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Airway Epithelial Regulation of Allergic Sensitization in Asthma

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Abstract

While many of the contributing cell types and mediators of allergic asthma are known, less well understood are the factors that influence the development of allergic responses that lead to the development of allergic asthma. As the first airway cell type to respond to inhaled factors, the epithelium orchestrates downstream interactions between dendritic cells (DCs) and CD4⁺ T cells that quantitatively and qualitatively dictate the degree and type of the allergic asthma phenotype, making the epithelium of critical importance for the genesis of allergies that later manifest in allergic asthma. Amongst the molecular processes of critical importance in airway epithelium is the transcription factor, nuclear factor-kappaB (NF-κB). This review will focus primarily on the genesis of pulmonary allergies and the participation of airway epithelial NF-κB activation therein, using examples from our own work on nitrogen dioxide (NO₂) exposure and genetic modulation of airway epithelial NF-κB activation. In addition, the mechanisms through which Serum Amyloid A (SAA), an NF-κB-regulated, epithelial-derived mediator, influences allergic sensitization and asthma severity will be presented. Knowledge of the molecular and cellular processes regulating allergic sensitization in the airways has the potential to provide powerful insight into the pathogenesis of allergy, as well as targets for the prevention and treatment of asthma.

Keywords

asthma; CD4⁺ T cells; dendritic cells; epithelium; NF-kappaB; SAA

1. Introduction

Afflicting greater than 34 million Americans (1) and costing over 18 billion dollars annually (2), allergic asthma is a primary public health concern. The incidence of asthma has been rising steadily in the United States over the past two decades (3) and there are many potential reasons for this increase. Allergic asthma is the result of an inappropriate adaptive immune response to an inhaled antigen and is typically characterized by airway inflammation with eosinophils and lymphocytes, increased levels of Th2 cytokines, circulating IgE, airway goblet (mucus-producing) cell metaplasia, and airway hyperresponsiveness (bronchoconstriction in response to inhalation of a specific (allergen) or non-specific (methacholine, cold air) agonist) (4). The immune response in allergic asthma is driven primarily by CD4⁺ T helper type 2 (Th2) lymphocytes (4), activation of which is both necessary (5) and sufficient (6) to induce all of the features of allergic asthma in mice.

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Th17 cells producing IL-17A, IL-17F, and IL-22 are pathogenic in severe asthma involving neutrophilia (7) and a steroid-unresponsive form of the disease (8). Despite knowing much about effector mechanisms in allergic asthma that promote airway hyperresponsiveness, eosinophilia or neutrophilia, IgE, and mucus production, still poorly understood are the mechanisms that allow for initial allergen sensitization. Allergic sensitization, the act or process of inducing an acquired allergy, requires activation of antigen-presenting cells, such as dendritic cells (9) that induce the expansion of naïve CD4⁺ T cells (10) and influence their differentiation into Th2 or Th17 effectors. Importantly, pulmonary epithelium can direct the activities of dendritic cells and, thereby, affect CD4⁺ T cell activities that are critical for qualitatively shaping the type of allergic response manifest upon subsequent encounter with inhaled antigens. Especially important in airway epithelium is the transcription factor, NF- κ B, which we have reported substantially influences the development of allergic asthma during both allergen challenge and during allergen sensitization (Fig 1).

2. Nitrogen dioxide and allergic asthma

Nitrogen dioxide (NO₂) is a free-radical gaseous (11) component of indoor and outdoor air pollution generated during combustion processes, such as the operation of motor vehicles and biomass burning (12–14). In humans, NO₂ can cause acute lung injury (15) and trigger asthma exacerbations, particularly in children (14, 16–21). Concentrations of NO₂ above 5ppm cause lung damage (22, 23), whereas lower concentrations (100–400ppb) contribute to poor respiratory health (24) and exacerbate existing asthma (25, 26). We and others have demonstrated using mouse models that NO₂ exposure causes pathologic changes resembling acute lung injury (27) and that NO₂ inhalation can augment the degree of allergic airway inflammation and prolong allergen-induced airway hyperresponsiveness (28, 29). Asthmatics experience an enhanced reaction to inhaled allergen in the presence of NO₂ (18) and living in areas with high ambient NO₂ concentrations is correlated with an increased likelihood for developing asthma (16, 30). In addition to exposure originating from exogenous sources, NO₂ is also generated endogenously, typically as part of an immune response intended to clear infectious and environmental threats to homeostasis (reviewed in (11)). For example, during viral infection, NO₂ levels are elevated in the lungs, as endogenously produced nitric oxide (NO) and superoxide (O₂^{•-}) (31) combine to form NO₂ (32). Respiratory viral infections in childhood are associated with the development of asthma (33) and mouse models demonstrate that pulmonary virus infection induces dendritic cell activation and can cause allergic sensitization (34). Understanding the cellular and molecular mechanisms by which NO₂ exposure, whether from the environment or generated endogenously, contributes to allergic sensitization and asthma has important implications for public health.

3. Nitrogen dioxide-promoted allergic sensitization

It remains incompletely understood how and why in an otherwise healthy lung, a cascade of events is initiated to allow innocuous inhaled antigens to initiate an allergic reaction that can manifest in the pathophysiological features of allergic asthma upon subsequent antigen exposure. We developed a mouse model of NO₂ exposure followed by inhalation of ovalbumin to study mechanistically the effects of NO₂ on allergic sensitization. We have reported that NO₂ acts as an adjuvant, promoting the development of allergic asthma features including airway eosinophilia, airway hyperresponsiveness, antigen-specific IgE and IgG1, and antigen-specific CD4⁺ T cells that exhibit a mixed Th2 (35) and Th17 cytokine profile (36). We have also shown that NO₂ inhalation has a substantial impact on pulmonary CD11c⁺ dendritic cells, as reflected by antigen uptake and processing, cytokine production, upregulation of maturation markers, migration to the lung-draining lymph node, and

improved ability to stimulate CD4⁺ T cells (36). Additionally, CD11c⁺ cells are critical for NO₂-promoted allergic sensitization, as depleting this population during sensitization diminishes multiple features of allergic asthma in mice (36). It is now appreciated that several environmental agents, including NO₂ and ozone (37, 38), bacterial endotoxin (39, 40), respiratory viral infections (34), and ambient particles (41–49) are capable of functioning as adjuvants, promoting pulmonary allergic responses. Importantly, like NO₂ (27, 35), many of these promoters of allergic responses have the capacity to stimulate lung epithelium.

4. Airway epithelium in allergic sensitization

As the first line of defense against inhaled insult, the airway epithelium participates both in maintaining barrier function and initiating innate immune responses. Epithelial cells express cell-surface and intracellular pattern-recognition receptors that enable them to detect microbial infection, danger (such as by inhaled oxidant gasses and respirable particulate matter), and cellular damage to synthesize pro-inflammatory cytokines that recruit and/or activate other innate and adaptive immune cells (50). For instance, house dust mite (HDM) extracts (51) induce allergic asthma via TLR4 expressed on airway structural cells. The Derp2 allergen in HDM extract has structural homology to the TLR4-associating molecule, MD-2, and can directly interact with TLR4 to initiate signals required for allergic sensitization and the development of allergic airway disease (52). Activation of the airway epithelium influences dendritic cell recruitment, maturation, and CD4⁺ T cell polarization by producing various cytokines that promote allergic sensitization and subsequent inflammation (reviewed in (53–55)). These include the Th2-polarizing cytokine IL-6 (56–59), chemokines including CCL20 (MIP-3α) and β-defensins that recruit immature and Th2-inducing dendritic cells to the lung (60–62), and the dendritic cell-activating or Th2-differentiating cytokines thymic stromal lymphopoietin (TSLP), GM-CSF, IL-1, IL-33, osteopontin, and IL-25 (53, 63–65). Importantly, the transcription of many of these lung epithelial-derived mediators is regulated by NF-κB. Pulmonary dendritic cell recruitment and activation are tightly linked since activation of the resident pulmonary dendritic cell population causes them to downregulate the peripheral homing receptor, CCR6, allowing the basal expression of CCR7 to instruct chemotaxis towards the CCL19/CCL21 gradient emanating from the draining lymph node (66). In response to inflammatory stimuli, pulmonary epithelial cells secrete chemokines to recruit dendritic cells to the lung (61, 62), including CCL20 (MIP-3α) and β-defensins, ligands of CCR2/CCR6 expressed by immature dendritic cells (53). As DC activation is regulated by pro-inflammatory cytokines (TNFα, IL-1β), these mediators also induce an effect on surrounding tissue that causes release of chemotactic cytokines, including CCL20 from epithelial cells, that influence the recruitment of inflammatory monocytes/dendritic cells to encourage repopulation of the tissue with these cells when activated/matured dendritic cells migrate towards draining lymph nodes. DC maturation is profoundly affected by pulmonary epithelial-derived GM-CSF (67), which we have reported is expressed by the lungs (68) and secreted into the lavageable airspaces (69) of transgenic mice following airway epithelial NF-κB activation. The actual site of pulmonary dendritic cell – T cell interaction that leads to allergic sensitization remains to be definitively addressed. The priming of CD4⁺ T cells in the lungs (outside of lymph nodes) has only truly been demonstrated by Dr. Bottomly's group using peripheral lymph node-deficient lymphotoxin alpha knockout mice (70), which are not representative of the lymph node anatomy of a normal mouse.

5. Airway epithelial NF- κ B: providing transcriptional control of allergic sensitization

Of the many signaling cascades activated by airway epithelium in response to stimulation, nuclear factor-kappaB (NF- κ B) family members are critical regulators of innate and adaptive immunity. NF- κ B is activated by cytokines, mitogens, physical and oxidative stress, infection, and microbial products (71). The canonical and non-canonical NF- κ B cascades are initiated by upstream kinases, IkappaB kinases (IKK), which are inducibly phosphorylated by kinases further upstream, including the IL-1 Receptor-Associated Kinases (IRAKs) and NF- κ B-Inducing Kinases (NIKs) (72). Canonical NF- κ B activity is tightly controlled by the inhibitory protein, I κ B α , which is normally present in the cytosol complexed to NF- κ B dimers. Upon cellular stimulation, the IKK complex, composed of IKK α , IKK β and IKK γ , becomes activated for IKK β to phosphorylate I κ B α at serines 32 and 36, inducing its ubiquitination and degradation through the 26S proteasome pathway (73–75). I κ B α degradation exposes the nuclear localization sequence of NF- κ B, facilitating its translocation to or retention in the nucleus, interaction with co-activator complexes, and transcriptional upregulation of genes downstream of the κ B motif (71).

We have reported that NF- κ B is active in the airway epithelium of mice exposed to NO₂ (27, 35) and have found that NO₂ exposure induces NF- κ B activity in airway epithelial cells *in vitro*, as reflected by IKK activity and NF- κ B-dependent reporter gene expression (unpublished results). To directly test the function of airway epithelial NF- κ B in allergic sensitization, we developed an inducible transgenic mouse model ($\text{C}_{\text{A}}\text{IKK}\beta$) in which doxycycline (dox) administration transiently activates IKK β , and thereby NF- κ B, specifically in airway epithelium (69). While airway epithelial IKK and NF- κ B activation are the initial signaling events in this model, there are most certainly additional cells and signaling cascades activated as a consequence of IKK transgene expression. Previously, we demonstrated that induction of airway epithelial NF- κ B activity for 7 days leads to an acute inflammatory environment characterized by airway neutrophilia, airway smooth muscle thickening, and robust induction of inflammatory cytokines in the bronchoalveolar lavage fluid (BAL), including KC, G-CSF, GM-CSF, IL-12p40, and MCP-1 (69). Additional studies demonstrated that, similar to exposing mice to inhaled NO₂, only a short period of airway epithelial NF- κ B activation (48 hours) was required to sensitize these mice to an innocuous inhaled antigen (ovalbumin) (68). When challenged with inhaled ovalbumin several weeks later, these mice generated a mixed Th2/Th17 allergic response accompanied by methacholine hyperresponsiveness, airway eosinophilia and neutrophilia, enhanced *Muc5ac* gene expression and mucus metaplasia, and production of IL-5, IL-13, and IL-17 from restimulated CD4⁺ T cells (68). Flow cytometric analysis of dendritic cells from the lung-draining mediastinal lymph nodes of mice following a 48-hour induction of airway epithelial NF- κ B activation demonstrated phenotypic evidence of maturation (68). These alterations took place at a time when the transgene was no longer expressed, implicating the induced adaptive immune response as the causal pathogenic mediator at this time point. Furthermore, transient activation of airway epithelial NF- κ B did not alter epithelial permeability or induce pulmonary expression of the Th2-skewing cytokines *IL-6*, *TSLP*, *IL-25*, or *IL-33*, but did induce expression of *CCL20*, *GM-CSF*, *IL-23*, and *Serum Amyloid A (SAA)*3 (68). Thus, in our transgenic model pulmonary dendritic cells respond to signals from the epithelium and participate in the generation of allergic immune response.

It appears that the majority of the epithelial-derived products (and/or those expressed by the lung or present in BALF of mice in which airway epithelial NF- κ B activity have been selectively induced) are generally capable of inducing neutrophilic inflammation. How then might airway epithelial NF- κ B activities skew CD4⁺ T cells towards a Th2 phenotype? In addition to the plethora of aforementioned airway epithelial-derived mediators (IL-6 (56–

59), TSLP, IL-33, osteopontin, and IL-25 (53, 63–65), it has been reported that the lung consists of over 40 distinguishable cell types (76) and it has been proposed that the tissue is in quantitative and qualitative control of adaptive immune responses (77, 78). Therefore, it is not unreasonable to predict that additional pulmonary cell types besides the epithelium, as well as additional intracellular signaling cascades in addition of NF- κ B, participate in the modulation of CD4⁺ T cell responses towards Th2. For instance, invariant natural killer T (iNKT) cells are an innate-like population of CD4⁺ T cells that rapidly secrete cytokines upon stimulation, are an early source of IL-4 that can promote Th2 polarization, and have been implicated in the pathology of allergic airway disease (79, 80). In addition, akin to nuocytes (81, 82) and lineage-negative multipotent progenitor-type 2 (MPP^{type2}) cells (83, 84), there may be an additional “innate” cellular source for Th2-inducing cytokines that remain undiscovered. Finally, the IL-12p40 subunit, which we have previously reported is substantially augmented in the BALF of mice in which NF- κ B activity is selectively induced in airway epithelium (69), can form homodimers that antagonize the Th1- and Th17-inducing capacity of IL-12p70 (a heterodimer of IL-12p40 and IL-12p35) (85–87) and IL-23 (a heterodimer of IL-12p40 and IL-12p19) (88), respectively. Whereas a complete understanding of the involvement of pulmonary cells in promoting Th2 responses remain enigmatic, these processes may provide potential opportunities for intervention. Defining the epithelial-derived mediators that influence dendritic cell activities is of critical importance to better understand the pathogenesis of allergic sensitization and asthma exacerbation.

6. Serum Amyloid A (SAA) as an inflammatory mediator

Acute phase responses are induced in an attempt to eliminate pathogens, alter host metabolism, and transition between innate and adaptive immunity. SAA is an acute phase protein that is upregulated over 1000-fold in response to infection, exposure to microbes and microbial products such as LPS, inflammatory cytokines, glucocorticoids, and SAA itself (89, 90). Of relevance to the lung, SAA has been reported as a biomarker for a causal mediator in sarcoidosis (91), COPD (92, 93), and asthma (94–96). In mice, the *Saa1* and *Saa2* genes are expressed predominantly in the liver, whereas *Saa3* is expressed in other tissues, including the lung (89). In humans, the *SAA1* and *SAA2* genes regulate pulmonary SAA production and are expressed mainly by the epithelium (97). The transcriptional regulation of extra-hepatic SAA involves three motifs in the *Saa3* promoter, termed “SAA-activating sequences” (SAS) (98). *Saa3* expression is regulated by the transcription factor, SAA-Activating Factor (SAF)-1. Mitogen-activated protein kinases, protein kinase C, and protein kinase A are capable of phosphorylating SAF at specific serines, causing a conformational change and nuclear translocation. In the nucleus, SAF binds DNA at SAS motifs in the *Saa3* promoter, inducing *Saa3* transcription (99, 100). NF- κ B also participates in transcriptional regulation of the *Saa3* gene (101, 102). SAA can promote leukocyte chemotaxis through the Formyl Protein Receptor 2 (FPR2) (103) and can activate myeloid cells via Toll-Like Receptor (TLR)2 (90, 104, 105) to induce the production of cytokines such as G-CSF (106), IL-8 (107, 108), IL-1 α (90) and IL-1 β (90, 105, 109). In addition, indirect effects of SAA on other cell types can lead to the secretion of cytokines that also affect neutrophil chemotaxis and activation (107, 108). Finally, SAA has been implicated as an important mediator for Th17 polarization in the gut induced by colonization with apathogenic segmented filamentous bacteria (110).

7. IL-17 in severe allergic asthma

IL-17 is elevated in the lungs, BAL fluid, sputum, and serum of asthmatics (111, 112), wherein the levels positively correlate with disease severity, including the magnitude of airway hyperresponsiveness (113–116) and neutrophilia (117). In mouse models, inhalation-mediated allergen sensitization is associated with the generation of Th17 cells (40, 118),

which are sufficient to drive neutrophilic airway inflammation, AHR, and glucocorticoid-resistant disease – all characteristic of severe allergic asthma (8). While IL-17-producing CD4⁺ T cells can be generated in a number of ways, they are strongly influenced by inflammatory cytokines, including IL-1 β (119–124). Because IL-1 β lacks a conventional signal sequence (125), it requires two sequential signals to enable secretion (126). Stimulation of pattern recognition receptors provides “signal 1” by inducing transcription and translation of the 31kDa pro-IL-1 β (126, 127). IL-1 β release is regulated by cleavage of the pro- form to the 17kDa secreted form through “signal 2”. This caspase-1-dependent IL-1 β cleavage is facilitated by the cytosolic assembly and activation of an inflammasome, a ~700kDa multi-protein complex typically comprised of NOD-Like Receptors (NLR), with or without ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) linker molecules, and active caspase-1 (127, 128). The best-studied inflammasome-assembling platform is comprised of the NOD-Like Receptor containing a Pyrin domain (Nlrp3), which recruits caspase-1 via the linker ASC (129). Whereas inflammasome activation is potently induced during infection by microbes and microbe-derived molecules, endogenous molecules including amyloidogenic proteins (130, 131) can also activate the Nlrp3 inflammasome and induce IL-1 β secretion. We (90) and others (105) have recently reported that SAA activates the cytoplasmic molecule, Nlrp3, to promote IL-1 β secretion. We have also recently published that recombinant SAA can act as an adjuvant to sensitize mice to ovalbumin and elicit a Th2/Th17 response. In addition, we have shown that our $\text{C}_{\text{A}}\text{IKK}\beta$ mice induce *Saa3* expression in the lung (90). Taken together, these data identify *Saa3* as a candidate epithelial-derived mediator capable of stimulating dendritic cells to promote Th17 polarization. Aluminum-containing salts such as aluminum hydroxide (Alum) are among the best-studied adjuvants used to promote humoral and Th2 responses. Eisenbarth *et al.* demonstrated an important direct contribution of Nlrp3 for the induction of an allergic phenotype in response to antigen sensitization using an Alum-containing adjuvant (132), whereas Kool *et al.* have reported an indirect (via the generation of uric acid) contribution of Nlrp3 activation in the effects of Alum-containing adjuvants (133, 134). However, this group has also described an Nlrp3-independent effect for uric acid in the induction of Th2 responses following inhalation of innocuous antigen or house dust mite allergen (135). Furthermore, another recent paper provides contrasting data demonstrating that Nlrp3 is dispensable for the immunologic and physiologic effects in similar models of asthma (136). An important caveat to be considered is that none of the aforementioned papers measured IL-17. Whereas there is little argument that Nlrp3 is involved in the generation of bioactive IL-1 β in response to a variety of molecules, including those that can induce Th2 responses, and that IL-1 β can promote Th17 responses (119, 121–124), an integrated role for the SAA/Nlrp3/IL-1 β /IL-17 axis in severe allergic asthma remains to be fully explored.

8. Epithelial-derived SAA in the origin and exacerbation of severe allergic asthma

Since short-term exposure to NO₂ or NF- κ B activation in airway epithelial cells induces acute neutrophilia (27), which has been associated with the induction of an acute phase response (89), we measured expression of *Saa3*, the SAA inducibly expressed in mouse lungs (137), following exposure to 15ppm of NO₂ for 1 hour. The *Saa3* increases we have reported (90) were reflected by elevated SAA protein expression localized to the airway epithelium (Fig 2) in response to NO₂. We corroborated the expression of SAA3 in airway epithelium by positive staining for phosphorylated SAA-Activating Factor-1 (SAF) (Fig 2), a transcription factor regulating *Saa3* expression (138). As anticipated, NO₂ exposure did not alter lung epithelial levels of total SAF-1 (Fig 2). These results suggest that lung

epithelial-derived SAA may function downstream of NO₂ in promoting pulmonary inflammation and polarizing antigen-specific Th2/Th17 responses.

Our work (35, 36, 68, 90) and that of others indicates that mixed Th2/Th17 responses are generally initiated following allergen exposure via the lungs (40, 139, 140). To examine whether SAA3 may be a common link between several of these allergen-sensitizing regimens, we exposed C57BL/6 mice to low-endotoxin ovalbumin, NO₂, low-dose LPS, recombinant SAA (apoSAA), or vehicle control. In addition, transgenic mice with airway epithelial NF- κ B activity or transgene-negative littermate controls were provided doxycycline for 3 days. Finally, mice received an intraperitoneal injection of the Th2-polarizing adjuvant aluminum hydroxide (Alum) or saline. We then measured *Saa3* expression in the lung by Q-RT-PCR and conducted SAA immunostaining from paraffin-embedded lung sections. Our results (Fig 3A) demonstrate that each of the Th2/Th17-inducing regimens (airway epithelial NF- κ B activation, NO₂ exposure, and LPS or apoSAA inhalation) induce pulmonary *Saa3* expression, whereas inhalation of ovalbumin or injection of Alum do not. Furthermore, the airway epithelium is a major source of SAA3 subsequent to several pulmonary stimuli (Fig 3B). These data suggest that inhalational Th2/Th17 allergic sensitization regimens may converge on pulmonary epithelial SAA3.

We have recently published that administration of recombinant human apoSAA, a molecule with functional homology to mouse SAA3 (141), mimicked the effects of NO₂ or NF- κ B activation by inducing BAL neutrophilia and the production of inflammatory cytokines, including neutrophilic chemokines and Th17-polarizing mediators (90). To test the ability of SAA to promote allergic sensitization to an innocuous inhaled antigen, we administered apoSAA to C57BL/6 mice by aspiration immediately prior to ovalbumin nebulization on days 0 and 6. Following subsequent allergen challenge on days 13–15, we measured increased eosinophils and lymphocytes in BALF and the production of IL-13 and IL-17 upon restimulation of antigen-specific CD4⁺ T cells (90).

To examine the effects of SAA on antigen-presenting cells, we exposed bone marrow-derived dendritic cells to SAA and measured upregulated expression of the maturation markers CD80, CD86, OX40L, and MHCII as well as secretion of Th17-polarizing mediators, including IL-1 α , IL-1 β , IL-6, IL-23, and PGE₂ (90). Polyclonal stimulation of CD4⁺ T-cells in the presence of conditioned medium from SAA-exposed BMDCs induced the production of IL-17, but not IL-4 or IFN γ . Importantly, IL-17 production was not induced by exposing CD4⁺ T cells directly to SAA (90). Polyclonal stimulation of splenocytes in the presence of SAA augmented IL-17 production, which was inhibited by the addition of anakinra (recombinant IL-1 receptor antagonist) or by using cells from Nlrp3- ASC-, or Caspase-1-deficient mice (90). Given the importance of IL-1 in Th17 polarization, we subjected wildtype and IL-1R α -/- mice (deficient specifically in the capacity to respond to IL-1) to SAA-promoted allergic sensitization and subsequently challenged them with ovalbumin. The bronchoalveolar lavage fluid from IL-1R α -/- mice contained fewer eosinophils and lymphocytes than wildtype mice. All mice sensitized with SAA plus ovalbumin generated IL-5 and IL-13, whereas splenocytes from IL-1R α -/- mice produced no IL-17 following *in vitro* restimulation (90). These data indicate that signaling through IL-1R α is required specifically for Th17 polarization in inhalational allergic sensitization mediated by SAA.

The first signal required for IL-1 β production is often through pattern recognition receptors to induce pro-IL-1 β . TLR2 is a receptor for SAA (104, 106) that can promote the production of IL-23 and IL-1 β (142) and mixed Th2/Th17 responses (143). We have demonstrated the requirement for TLR2 and its adaptor protein, MyD88, for NO₂-promoted allergic sensitization (35). Using bone marrow-derived dendritic cells and peritoneal exudate

macrophages, we demonstrated that IL-1 β secretion was almost completely absent in TLR2 $^{-/-}$ and MyD88 $^{-/-}$ cells, whereas the response to SAA was still robust using cells from TLR4-deficient mice (90). Furthermore, SAA-induced airway neutrophilia was significantly blunted in TLR2-deficient mice and in wildtype mice treated with anakinra (90). In addition to “signal 1”, IL-1 β secretion generally requires inflammasome activation to cleave pro-IL-1 β . Therefore, we exposed wildtype, Nlrp3 $^{-/-}$, ASC $^{-/-}$, and caspase-1 $^{-/-}$ macrophages to SAA. Each of the Nlrp3 inflammasome components was required for the secretion of IL-1 β . Niemi *et al.* also recently reported the capacity of SAA to activate TLR2 and Nlrp3 in myeloid cells (105). As has been demonstrated with several Nlrp3 agonists that are endocytosed and form macromolecular complexes within endosomes (reviewed in (144)), Niemi *et al.* found that cathepsin activities were required for SAA-induced IL-1 β secretion (105). Using a pharmacologic approach to (non-specifically) block cathepsin activities, we also observe a significant reduction of SAA-induced IL-1 β secretion (Fig 4A). Importantly, up to 50 μ M cathepsin inhibitor had no effect on cell viability nor did it diminish SAA-induced TNF α levels, secretion of which is not inflammasome-dependent (not shown). As other groups have implicated cathepsin-B as a modulator of amyloid-beta (130), cholesterol crystal- (145), asbestos- (146), silica-, and Alum-induced (147) IL-1 β secretion, we additionally employed a genetic approach to demonstrate that cathepsin-B-knockout peritoneal exudate macrophages and bone marrow-derived dendritic cells are fully capable of secreting IL-1 β in response to SAA (Fig 4B–C). Taken together, these data suggest that SAA can act both as an initiator of IL-1 β gene expression through the TLR2 pathway, and as a signal for inflammasome activation via the Nlrp3 pathway to induce IL-1 β secretion. However, the mechanism of SAA-induced Nlrp3 activation remains to be conclusively demonstrated.

Since airway epithelial NF- κ B activation, LPS aspiration, or NO $_2$ inhalation induce *Saa3* (Fig 3) and exacerbate pre-existing allergic airway disease (29, 69), we hypothesized that SAA too may exacerbate methacholine hyperresponsiveness when administered at the time of antigen challenge in allergically-sensitized mice. Therefore, we evaluated unsensitized mice that were injected only with Alum+saline during the sensitization regimen (Alum+saline) or Alum+ovalbumin-sensitized (Alum+ova) mice following intranasal challenge with low-endotoxin ovalbumin without or with SAA. Antigen challenge promoted airway hyperresponsiveness to inhaled methacholine in ovalbumin-sensitized mice and in Alum+saline mice administered SAA, which was exacerbated by the combination of allergic inflammation and SAA inhalation for each of the parameters measured, including airway resistance (R_N), tissue damping (G), and tissue stiffness (H) (Fig 5). SAA-exposed mice also demonstrated substantial bowing of the inspiratory limb of the pressure/volume curve as well as airway neutrophilia (not shown). These data suggest that the presence of SAA at the time of antigen challenge can exacerbate allergic airway disease even under conditions of allergen sensitization that promote Th2 responses. Therefore, it appears that SAA in the lung may be capable of eliciting immunologic and physiologic phenotypes consistent with severe allergic asthma, making pulmonary SAA an attractive therapeutic target that has recently been implicated as a mediator of steroid-resistant lung disease (93).

9. Summary

The complex interplay between airway epithelium and pulmonary leukocytes modulates allergic sensitization that predisposes to the development of allergic asthma. Therefore, a better understanding of the sequence of events leading to allergic sensitization, the involvement of resident and inflammatory cells, and the interactions that lead to airway epithelial NF- κ B activation and mediator secretion, as well as complementary pathways in epithelial and other lung cells, may provide crucial knowledge for the future development of

therapeutic interventions. The efforts of several laboratories aimed at better understanding these processes are providing novel insight bringing us ever-closer to achieving this goal.

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Figure 1. The influence of airway epithelial NF- κ B during allergen sensitization and challenge

We have developed novel transgenic and NO₂ exposure model systems to test the effects of airway epithelial NF- κ B activities in the process of allergen sensitization and during allergen challenge. **1)** We generated a transgenic mouse in which NF- κ B activation was inhibited specifically in non-ciliated airway epithelial cells by overexpressing a non-phosphorylatable I κ B α under control of the CC10 promoter (148). These mice demonstrated reduced inflammatory responses following allergen challenge as well as reduced expression of the dendritic cell chemokine CCL20 (149). **2)** Our work has revealed that NO₂ inhalation during allergen challenge exacerbated airway hyperresponsiveness to methacholine and protracted the duration of airway inflammation with neutrophils and eosinophils (29). **3)** We developed a dual transgenic mouse expressing *a)* a constitutively-active (resembling phosphorylated) IKK β (CAIKK β) under control of a promoter activated by a doxycycline-responsive transcription factor and *b)* the doxycycline-responsive transcription factor under control of the CC10 promoter. When airway epithelial NF- κ B was activated during allergen challenge, these mice produced elevated levels of IL-4, IL-17, and KC, displayed elevated lymphocytes in the bronchoalveolar lavage fluid, but showed no increase in airway hyperresponsiveness or mucus metaplasia (69). Instead, in the absence of allergen sensitization and challenge, these mice had pulmonary inflammation, were already hyperresponsive to methacholine, and displayed airway smooth muscle thickening (69). **4)** Our further work has revealed that NO₂ inhalation induced airway epithelial NF- κ B activation, which helped to protect against NO₂-mediated lung damage (27). NO₂ inhalation prior to first exposure to inhaled allergen induced allergic sensitization (35), the activation of CD11c⁺ dendritic cells, and Th2/Th17 cytokine production (36), and induced asthma like symptoms, including airway hyperresponsiveness, airway eosinophilia, Th2 cytokine synthesis, mucus metaplasia, and IgE production (35). **5)** CAIKK β mice also underwent allergic sensitization when concomitantly exposed to inhaled antigen, producing asthma-like symptoms (airway hyperresponsiveness, mucus metaplasia, airway eosinophilia) following subsequent allergen challenge. In addition, airway epithelial NF- κ B activation induced pulmonary dendritic cell activation and expression of several pro-inflammatory mediators, including *Saa3* (68). These data demonstrate that airway epithelial NF- κ B activity is sufficient to set into motion the events that culminate in allergic sensitization and the

development of allergic asthma and underscore importance of airway epithelial NF- κ B activity in the orchestration of innate and adaptive immune responses.

Figure 2. SAA is produced by lung epithelial cells following NO₂ inhalation

C57BL/6 mice were exposed to 15ppm NO₂ for 1 hour and were analyzed 24 hours later. Lungs were inflated with 4% paraformaldehyde, paraffin-embedded, sectioned, probed for SAA, SAA-Activating Factor-1 (SAF), and phospho-SAF using specific antibodies (Santa Cruz Biotechnology), and developed with diaminobenzadine to give a brown color. Data are representative of 5 mice/group.

Figure 3. *Saa3* is expressed in lungs and SAA is produced by airway epithelial cells of mice exposed to Th2/Th17-promoting regimens

Wildtype C57BL/6J or CAIKKβ transgenic mice (69) were fed doxycycline-containing chow for 3 days. C57BL/6J mice were instilled with low-endotoxin ovalbumin (OVA), exposed to 15ppm NO₂ for 1 hour, exposed to LPS or apoSAA, or were administered Alum via intraperitoneal injection. After 24 hours, lung *Saa3* gene expression was measured by Q-RT-PCR and is expressed relative to appropriate vehicle or genetic controls (A). N = 3/group.

*=p<0.05, ***=p<0.001 compared to control. Lungs were inflated with 4% paraformaldehyde, paraffin-embedded, sectioned, probed with an SAA antibody (Santa Cruz Biotechnology), and developed with diaminobenzadine to give a brown color (B). Arrows indicate areas of epithelial cell SAA3 immunoreactivity. Asterisks indicate entire airways with SAA immunoreactivity. Images are representative of >5 mice/group.

Figure 4. apoSAA-induced IL-1 β secretion does not require Cathepsin B activity

Thioglycollate-elicited peritoneal exudate macrophages from C57BL/6J mice were preincubated with cathepsin inhibitor III (Ac-LVK-CHO, Calbiochem) for 30 minutes and were treated with 1 μ g/ml SAA (Peprotech) for 16 hours. Peritoneal exudate macrophages (B) or bone marrow-derived dendritic cells (C) from wildtype C57BL/6J (WT) or Cathepsin-B-deficient (CathB^{-/-}) mice (Jackson Laboratories) were treated with 1 μ g/ml SAA for 16 hours. Secretion of IL-1 β into media was measured by ELISA.

Figure 5. apoSAA exposure during allergen challenge exacerbates airway hyperresponsiveness
C57BL/6J mice allergically sensitized with ImjectAlum (Pierce) and ovalbumin (Alum+ova) or Alum+saline on days 0 and 7 were challenged by intranasal instillation with either 100 μ g low-endotoxin ovalbumin (OVA, Hyglos), 10 μ g apoSAA (Peprotech), or OVA in combination with apoSAA on days 14, 15, and 16, and were subjected to inhaled methacholine challenge on day 17, as described (90). N=4mice/group. *= p <0.05, **= p <0.01 compared to Alum+ova/OVA at the 25mg/ml dose of methacholine.