X in the epithelium trended towards an association with airway hyperresponsiveness (AHR; linear regression analysis). Adapted from Hallstrand et al, Am J Resp Crit Care Med, 2013 [24].

Figure 6:

Scheme of the mechanism of $sPLA_2-X$ -mediated CysLT synthesis in eosinophils. Enzymatic activity of $sPLA_2-X$ causes the release of lysophospholipids (LysoPL) and free fatty acids (FFA), such as arachidonic acid (AA), from membrane phospholipids. Synthesis of CysLT is dependent upon downstream activation of $cPLA_2\alpha$ via Ca^{2+} flux and phosphorylation of $cPLA_2\alpha$ through $p38$ and JNK -dependent signaling. Activity of $sPLA_2-X$ can cause a Ca^{2+} flux in human eosinophils. The $sPLA_2-X$ and LysoPC-induced synthesis of CysLT was dependent on $p38$ and JNK activity, as inhibition of either prevented CysLT release in this context. Free AA released by $sPLA_2-X$ activity may also lead to transcellular synthesis of eicosanoids, as AA is readily taken up by cells. Adapted from Lai et al, J Biol Chem, 2010 [25].

Figure 7:

Expression and localization of PLA₂R1 in human endobronchial airway tissue. (A) *PLA2R1* expression from airway epithelial brushings was elevated in children with asthma compared to healthy non asthmatics (HNA = healthy non-asthmatic, AA = allergic asthmatic; N = 9/group, mean ± SEM) . (B-E) Immunostaining for PLA₂R1 reveals distinct staining in submucosal epithelium (B, 10x; C, 40x). Further analysis shows that columnar epithelial cells are also positive for PLA₂R1 immunostaining (D, 60x oil). Cytospins also show positive staining for PLA₂R1 in airway epithelial cells. AEC, airway epithelial cell; BL, basal lamina; SG, submucosal gland. Adapted from Nolin et al, Am J Resp Cell Mol Biol, 2016 [82]..

Figure 8:

Scheme depicting the role of sPLA₂-X as a key regulator of type-2 inflammation in asthma. The airway epithelium is the major source of sPLA₂-X, although other cells, including macrophages (MΦ), eosinophils (Eos) and mast cells (MC) can potentially contribute the the sPLA₂-X pool in the airways. Levels of sPLA₂-X are elevated in the airways of asthmatics and contribute to disease pathogenesis in part through release of lysophospholipids (LysoPL), free fatty acids (FFA), such as arachidonic acid (AA), and subsequent production of eicosanoids by eosinophils. In animal models, sPLA₂-X deficiency is protective, leading to less mucus production, decreased eosinophilia, impaired activation of MCs, fewer pathogenic macrophages in the lungs, and dampened IL-33 release in the airways that corresponds with decreased production of type-2 cytokines. Another possible consequence of increased sPLA₂-X levels is surfactant degradation, leading to surfactant dysfunction and airway closure.