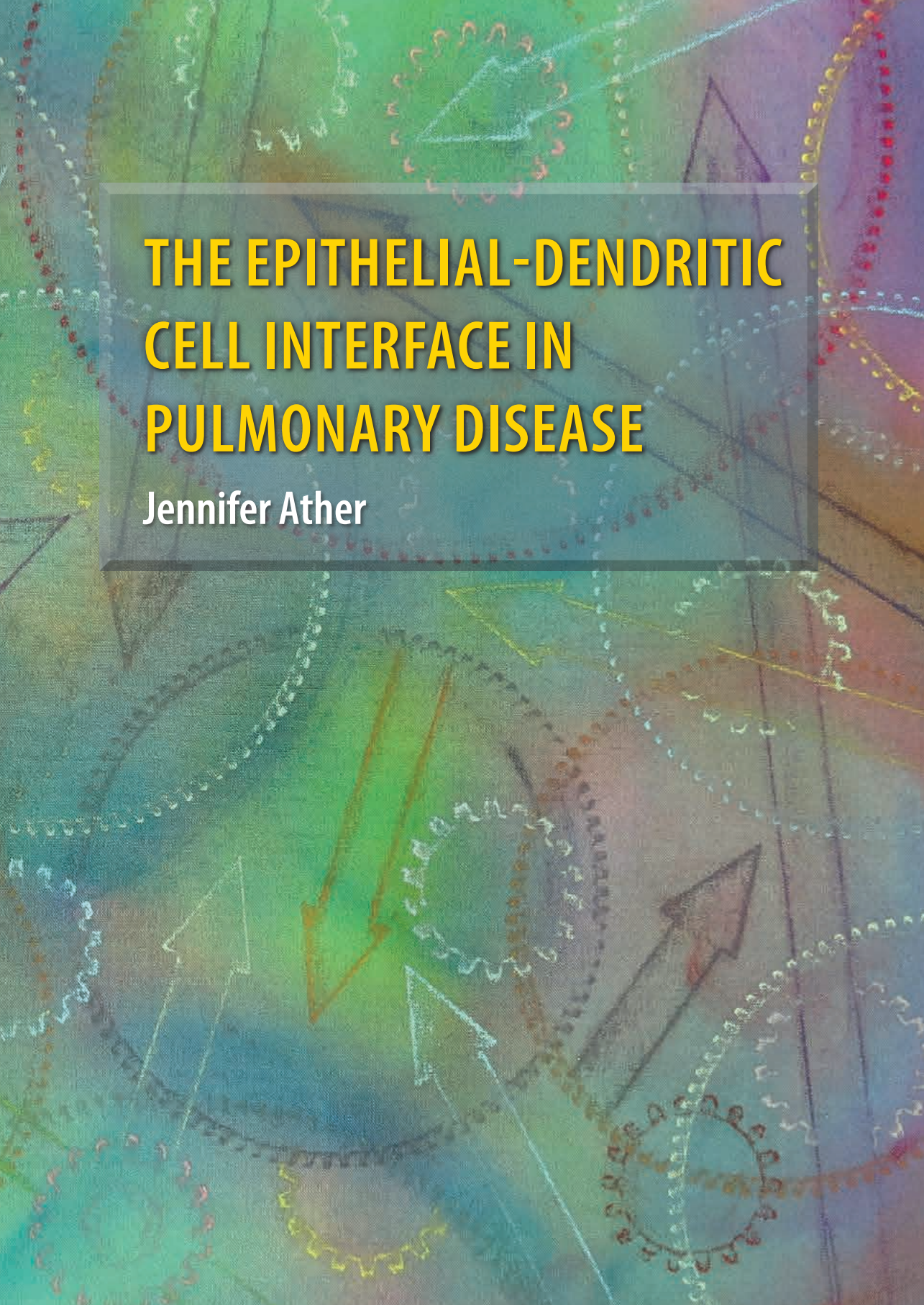
The epithelial-dendritic cell interface in pulmonary disease


THE EPITHELIAL-DENDRITIC CELL INTERFACE IN PULMONARY DISEASE
Jennifer Ather

The epithelial-dendritic cell interface in pulmonary disease

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by

Jennifer Ather

UNIVERSITAIRE PERS MAASTRICHT

Promoter

Prof. dr. E.F.M. Wouters
Prof. dr. Y.M. Janssen-Heininger

Committee

Prof. dr. B.W.W. Kramer
Prof. dr. B.N. Lambrecht
Prof. dr. A.J.M. van Oosterhout
Prof. dr. P. Savelkoul
Prof. dr. M. van Steensel

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CHAPTER 1

General introduction

1. Asthma and Allergic Airway Disease

The continued rise of asthma across the globe has greatly increased the need for improved understanding of this disease. Now recognized as a multi-faceted syndrome, asthma is phenotyped and endotyped according to differential patient responses, including the type and quantity of leukocyte infiltration, airways hyperresponsiveness, antigen-specific immunoglobulins, and responses to pharmaceutical intervention (1, 2). Allergic asthma can manifest in many different symptoms; with a key determinant being the type of T helper cell involved in the adaptive immune response. An increasing body of evidence has separated the "classic" T_H2 asthma phenotype, characterized by eosinophilia and increases in serum IgE, from the more severe T_H17 phenotype, characterized by airway neutrophilic influx, increases in interleukin-17 (IL-17), and poor response to inhaled corticosteroids (3).

An Alum/OVA asthma model in mice, involving sensitization by injection of the antigen ovalbumin (OVA) in the presence of the adjuvant aluminum hydroxide (Alum) and subsequent challenge by inhalation of nebulized or intranasally administered OVA, has long been used in the asthma research field as a powerful T_H2 model. Following antigen challenge, mice develop robust airway eosinophilia, mucus metaplasia, increases in OVA-specific IgE, increases in the T_H2 cytokines IL-4, IL-5, and IL-13, and airway hyperresponsiveness in response to inhaled methacholine challenge (4, 5). However, the relevance of a model that relies upon intraperitoneal injection for sensitization has been called into question, and a series of fully inhalational models has gained acceptance among researchers; these include the *Aspergillus fumigatus* model (6), several house dust mite (HDM) models (7), ragweed models (8, 9), cockroach antigen models (10), and using lipopolysaccharide (LPS) as an inhaled adjuvant along with OVA (11). Furthermore, several models have demonstrated the ability of oxidants, such as nitrogen dioxide (NO_2) (12) and cigarette smoke (CS) (13, 14), to sensitize mice to inhaled OVA. These new models of allergic asthma have also highlighted the role of the T_H17 phenotype and IL-17A in particular in the milieu of the asthmatic airway.

Inhalational models of allergic asthma have also begun to elucidate the participation of specific cell types in initiating and exacerbating the asthmatic response. Front and center among these respondents is the airway epithelium (15-18). Comprising the cellular barrier between the air we breathe and our bodies, the airway epithelium is uniquely poised to mount the first response to inhaled particulates and antigenic insults. The airway epithelium was originally considered to be a simple structural barrier, but it is now known that these cells not only initiate the innate immune response, but also provide polarizing signals that orchestrate adaptive immunity. A number of epithelial transcription factors are involved in these downstream signaling response to inhaled agonists, including nuclear erythroid 2

p45-related factor 2 (Nrf2) (19), Mitogen-Activated Protein kinases (MAPKs) (20), and nuclear factor-kappa B (NF-κB) (21).

1.1 NF-κB in the lung

Originally identified in B cells as an immunoglobulin enhancer binding protein (22), NF-κB quickly earned recognition as the "master regulator" of inflammation, modulating cytokine production, growth factor function, and other effector molecules in response to a wide variety of stimuli (21, 23). NF-κB is activated via a phosphorylation cascade induced downstream of numerous immune receptors, including Toll-like receptors (TLRs), B cell receptors (BCRs), T cell receptors (TCR), Tumor Necrosis Factor Receptor (TNFR), Interleukin-1 Receptor (IL-1R), and many others (24, 25). Once phosphorylated, NF-κB translocates to the nucleus and binds to genes that contain κB motif sequences, of which over 150 have been identified, and controls such diverse effects as cellular growth, differentiation, and protection against apoptosis (24, 26).

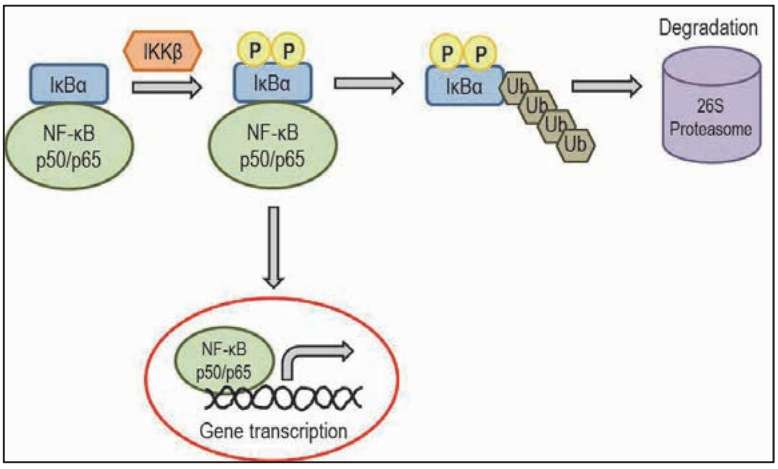

Figure 1. *Canonical NF-κB activation.*
The enzyme IKKβ phosphorylates (P) the repressor protein IκBα at serines 32 and 36. Phosphorylation of IκBα leads to its polyubituitination (Ub) and subsequent degradation by the 26S proteasome. Free NF-κB translocates into the nucleus and binds to DNA, driving gene transcription.

Mechanistically, NF-κB acts through two different and distinct pathways; the canonical pathway and the non-canonical pathway (25). In the canonical cascade (Fig.1), I kappa B kinase beta (IKKβ) is primarily activated, which phosphorylates the inhibitory I kappa B alpha (IκBα) protein. IκBα effectively sequesters NF-κB in the cytoplasm by binding to the Rel homology domain contained by all NF-κB fami-

ly members (23, 25). IKKβ phosphorylates IκBα at serines 32 and 36, leading to its subsequent polyubiquitination and degradation via the 26S proteasome. This in turn exposes the nuclear localization sequence of two NF-κB subunits, p65 (RelA) and p50, which form dimeric complexes that then translocate into the nucleus, bind to DNA, and trigger transcription of proinflammatory and pro-survival genes (23, 24).

NF-κB has been widely implicated in lung disease, including the pathogenesis of chronic infections, lung cancer, chronic obstructive pulmonary disease (COPD), and asthma. Not only has NF-κB activation been documented in human patients with asthma (27-29), but the same is true for multiple animal models of allergic airway disease (30, 31). Specifically, studies in which mice lack certain subunits of the NF-κB family have demonstrated abrogated responses to antigenic and allergic stimuli (4, 5). In previous studies, Poynter et al developed a CC10-IκBα$_{SR}$ transgenic mouse in which the IκBα protein was mutated, such that the critical serines 32 and 36 were mutated to alanines. This mutation renders the IκBα protein resistant to phosphorylation by IKKβ, thus abrogating the activation of NF-κB. These mice demonstrate a diminished response to both acute LPS challenge (32) and the Alum/OVA model (28).

1.2 Inflammasomes in the lung

A decade ago, an intracellular class of receptors that resembled disease-resistance plant R proteins was identified and named the Nucleotide Oligomerization Domain (NOD)-Like receptors (NLRs) (33). They possess a common structure, and based upon their homology to plant R proteins (Fig. 2), it was originally hypothesized that NLRs would prove to play important roles in apoptosis (which they do), however studies soon revealed that they were in fact pattern recognition receptors (PRRs) that modulated inflammatory responses to a variety of stimuli. Many of the NLRs were determined to function as scaffolds for the assembly of a complex of caspase-1 activating proteins termed “the inflammasome”, which ultimately served to activate members of the pro-inflammatory IL-1 family of cytokines, IL-1β and IL-18 (34).

While over 20 NLRs in humans and 30 in mice have been identified (35), only NLRP1, NLRP3, NLRP12, NLRC4, and AIM2 have been studied in the lung . NLRP1 was the first to be identified, and was found to be a crucial recognition molecule for anthrax lethal toxin (36). NLRP3 was initially identified as being critical in the immune response to Alum, although this was recently disproven; however, a role for NLRP3 has been demonstrated in other inhalational models of allergic airway disease (37). The role of NLRP12 is still up for debate, although a thorough study showed no role for this inflammasome in the house dust mite model (HDM) of asthma (38). NLRC4 has been thoroughly characterized as the intacellular sensor

for bacterial flagellin, and is a key modulator of gram-negative bacterial infection in the lung (39). AIM2 binds directly to double-stranded DNA from cytosolic viruses and bacteria via its unique HIN200 (hematopoetic expression, interferon-inducibility, nuclear localization, and a characteristic 200 amino acid sequence) domain.

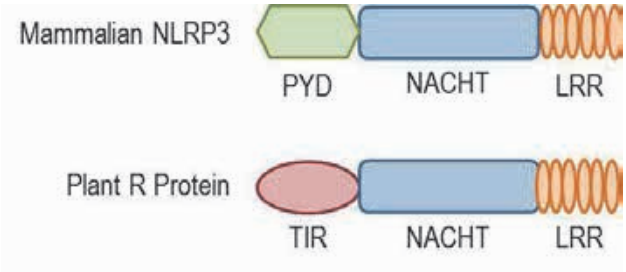

Figure 2. *The similar structures of the nod-like receptor NLRP3 and plant R proteins.*
PYD = Pyrin doman; TIR = Toll interleukin-1 receptor; NACHT = [NAIP – neuronal apoptosis inhibitory protein] ; NB-ARC = Nucleotide-binding adaptor shared by Apaf-1, R proteins, and CED-4; LRR = Leucine rich repeat sequence.

Most importantly, a better understanding has been reached as to the intracellular conditions and endogenous damage associated molecular patterns (DAMPs) that are directly responsible for inflammasome assembly and activation; conditions and molecules that become key in the setting of allergic asthma. Currently, the known mechanisms include induction of reactive oxygen species (ROS), changes in ion influx/efflux from the cell (K^+, Ca^{++}), and leakage of lysosomal contents, particularly the cathepsin family of proteases. (reviewed in (40)). Recent publications implicate mitochondrial reorganization in the cells as a trigger of NLRP3 activation (41, 42). These particular events are not unique to microbial or viral infections, but can also be induced by environmental pollutants and endogenous insults, thereby contributing to lung disease (43).

Overproduction of IL-1β has long been linked to chronic inflammatory diseases including Muckle-Wells syndrome (44, 45), multiple sclerosis (46, 47), atherosclerosis (48), rheumatoid arthritis (49), gout (50), diabetes (51), Alzheimer's disease (52, 53), and others. More recently, the emerging role for IL-1β as a T_H17-polarizing cytokine (54) has been of great interest in the field of asthma and allergy. IL-1 signaling has been determined to be critical for a T_H17 –asthma models in mice (55, 56). Though IL-1β is a crucial mediator of host defense in response to antigen insult, over-production or production in the context of auto-immunity can lead to severe tissue and organ damage.

Clinical data support a role for IL-1 receptor (IL-1R) signaling in asthma. IL-1β is elevated in lavage fluid from patients with status asthmaticus and in sputum from patients with neutrophilic asthma (57-59). IL-1β can both directly and indirectly impact the pathogenesis of asthma. In mouse models, IL-1β has been found to exacerbate airway hyperresponsiveness (60) and airway neutrophilia (61) and can thus contribute directly to the symptoms of asthma. In addition, evidence from *in vitro* and *in vivo* studies has implicated a requirement for IL-1R signaling in the development of T_H17 responses (62-65). In a mouse model of pulmonary fibrosis, the instillation of IL-1β into the airway is sufficient to induce IL-17 production upon restimulation of cells from lymph nodes that drain the lungs (66). Additionally, the NO_2/OVA model of allergic airway disease requires both IL-1 signaling and caspase-1 activation for allergic responses, and intranasal instillation of IL-1β is sufficient to drive a T_H17 asthma phenotype (37).

The T_H17 adaptive immune response has been linked to neutrophilic glucocorticoid-resistant asthma in humans and is correlated with disease severity (67-70). *In vitro* and *in vivo* data have supported a causal role for the T_H17 response in glucocorticoid resistance (71-73) and additional studies suggest that IL-1R signaling can synergize with IL-17 in the modulation of chemokine release from human bronchial epithelial cells and impact glucocorticoid responsiveness (74, 75). Given IL-IR's critical role in T_H17 development (62) and IL-1β's wide-ranging involvement in acute inflammatory processes, it seems that inflammasome activity is likely to be involved in at least some subsets of asthma. While the contribution of the inflammasome to T_H17 development has not been extensively studied in the lung, the NLRP3-IL-1R-T_H17 axis has been explicitly hypothesized to contribute to allergic airway disease pathogenesis (76).

It is clear that airway epithelial activation, NF-κB signaling, and the contributions of the inflammasome conspire to create an inflammatory environment that is conducive to allergic sensitization.

2. The Airway Epithelial-Dendritic cell interface

Dendritic cells are considered to be "professional" antigen presenting cells, and are critical both for innate immune cell development (77) as well as for the generation of adaptive immune responses (78). Mature DCs can engulf and process antigen, upregulate expression of MHC I and MHC II as well as co-stimulatory molecules necessary for interactions with naïve T cells, and migrate to draining lymph nodes in order to present processed antigen (79). Subsets of pulmonary dendritic cells lie in close proximity to airway epithelium, and are even capable of extending dendritic processes through the epithelium to sample the airway lumen for antigen. In this way, the two cell types work together to monitor inhaled material for potential

harm and, as such, orchestrated crosstalk between these cells is of critical importance for mounting an immune response. It should come as no surprise that the mediators secreted by the airway epithelium heavily influence dendritic cell responses, and thus modulate antigen presentation and the resulting adaptive T cell response.

2.1 Acute inflammatory mediators secreted by the epithelium

Airway epithelial cells express a variety of innate cell surface receptors, including TLRs, TNFRs, and more (16). The binding of a harmful ligand to these receptors triggers a downstream cascade of gene transcription and protein production that can result in polarizing cytokine secretion. Airway epithelium generate a wide variety of innate effectors, including cytokines, chemokines, growth factors, and defensins. These include interleukins, such as IL-6, IL-12, IL-25 and IL-33; chemokines such as KC, MIP-2, and CCL20; growth factors such as G-CSF, GM-CSF, TSLP, and TGF-β, and defensins; i.e. β-defensin (16, 80). Epithelial-derived IL-12p40 can drive T_H1 responses, as in the case of viral infection. IL-6, IL-25, and IL-33 drive T_H2 adaptive responses, and IL-6, IL-1β, Serum amyloid A (SAA) and (in mice) TGF-β will collaborate to induce a T_H17 phenotype.

Serum amyloid A

Serum amyloid A was discovered well over thirty years ago, characterized as a 12 kDa acute phase protein produced by the liver in response to injury or infection that could circulate in the serum bound to high density lipoprotein (HDL) (81). It proved a rapidly and robustly induced biomarker of inflammation, upregulated over 1000-fold in the blood in response to infection (82). It also gained notoriety as a precursor protein for β-amyloid (81, 83), the culprit misfolded protein in the amyloid plaques found in the brain tissue of Alzheimer's patients. It was soon acknowledged to play a role in multiple inflammatory diseases, including atherosclerosis (84), rheumatoid arthritis (85), and type 2 diabetes (86). Further studies demonstrated that, far from being a mere "biomarker" of infection, SAA could contribute directly to the pathology of these diseases through cytokine-like affects. SAA is a ligand for both TLR2 (87) and Formyl peptide receptor-like 1 FPRL-1/FPR2 (88), and instillation of recombinant apo-SAA into the lung results in rapid and robust neutrophilia, as well as increased induction of the proinflammatory cytokines IL-1β, IL-6, and TNFα (89).

Multiple isoforms of SAA have been found. In humans, SAA1 and SAA2 are closely related (over 90% homologous in nucleotide sequences) and though most commonly expressed in the liver, can also be acutely expressed by monocytes and other cells such as gut and airway epithelium (90). For many years, SAA3 in humans was believed to be a pseudogene, though recent research has found func-

tional SAA3 in the human mammary gland (91), and SAA4 is known as Constitutive SAA (C-SAA). Though C-SAA is similar in structure to SAA1 and 2, there are significant differences in the promoter region, and C-SAA is not upregulated during inflammation, but functions at basal homeostatic levels and contributes to reverse cholesterol transport (92, 93).

In mice, SAA1 and 2 are also most heavily expressed in the liver, though they can also be expressed extrahepatically in other tissues and in leukocytes. Unlike humans, in mice and other non-human mammals, SAA3 is the functional isoform most commonly expressed extrahepatically (94). There is little homology between murine SAA3 and SAA1 and 2; in fact, murine SAA3 is as similar to human SAA as it is to murine 1 and 2 (90). SAA4 is a pseudogene in mice, and they possess a fifth isoform (SAA5) that functions as their constitutive form.

Several transcription factors have been documented to regulate SAA expression in the liver, among them NF-κB and C/EBP (95); however, several studies have implicated a family of transcription factors, the serum amyloid activating factors (SAFs), as critical modulators of acute, extra-hepatic SAA production seen in chronic inflammatory conditions. Expression levels of SAF-1 are undetectable in normal tissue, but abundant in tissues of patients with amyloidosis, and this corresponds (in humans) with levels of SAA1. SAF-1 binds directly to a consensus sequence in the SAA promoter, and its transactivating ability appears to require phosphorylating events by MAP kinases (96) and Phosphokinase C (PKC) (97), as well as conformational changes by Phosphokinase A (PKA) (98, 99). Transgenic mice that overexpress SAF-1 develop spontaneous amyloidosis at 12-14 months, and at younger ages can be easily induced to develop amyloidosis by injection with azocasein (100). These mice are also more susceptible to developing arthritis in response to *Borellia burgdorferi*, the causative agent of Lyme Disease (101). Of particular significance in these studies was the revelation that *localized* production of SAA1 was responsible for the formation of amyloid lesions; previously, it was assumed that amyloid deposits occurred secondary to high circulating levels of SAA1 produced by the liver. Similar to SAF-1, the splice variant SAF-3 is also upregulated in cases of inflammation and possesses greater transcriptional activation potential than the previously-identified SAF-1 (102). The splice variant SAF-2 appears to act as a negative regulator of transcription (103).

In addition to transcriptional regulation, SAA is also subject to post-translational modification. The amyloid lesions for which SAA is the precursor are comprised of the 76-residue amino terminal fragment of SAA that has a β-sheet conformation, and it is thought that these fragments are generated by incomplete or inappropriate proteolysis (104, 105). Whereas SAA1 is the predominant isotype found in *human* amyloid lesions, in mice only the SAA2 protein is found in amyloid deposits caused by chronic inflammation. Interestingly, CE/J mice are amyloid

resistant, unique in that they produce a form of SAA that is a "hybrid" of the normal murine SAA1 and 2, lacking a key amino acid sequence at positions 7 and 8 (106).

SAA cleavage sites vary, depending on tissue location, resulting in peptides that are 45-95 amino acids long. Both monocytes and neutrophils can degrade acute SAAs; monocytes by endocytosing the protein and performing proteolysis in endosomes, neutrophils by extracellularly releasing elastase. There is also a body of evidence linking cathepsins to SAA degradation. Cathepsins D and L are effective at degrading SAA, and in tissues that contain an abundance of these types of cathepsins, SAA degradation is more rapid and complete, and as a result, amyloid lesion load is lower (107, 108). In contrast, Cathepsins B and K have been implicated as being far less effective, generating aberrant N-terminal fragments of SAA that go on to form amyloid deposits (107, 108). As Cathepsin B is the primary isotype found in glial cells, it has been hypothesized that is it responsible for the inappropriate cleavage of SAA that leads to Alzheimer's Disease (109).

While most studies of amyloidosis focus on the N-terminal fragments of SAA, the C-terminal end has other innate immune functions. Early studies implicated the C-terminus as the binding region for neutrophils (110). Other functions have been proposed for SAA as well, including T cell migration and adhesion (111), opsonization and enhanced clearance of gram negative bacteria (112), and dysregulated cholesterol transport (113). Further, SAA enhances the activity of Phospholipase 2 (PLA_2) (114), which hydrolyzes arachidonic acid into eicosanoids. Recombinant SAA induces upregulation of Prostaglandin E2 (PGE_2), which can act upon dendritic cells to drive production of IL-23 and subsequent expansion of T_H17 cells (115).

2.2 Epithelial damage and barrier dysfunction

The airway epithelium must not only initiate innate immune signaling, but also maintain healthy barrier function. Lung injury or trauma that compromises the epithelial barrier can lead to more invasive infections and decreased lung function.

Nitrogen Dioxide

In addition to acting as the first responder to bacterial, viral, and allergen insult, the airway epithelium also orchestrates the response to oxidant stress (116-119) in the form of inhaled pollutants such as nitrogen dioxide (NO_2). NO_2 can affect the respiratory system in two ways; as an inhaled air pollutant, or as an endogenously generated product of inflammatory cells, such as neutrophils and eosinophils. NO_2 is absorbed all along the respiratory tract, and exposure in humans has been linked with induction of allergic asthma (120). Experiments have been performed in which human bronchial epithelial cells were exposed to acute doses of NO_2, resulting in the production of the inflammatory cytokines IL-8, IL-1β, and TNF-α, and also cytotoxicity (119, 121). NO_2 inhalation at high doses serves as a model of

acute lung injury. Mice exposed to 25 ppm of NO_2 for 6 hours a day for three days, show a robust inflammatory response, characterized by the influx of inflammatory leukocytes into the airways, increased production of chemokines, cytokines, and other inflammatory mediators, increased serum protein and lactate dehydrogenase leakage into the airways, and thickening of the airway walls. This injury is reversible and decreased by day 5, and it is completely resolved 20 days after cessation of the 3-day exposure period (122).

Epithelial alterations in the asthmatic lung

Damage to the airway epithelium is a common outcome of allergic airway disease, and may function to amplify the inflammatory process (29, 123, 124). Though atopy is often a symptom of asthma, it is not ubiquitous; that is, atopy does not always lead to asthma. To examine why this is so, studies have focused on "susceptibility" genes, many of which are epithelial-specific (IL33, SMAD3, ORMDL3) rather than related to atopy or IgE expression and function (i.e. FCER1A, STAT6, IL13). The theory arising from this literature is that the epithelium of asthmatics may be inherently defective, particularly in the formation of tight junctions between epithelial cells (124).

A basal dysfunction in epithelial formation may account for the airway remodeling that occurs in asthmatic patients. Two hallmarks of remodeling observed in asthma are subepithelial fibrosis and airway smooth muscle (ASM) thickening (125), the latter of which may be due to hypertrophy or, as was recently hypothesized, due to increased ASM cell migration in response to chemokines secreted by the epithelium (107). Both RANTES and IL-8, two cytokines secreted by epithelium, can induce ASM cell migration (125). Sputum from asthmatic patients often contain "creola bodies", which are clumps of uncleared epithelial cells sloughed from the airways (126, 127). In addition, the composition of the airway epithelium also differs between healthy subjects and those with asthma, with the asthmatic lung containing more resident stem cells, basal cells, and fewer ciliated cells (128). This lends credence to the theory that epithelial malformation, rather than simply T_H2 and serum IgE components, create the setting for asthma to take hold. The epithelial abnormalities encourage airway *repair*, but not regeneration, which allows for allergen penetration and leads to ongoing, localized inflammation (128).

In addition to structural abnormalities, the asthmatic airway epithelium also differs in its expression of pattern recognition receptors (PRRs). Airway epithelium express a range of functional TLRs (at least TLR1 through TLR10) (129) and expression of these receptors can be upregulated in response to insult or injury. Studies have demonstrated that challenge with respiratory syncytial virus (RSV) or exposure to cigarette smoke upregulate the expression of TLR4 on the surface of the epithelium, rendering these cells more sensitive to endotoxin (130, 131), and likely allergens, such as Derp2, that trigger TLR4 (132).

It is also of note that there is an emerging body of literature focused on the lung microbiome; particularly the striking differences between the microbiota that colonize healthy lungs vs. asthmatic lungs (124). The role of commensal bacteria in the development of the immune system has been strongly documented in the gut (133), with emerging roles for microbiota-induced SAA in skewing towards a T_H17 response in the mouse intestine (134). Until recently, the healthy lung was considered to be a sterile environment, but this has proven not to be the case (135). The lungs of healthy, non-smokers contain a small total number of lung commensals, whereas the lungs of asthmatic patients support greater bacterial burden of diverse colonies (136). One intriguing finding of asthmatic cohort studies indicates that steroid-unresponsiveness, characteristic of severe asthma, may be the result of respiratory colonization by the steroid-degrading *Comamonadaceae* family (137).

3. Dendritic cells in the lung

There are no dendritic cells present in the airways at birth; in time, immature DCs from the bone marrow are mobilized and recruited to the lung, likely in response to damage, microbial infiltration, commensal activation, and irritants that activate the epithelium (124). The airway epithelium is a prime source of secreted chemokines that recruit dendritic cells, including CCL5, CCL20 (Mip-3α), and CCL127.

The lung network of dendritic cells, which can be influenced and polarized depending on the secretions and innate responses of the epithelium, consist of three main populations; plasmacytoid DCs (pDCs), conventional myeloid DCs (cDCs) and monocyte-derived DCs (monoDC). The pDCs are generally Siglec $H^+CD11c^+CD11b^+$, and perform distinct functions depending on the situation. Compared to their myeloid counterparts, they are poor at phagocytosis and thus also poor at exogenous antigen presentation (for this reason they are not considered to be "professional" antigen presenting cells) (138). However, pDCs are better at presenting endogenous antigen, and preferentially express intracellular TLR7 and TLR9 (138, 139). This makes pDCs key in the reponses to viruses, acting as the main viral antigen-presenting cell, secreting large amounts of type I interferons, and driving $CD8^+$ T cell activation and subsequent viral clearance by cytotoxic T lymphocytes (138). Conversely, they also promote inhalational tolerance in response to innocuous inhaled antigen; adoptive transfer of antigen-pulsed pDCs induces tolerance to OVA (140), and lack of pDCs results in increased susceptibility to allergic sensitization (141).

The conventional myeloid DCs are further divided into two distinct subsets: the $CD103^+$ subset, which lies in close contact with the airway epithelium and samples inhaled antigen, and the $CD11b^+$ subset which exist mainly in parenchy-

mal spaces (142, 143). MonoDCs are a $CD11c^+$, $CD11b^+FCERI^+Ly6C^+CD64^+$ subset. $CD11b^+$ mDCs and monoDCs function similarly to take up allergen and migrate to the draining lymph nodes, where they present to T cells (144). Studies have shown that $CD11b^+$ mDCs are primarily more efficient at generating T_H2 adaptive responses, whereas $CD103^+$ presentation to naïve T cells results in greater amounts of IFNγ and IL-17 (145).

3.1 Dendritic cell maturation and antigen presentation

In the absence of activating stimuli, dendritic cells that sample harmless antigen will present it to T cells without any co-stimulatory molecule involvement, which results in the expansion of an antigen specific, $Foxp3^+CD25^{HI}$ T_{REG} population that is immunosuppressive (146). However, if the antigen encountered is of pathogenic origin, or if an innocuous antigen is encountered in the presence of a PAMP or in the setting of a DAMP, it will stimulate pathogen recognition receptors (PRRs) on the cell surface, such as TLRs, that will trigger a cascade of immunostimulatory signaling. These signals in dendritic cells result in the upregulation of co-stimulatory molecules on the cell surface, including CD40, CD80, CD86, MHC II, and OX40L. The processed antigen is then presented to the naïve T cell in conjunction with co-stimulatory signals and inflammatory cytokine production, leading to the expansion of antigen specific T effector cells (79). In the context of allergic asthma, cytokines produced by the airway epithelium act upon dendritic cells and determine the "education" of the naïve T cells. For instance, high levels of airway epithelial IL-25, TSLP, and IL-33 are known to encourage DCs to drive a T_H2 response (147), whereas TLR7 and TLR8 agonists induce DCs to produce high levels of IL-12p70 and promote T_H1 expansion (148). Fungal infections, in particular, induce strong IL-1β, IL-6, and IL-23 production from DCs themselves, which drives a T_H17 response (149, 150). The T effector cells then orchestrate the destruction/removal of the invading pathogen.

Difficulties arise, however, when these anti-pathogenic responses are generated in response to an antigen that is either non-pathogenic or "self"-generated, as in the case of allergy and autoimmune diseases, respectively. In these cases, recognition of a harmless antigen *should* result in tolerance, but fails to do so.

3.2 Tolerance vs. Allergy

The majority of animal models of allergic asthma have two distinct temporal components required to generate disease: a sensitization phase and a challenge phase. Sensitization is required to expand the small number of naturally circulating, antigen-specific T cells, and polarize them in a pathogenic lineage, and is accomplished by delivering the antigen to the animal in the context of an adjuvant (i.e. aluminum

hydroxide, LPS, etc). Sensitization results in a more rapid and numerically larger expanded population of antigen-specific $T_H1/T_H2/T_H17$ cells. The challenge phase provides only the antigen, and stimulates the $T_H1/T_H2/T_H17$ populations to effect detrimental responses (151). These models represent a situation in which the individual (mouse) has not before encountered this harmless antigen.

However, an alternative "real-world" situation involves generation of an allergic airway response to an innocuous antigen that the individual *has* encountered previously, and to which he or she was not previously allergic. That is, the new inflammatory responses occur despite antigen-specific T_{REG} cells being present; this can be referred to as "breaking tolerance". How this occurs represents a very different problem from naïve sensitization, and may have much to do with changes in the resident dendritic cells.

In a seminal paper, Odemuyiwa *et al*. reported that mice with previous inhalational exposure to OVA could not later be sensitized to this antigen, even when delivered concurrently with an adjuvant (152). This paper also established the importance of indolamine-2,3-dioxygenase (IDO) and tryptophan catabolism in maintaining tolerance and preventing allergic sensitization. Furthermore, a simple adoptive transfer of T_{REG} cells can confer resistance to allergic sensitization (153). The "breaking" of tolerance requires exposure to a previously-tolerated antigen concurrent with a new inflammatory signal, such as a Toll-like receptor agonist (154). There is support in the literature for the idea that dendritic cell phenotype plays a role in generating tolerogenic responses, specifically that tolerizing regimens encourage expansion of tolerogenic plasmacytoid DCs in the lung (155). Plasmacytoid DCs often promote tolerogenic responses due to the fact that they are poorly phagocytic and thus not efficient processors and presenters of *exogenous* antigen, and also because they express high levels of IDO (138).

4. Apoptosis and the resolution of inflammation in the lung

Apoptosis is a critical process in tipping the balance between chronic inflammation and resolution. As a form of programmed cell death, apoptosis changes the morphologic and molecular structures of cells, causing cytoskeletal rearrangement, chromatin condensation, DNA fragmentation, and formation of apoptotic bodies (156). Unlike necrosis, the goal of apoptosis is to control the death of cells such that they do not release inflammatory mediators into the extracellular milieu. The critical caspases involved, once thought to trigger apoptosis, are emerging instead as gate-keepers of cellular contents, including HMGB1, S100 proteins, and cathepsins that might otherwise act as DAMPs (157).

NF-κB is most commonly associated with anti-apoptotic effects, and indeed, deficiency in p65 (RelA) results in an embryonic lethal phenotype due to excessive

apoptosis of developing hepatocytes (26). Similar phenotypes are present in mice that are deficient in IKKα, IKKβ, and IKKγ (NEMO) (26). NF-κB drives the expression of pro-survival genes such as Gadd45β, Bcl-2, xIAP, cIAPs, and c-FLIP, and over-expression of NF-κB (and these survival genes) is commonly seen in cancer (24, 158, 159). It has also been demonstrated that inhibition of NF-κB increases tumor killing in conjunction with traditional cancer therapy (160). NF-κB can also override pro-apoptotic signals induced by TNFα (161, 162) and the c-Jun N Terminal Kinase (JNK) signaling pathways (163, 164). Prolonged JNK activation and subsequent apoptosis have been demonstrated in models where NF-κB is deficient (165).

In cases of acute lung injury or infection, the influx of leukocytes can prove detrimental if apoptosis is dysregulated. Neutrophils in particular have a short lifespan (2-3 days) and function against invading pathogens via apoptosis and respiratory burst, releasing factors that not only elicit antimicrobial effects, but that can be harmful to host tissue as well. Neutrophil elastase, a 29 kDA serine protease stored in neutrophil granules and released upon burst, can degrade almost all extracellular matrix, plasma proteins, and protease inhibitors, as well as activate matrix metalloproteinases (MMPs) that can further break down host tissue (166). As mentioned in Section 1.2, neutrophil elastase is also emerging as an activator of IL-1β in certain contexts (167). It has been suggested that improper neutrophil apoptosis may contribute to lingering inflammatory conditions (156), and is has been hypothesized that the pro-survival factors induced by NF-κB can have direct anti-apoptotic effects on neutrophils themselves, thus overriding the normal programmed cell death of these cells and contributing to a chronic inflammatory state (168).

4.1 DC lifespan regulates T cell death and resolution of inflammation

The resolution of an effector T cell response also requires strictly regulated dendritic cell apoptosis. Under homeostatic conditions, naïve dendritic cells that do not encounter antigen will undergo caspase-3 dependent apoptosis. Those that do encounter antigen, and mature to present that antigen to T cells, will initiate a "molecular timer", that ensures proper apoptosis and an end to antigen presentation (169). It is believed that the short lifespan of mature DCs is designed to limit antigen availability to T cells (123). Significant DC apoptosis results in immunosuppression; in fact, apoptotic DCs can be taken up by viable DCs and result in tolerance via generation of antigen-specific T_{REG} cells (170). Lack of dendritic cell apoptosis, therefore, can either lead to autoreactive T and B cells (in the case of dysregulated naïve cell apoptosis) or to chronic adaptive immune responses in which the DC-antigen-T cell interaction persists (171).

4.2 The role of HSP70 in the regulation of DCs

The intracellular protein, heat shock protein 70 (HSP70), performs a number of roles within the cell, particularly in dendritic cells. As the name suggests, HSP70 is a DAMP that responds to cell trauma, particularly heat shock, but also changes in cellular stretch, oxidative stress, or nutrient availability. HSP70 is widely expressed and highly conserved across species and cell types, and can function both as a cytokine and as a chaperone molecule for protein complexes; for this reason, it has been aptly dubbed a "chaperokine". In company with another HSP, HSP90, HSP70 forms the chaperone complex that is responsible for antigen loading on MHC II (172), and changes in the HSP70/HSP90 balance can lead to altered antigen presentation (173).

In addition, HSP70 is overexpressed in cases of dysregulated apoptosis (174), most notably in tumor cells. The increased expression of HSP70 is indicative of adenocarcinomas, and inhibition of HSP70 can lead to increased tumor killing and greater efficacy of chemotherapeutics (175). Though normally found in the cell, HSP70 can be found in the extracellular space, particularly in cases of cell-membrane permeabilization (necrosis) and in some cases it can also be secreted. Extracellularly, it can function as a cytokine, binding to a number of receptors including TLR2, TLR4, CCR5, and CD40 (176) and enhancing the secretion of TNFα, IL-1β, IL-6, and IL-12 (177) via activation of NF-κB (172, 178). In the lung, HSP70 has been linked to epithelial breakdown and oxidant stress. HSP70 levels are not only increased in the sputum and plasma of asthmatic patients, but levels correlate with the severity of disease and may act as a predictor of airway obstruction (179). Increases in HSP70 are documented in response to cigarette smoke exposure in mice (180) and in patients afflicted with COPD (181).

The basal function of HSPs is to respond to heat stress in cells, and to fold heat-denatured proteins that could otherwise act as DAMPs and initiate caspase-dependent apoptosis; in that sense, their purpose is to repair damage in the cell and avert death (176). But in addition to orchestrating repair within the cell, HSP70 can also be released, either following necrosis, or as a signaling molecule. Once released from the cell, HSP70 can bind to a number of different cell receptors, including CD91 (182), CD40 (183), TLR2 (184), TLR4/CD14 (184), CCR5, and several scavenger receptors (185, 186), which may function to internalize HSP70 (176). The first report of HSP70 release was from neuronal cells (187), but soon it was found to be secreted from other cells and tissues, including brown adipose tissue, leukocytes, and epithelium. In addition, HSPs are also released from pathogens; prokaryotic HSPs function as innate stimuli to drive inflammation and clearance (176) Notably, HSP70 is not secreted by the classical pathway; instead, evidence indicates that it is released either by direct cell lysis (necrosis) or in secretory vesicles (176).

Aims and outline of the thesis

The interplay between airway epithelial inflammatory signaling, dendritic cell maturation, and T cell responses in allergic asthma highlights the complexity of the immune system. Inappropriate hyperresponsiveness to an innocuous antigen requires dysfunction at all three steps, in the form of proinflammatory NF-κB signaling, immunostimulatory antigen presensation by dendritic cells, and poor resolution of the adaptive T cell response. Alterations that occur in the structure of the airway epithelium, as well as in dendritic cell apoptosis, may contribute to a chronic, unresolved inflammatory state in the asthmatic lung.

The chapters that comprise this thesis aim to describe the consequences of NF-κB activation specifically in the airway epithelium, and the role of this transcription factor in initiating and exacerbating allergic sensitization. Chapter 2 details the characterization of a transgenic mouse expressing a doxycycline-inducible, constitutively active form of IKKβ ($_{CA}$IKKβ) in the non-ciliated airway epithelial cells of the lung. Chapter 3 expands upon a model of acute lung injury, in the form of NO_2 inhalation, and the participation of airway epithelial NF-κB activation therein. In chapter 4, the $_{CA}$IKKβ transgenic mouse is utilized in a novel model of NF-κB induced allergic sensitization. Chapter 5 narrows the focus of allergic sensitization to the effects of a single mediator, serum amyloid A, and in Chapter 6, the transgenic $_{CA}$IKKβ mouse is used to provide insight into the cellular and moleculr mechanisms of overcoming inhalational tolerance, with special attention paid to the role of dendritic cell activation and maturation in polarizing allergic T cell responses. Finally, in Chapter 7, the role of dysregulated dendritic cell apoptosis is examined in the context of generating T_H17 responses.

A discussion of the results of this thesis will be presented in Chapter 7, and Chapter 8 will summarize the overall findings.

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CHAPTER 2

Nuclear factor-κB activation in airway epithelium induces inflammation and hyperresponsiveness

Cristen Pantano, Jennifer L. Ather, John F. Alcorn, Matthew E. Poynter, Amy L. Brown, Amy S. Guala, Stacie L. Beuschel, Gilman B. Allen, Laurie A. Whittaker, Mieke Bevelander, Charles G. Irvin, and Yvonne M. W. Janssen-Heininger

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ABSTRACT

Rationale: Nuclear factor (NF)-κB is a prominent proinflammatory transcription factor that plays a critical role in allergic airway disease. Previous studies demonstrated that inhibition of NF-κB in airway epithelium causes attenuation of allergic inflammation.

Objectives: We sought to determine if selective activation of NF-κB within the airway epithelium in the absence of other agonists is sufficient to cause allergic airway disease.

Methods: A transgenic mouse expressing a doxycycline (Dox)-inducible, constitutively active (CA) version of inhibitor of kB (IkB) kinase-beta (IKKβ) under transcriptional control of the rat CC10 promoter, was generated.

Measurements and Main Results: After administration of Dox, expression of the CA-IKKβ transgene induced the nuclear translocation of RelA in airway epithelium. IKKβ-triggered activation of NF-κB led to an increased content of neutrophils and lymphocytes, and concomitant production of proinflammatory mediators, responses that were not observed in transgenic mice not receiving Dox, or in transgene negative littermate control animals fed Dox. Unexpectedly, expression of the IKKβ transgene in airway epithelium was sufficient to cause airway hyperresponsiveness and smooth muscle thickening in absence of an antigen sensitization and challenge regimen, the presence of eosinophils, or the induction of mucus metaplasia.

Conclusions: These findings demonstrate that selective activation of NF-κB in airway epithelium is sufficient to induce airway hyperresponsiveness and smooth muscle thickening, which are both critical features of allergic airway disease.

Introduction

Allergic airway disease is characterized by eosinophilic inflammation and airway remodeling, which can induce mucus metaplasia, alterations in smooth muscle and blood vessel compartments, extracellular matrix deposition, and airway hyperresponsiveness (AHR) to inhaled bronchoconstricting agonists. The airway epithelium has also been suggested to play a causal role in initiating inflammation after exposure to antigen.

One critical regulator of inflammatory and immune processes is the transcription factor, nuclear factor (NF)-κB. NF-κB plays a causal role in the secretion of proinflammatory mediators from pulmonary epithelial cells exposed to various insults (188-190). NF-κB is sequestered in the cytoplasm through binding to inhibitor of κB protein (IκB) a. Phosphorylation of IκBα on serines 32 and 36 by the IκB kinase (IKK) signalsome leads to the ubiquitination and degradation IκBα, leading to the nuclear localization NF-κB, and enhanced transcriptional activation of NF-κB–controlled target genes (191). IKK exists as a complex consisting of three proteins: IKKα, IKKβ, and IKKγ. IKKα and IKKβ contain enzymatic activity, whereas IKKγ is a regulatory component of the signalsome that regulates protein–protein interactions. In the canonical pathway of NF-κB activation, which is activated by a wide array of stimuli, such as TNF-α, T cell receptors, and Toll-like receptors, IKKβ induces phosphorylation of IkBα, leading to transcriptional regulation of NF-κB target genes controlled by RelA/p50 dimeric complexes (191).

NF-κB has been widely implicated in the pathogenesis of asthma, and evidence for activation of NF-κB in bronchiolar epithelium is present in animal models of allergic airways disease (192), as well as in patients with asthma (193, 194). Furthermore, mice deficient in the NF-κB family member, c-Rel, or p50, have decreased pulmonary inflammation and AHR in a model of allergic airway disease (30, 31, 195). Through the use of transgenic mice and conditional ablation strategies, we and others recently demonstrated that activation of NF-κB within airway epithelium is necessary to induce airway inflammation after sensitization and challenge with ovalbumin (OVA) (196, 197). Although the aforementioned studies demonstrate that activation of NF-κB plays a causal role in allergic disease in mice, they did not address whether activation of NF-κB within airway epithelium is sufficient to drive allergic airway disease independent of other stimuli. Therefore, the goal of the present study was to create a transgenic mouse model in which activation of the canonical NF-κB pathway within the airway epithelial compartment is inducibly regulated, in order to determine its contribution to features of allergic airway disease. Transgenic mice in which a constitutively active (CA) version of IKKβ (CA-IKKβ) is expressed under the control of the CC10 promoter and the tetracycline operon (TetOP) were generated in order to activate the canonical NF-κB pathway within airway epithelium in response to administration of doxycycline

(Dox). Some of these studies have been previously reported in the form of an abstract (198).

Materials and Methods

Generation of CC10-Tetracycline-inducible CA-IKKβ transgenic mouse.
Two independent lines (33 and 50) of bi-transgenic mice expressing CC10-M2-hGHpA, and Tet-OP-CA-IKKβ constructs were generated in order to induce expression of constitutively active IKKβ in bronchiolar epithelium, following administration of doxycycline (Dox). Mice were backcrossed for at least 5 generations into C57BL/6J mice and transgene negative (WT) littermates were used as controls. The Institutional Animal Care and Use Committee at the University of Vermont gave approval for all studies.

Culture of primary mouse tracheal epithelial cells.
Primary mouse tracheal epithelial (MTE) cells were isolated from CA-IKKβ positive mice and littermate controls, as described elsewhere (199).

Analysis of transgene expression.
RNA from whole lung was extracted and reverse transcribed and PCR analysis for CA-IKKβ cDNA expression performed using the forward primer used in genomic analysis and an hGHpA intron spanning reverse primer; GAGCAGGCCAAAAGCCAGGA.

Bronchoalveolar lavage (BAL).
BAL fluid was immediately collected, and cell counts evaluated, as described previously (200).

Histological and immunofluorescence analysis.
Following euthanasia and BAL, lungs were fixed with 4% paraformaldehyde , for the generation of paraffin sections and hematoxylin and eosin staining to evaluate histopathology (200). For evaluation of nuclear localization of RelA, frozen lung sections were prepared, or MTE cells were fixed, stained (200) and analyzed by confocal microscopy. To evaluate smooth muscle changes, sections were stained with an α-SMA antibody (Sigma).

Analysis of NF-κB driven inflammatory genes.
cDNA was synthesized from MTE cells or pulverized lung tissue and expression of KC, MIP-2, and CCL20 mRNAs were quantified by real time TaqMan PCR. Cytokine levels in the BAL fluid or cell culture media were assessed by a 23-cytokine Bioplex assay (Bio-Rad, Hercules, CA).

Model of allergic airway disease.
Allergic airway inflammation was induced as previously reported (192). Mice were injected intraperitoneally with 40 μg of ova plus Alum on days 1 and 7. WT or CA-IKKβ transgenic mice were given Dox starting on day 7 and continuing until euthanization. Mice were subjected to aerosolized 1% ova in sterile PBS for 30 minutes on days 14-16, and euthanized 2 days following the final ova challenge.

Respiratory mechanics and determination of AHR.
Anesthetized mice were mechanically ventilated for assessment of respiratory mechanics using the forced oscillation technique as previously described (5, 201), (*flexi*Vent, SCIREQ Inc. Montreal, QC, Canada).
Statistical analyses. All experiments were repeated at least once. Data are presented as mean value ± standard error of the mean, and were subjected to ANOVA or Student's t Test where appropriate. Analyses with resultant $p < 0.05$ were determined significant, except where noted.

Results

Characterization of doxycycline (Dox)- inducible CC10 -CA-IKKβ transgenic mice.
To examine whether activation of the canonical NF-κB pathway in airway epithelium is sufficient to induce features associated with allergic airway disease, bi-transgenic mice were generated in which constitutively active IKKβ, SS171/181EE (CA-IKKβ), was expressed under the control of the Tetracycline operon, (TetOP), and expression was targeted to non-ciliated airway epithelial cells using the rat CC10 promoter to express the tetracycline transactivator, (tTA-M2) (196, 202, 203). Two lines of CC10-Tet-inducible CA-IKKβ (CA-IKKβ) mice (33 and 50) were further characterized. Both lines had similar levels of TetOP-CAIKKβ and CC10-rtTA transgene integration, as determined by slot blot analysis (data not shown). To determine inducibility of the CA-IKKβ transgene, mice were fed Dox-containing chow and cDNA was synthesized from whole lung RNA. Through the use of an hGH pA-intron spanning reverse primer, we differentiated between genomic and mRNA expression of CA-IKKβ. As shown in Figure 1A, administration of Dox for one, two, three or seven days induced CA-IKKβ mRNA in transgene positive animals. In absence of Dox, a low level of CA-IKKβ mRNA was apparent within the lung, indicating some leakiness of the transgene. To ensure that the transgene was being induced specifically in the lung, cDNA was generated from lung, heart, thymus, liver, spleen, kidney and uterus from a transgene positive mouse administered Dox for one week. As demonstrated in Figure 1A (lower panel), mRNA expression of CA-IKKβ was only detected in the lung. These data demonstrate that Dox induced expression of CA-IKKβ mRNA using the Tet-on (tetracycline responsive) expression

system, and that the expression of the transgene was specific to the lung. Furthermore, expression of the CA-IKKβ resulted in marked increases in content of IKKβ protein in lung homogenates, whereas no IKKβ protein was detected in CA-IKKβ–transgenic mice that did not receive Dox or in the other control groups, because levels of endogenous IKKβ were below the level of detection (Figure 1B, *upper panel*, and data not shown). Serine 536 of RelA is a direct target of phosphorylation by IKKβ and is required for transactivation of NF-κB–dependent genes (204). We therefore assessed phosphorylation of RelA at serine 536, the target of IKKβ in lung homogenates. Results in Figure 1B (*lower panel*) demonstrate increases in levels of phosphorylated RelA in CA-IKKβ–transgenic mice that received Dox, whereas no clear differences were apparent in any of the other groups. Nuclear localization of the transcriptionally active NF-κB subunit, RelA, is a hallmark of activation of the canonical pathway. To determine if induction of CC10–CA-IKKβ led to NF-κB nuclear localization in airway epithelium, transgene-negative WT littermate control mice and CA-IKKβ–transgenic mice received Dox for 3 days and RelA nuclear localization was determined by immunofluorescence and confocal microscopy. As seen in Figure 1C, CA-IKKβ mice that received Dox displayed marked RelA nuclear localization, predominantly in the airway epithelium, whereas no nuclear translocation of RelA was observed in CA-IKKβ–transgenic mice not receiving Dox, or in WT mice that received Dox.

To verify expression of the CA-IKKβ transgene specifically in proximal airway epithelium, primary tracheal epithelial cell cultures were established from CA-IKKβ mice or WT littermates, because the CC10 promoter is expressed in tracheal epithelial cells (205). In response to *in vitro* treatment with Dox, CA-IKKβ mRNA expression increased in MTE (Figure 2A). In comparison with WT cells, nuclear presence of RelA was already apparent in MTE cultures derived from CA-IKKβ mice, in the absence of Dox, although Dox administration led to additional increases of nuclear RelA (Figure 2B). Evaluation of NF-κB–dependent cytokines in the culture medium of MTE cells derived from CA-IKKβ or WT mice demonstrated that marked increases in IL-3, IL-6, granulocyte colony–stimulating factor, granulocyte-macrophage colony–stimulating factor, and regulated upon activation, normal T-cell expressed and secreted (RANTES) occurred in CA-IKKβ–transgenic cells. As seen in Table 1, increases in levels of these cytokines in CA-IKKβ cells compared with WT cells occurred in the absence of Dox, which only effectuated marginal additional increases. These data are consistent with the observed expression of IKKβ (Figure 2A) and nuclear RelA (Figure 2B) under these conditions, and indicate apparent leakiness of the Tet-on system in the culture model, or presence of Dox derivatives in the cell culture medium.

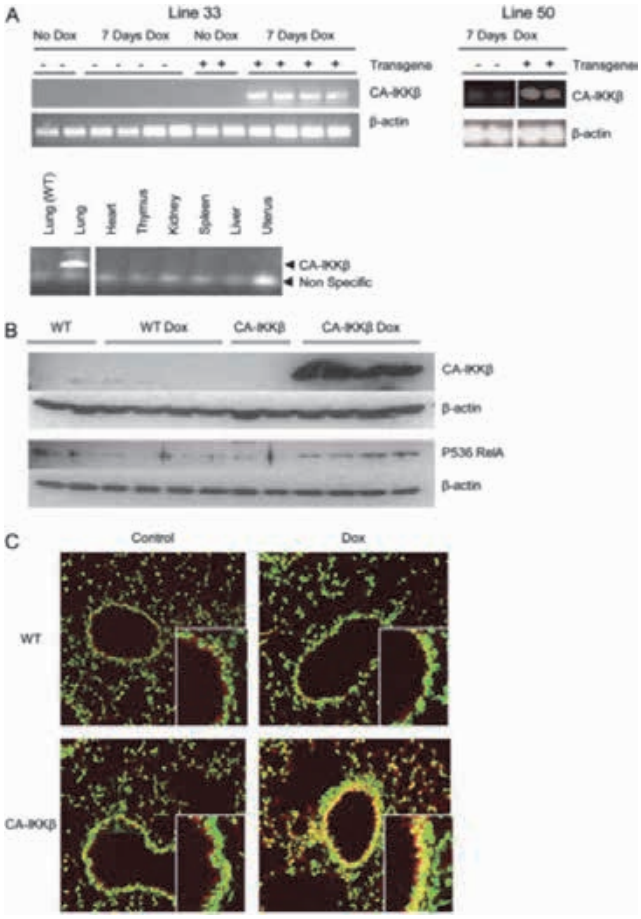

Figure 1. Constitutively active (CA) inhibitor of κB (IκB) kinase (IKK) β transgene expression in airway epithelium is inducible after doxycycline (Dox) administration. (A) Polymerase chain reaction (PCR) analysis of CA-IKKb cDNA induction: cDNA was generated from wild-type (WT) and CA-IKKβ mice from two independent lines given Dox for 7 days, and transgene expression was assessed by PCR. β-Actin mRNA levels were evaluated as a loading control; (lower panel) cDNA was generated from the heart, thymus, kidney, spleen, liver, and uterus of a CA-IKKβ mouse receiving Dox for 1 week, and CA-IKKβ expression was determined by PCR. Irrelevant lanes were cut from the gel. (B) Evaluation of IKKβ and serine 536 phosphorylated RelA (P536 RelA) levels in lung homogenates from control or CA-IKKβ mice in the presence or absence of Dox feeding for 1 week. Two independent samples from WT or CA-IKKβ groups (without Dox) were evaluated, whereas four independent samples from WT or CA-IKKβ mice that received Dox were processed. Gels were run and reprobed for b-actin as a loading control. Note that the lack of detection of endogenous IKKβ in the control groups is due to the short exposure time that is necessitated to visualize IKKβ in the CA-IKKβ–expressing mice. (C) Lung sections from WT and CA-IKKβ mice that received Dox for 3 days or fed regular chow were assessed for immunolocalization of RelA using confocal laser scanning microscopy. Nuclei were visualized with Sytox (green) and RelA was visualized using a Cy3-conjugated secondary antibody (red). Nuclear localization is indicated by overlap of fluorophores, resulting in a yellow color. Original magnification, 3200; inset: enlargement of bronchiolar epithelial region.

Table 1. Primary epithelial cells from WT and CA-IKKβ mice were exposed *in vitro* to 10 μg/ml of Dox for 24h, or left untreated, and levels of cytokines in medium were assessed by Bioplex analysis. Levels of Eotaxin, IFN-γ, IL-1β, IL-2, IL-4, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, KC, MCP-1, MIP-1α, MIP-1β, and TNF-α were not different between any of the experimental groups (data not shown). *= significance (p<0/05) when compared to wild type mice.

	WT	WT Dox	CA-IKKβ	CA-IKKβ Dox
G-CSF	891.7 ± 105.3	823.8 ± 86.3	1260.4 ± 205.6*	1465.8 ± 182.7*
GM-CSF	1511.9 ± 593.5	1586.9 ± 427.0	3113.8 ± 450.9*	3395.3 ± 350.9*
IL-1α	54.9 ± 3.1	49.5 ± 5.3	73.9 ± 8.7*	82.0 ± 5.9*
IL-3	6.2 ± 0.7	6.5 ± 0.6	13.8 ± 1.8*	13.3 ± 1.5*
IL-6	7.1 ± 0.8	6.2 ± 1.0	10.3 ± 1.6*	10.2 ± 1.2*
RANTES	274.8 ± 114.5	325.3 ± 132.3	548.1 ± 87.1*	637.7 ± 45.8*

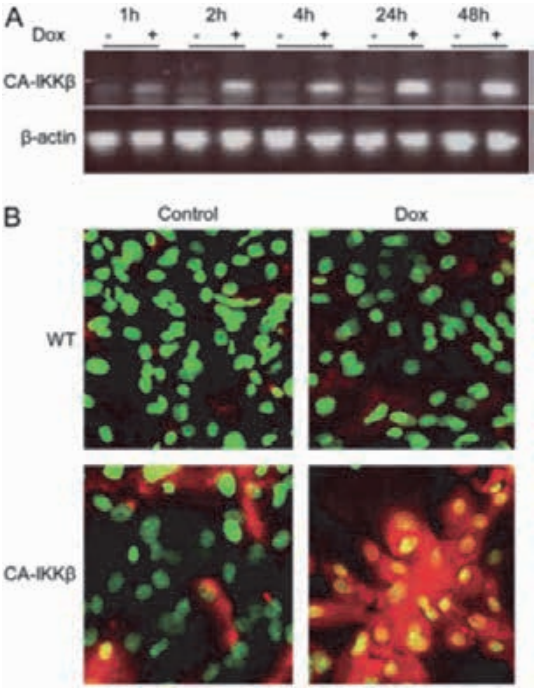

Figure 2. Primary tracheal epithelial cells isolated from CA-IKKβ–transgenic mice demonstrate induction of the CA-IKKβ transgene, nuclear factor (NF)-κB nuclear localization, and production of proinflammatory cytokines. (A) cDNA was generated from primary tracheal epithelial cell cultures from CA-IKKβ and wild-type (WT) mice. Cultures were treated with 10 μg/ml of doxycycline (Dox) and harvested at 1, 2, 4, 24, and 48 hours. Transgene expression was assessed as described in Figure 1. (B) Primary epithelial cell cultures from WT and CC10–CA-IKKβ mice were treated with 10 μg/ml of Dox for 24 hours and RelA nuclear localization was determined by confocal laser scanning microscopy. Nuclei were visualized with Sytox (green) and RelA was visualized using a Cy3-conjugated secondary antibody (red). Nuclear localization is indicated by overlap of fluorophores, resulting in a yellow color. Original magnification, ×200.

CA-IKKβ transgene expression is sufficient to cause airway inflammation. We next addressed the impact of selective activation of the canonical NF-κB pathway in airway epithelial cells in the inflammatory process. CA-IKKβ mice that received Dox for 3 days, 7 days, or 1 month exhibited increases in total cell counts recovered from BAL fluid (Figure 3A) as compared with WT littermates receiving Dox, or CA-IKKβ mice not receiving Dox. Differential cell counts revealed that transgene activation led to increases in macrophages, neutrophils, and lymphocytes in CA-IKKβ mice (Figure 3B). Levels of neutrophils were highest after 3 days of Dox, whereas lymphocyte levels were highest after 1 month. No eosinophils were observed in BAL fluid at any of the time points evaluated (Figure 2C). The observed inflammatory responses were greater in transgene line 33 compared with line 50 (Figures 3A and 3B) Evaluation of lung histopathology revealed peribronchiolar inflammation in both lines of CA-IKKβ–transgenic mice that received Dox, in association with apparent thickening of the bronchiolar epithelium (Figure 3C), whereas no overt histological changes were apparent in CA-IKKβ mice not fed Dox or in WT littermate control animals receiving Dox.

Expression of NF-κB–driven proinflammatory genes in lung tissue was evaluated by performing real-time PCR analysis on cDNA generated from lung homogenates. Figure 4 demonstrates that the mRNA levels of MIP-2 and KC, which are important in neutrophil recruitment, and CCL20, a dendritic cell and T cell chemoattractant, were significantly increased in lung tissue of CA-IKKβ mice that received Dox. Increases in levels of KC and granulocyte colony–stimulating factor were also detectable in BAL fluid from CA-IKKβ mice in response to administration of Dox (Table 2). Note that Dox administration to WT mice, or lack of Dox administration to CA-IKKβ mice, did not result in increased expression or production of proinflammatory mediators. Collectively, these findings demonstrate that Dox-dependent expression of the CA-IKKβ transgene construct in airway epithelium leads to NF-κB activation, expression of NF-κB–dependent proinflammatory mediators, and an inflammatory process characterized by macrophages, neutrophils, and lymphocytes.

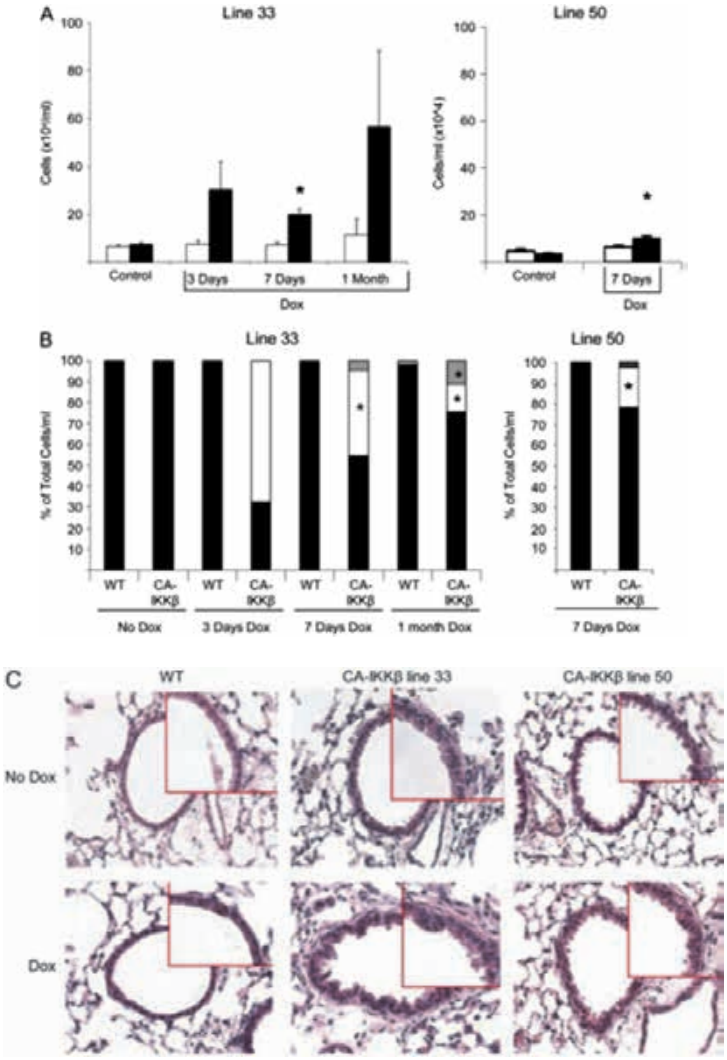

Figure 3. *CA-IKKβ transgene induction results in pulmonary inflammation.* Cell counts (*A*) and differentials (*B*) from the bronchoalveolar lavage (BAL) fluid of wild-type (WT) or CA-IKKβ mice were assessed after administration of doxycycline (Dox) for 3 days, 7 days, or 1 month. *Lines 33* and *50* represent transgenic mice obtained from two independent founders. Data in (*A*) are presented as mean (±SEM); *open bars*, WT; *solid bars*, CA-IKKβ. (*B*) *Gray bars*, lymphocytes; *open bars*, neutrophils; *closed bars*, macrophages. On average, three mice were included per group per time point, with the exception of the group of CA-IKKβ–transgenic mice receiving Dox, which contained seven mice/group/timepoint. (*C*) Representative hematoxylin–eosin sections from transgene-negative littermates and two lines of CA-IKKβ mice that received Dox for 7 days. Original magnification, ×200; *insets*, ×400.

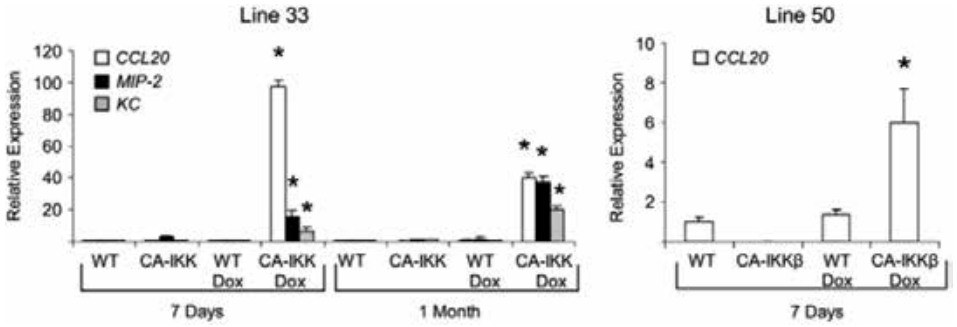

Figure 4. *CA-IKKβ activation leads to the increased expression of proinflammatory genes.* cDNA was prepared from lung homogenates from wild-type (WT) and CA-IKKβ mice given doxycycline (Dox) for 7 days or 1 month for assessment of mRNA expression of inflammatory genes by quantitative real-time polymerase chain reaction. On average, three mice were included per group per time point, with the exception of the group of CA-IKKβ–transgenic mice receiving Dox, which contained seven mice/group per timepoint.

Table 2. Cytokine levels (pg/ml) in BAL fluid from transgene negative (WT), or CA-IKKβ mice after one week of Dox administration. Cytokines in medium were assessed by Bioplex analysis. IFNγ, MIP-1α. IL-2, IL-3, IL-4 were non-detectable and Eotaxin, IL-10, IL-12p70, IL-13, IL-17, IL-1α, IL-1β, IL-5, IL-6, IL-9, MIP-1β, and TNF-α were not different between any of the experimental groups (data not shown). *= significance ($p<0.05$) when compared to wild type and CA-IKKβ mice not receiving Dox.

	WT	WT Dox	CA-IKKβ	CA-IKKβ Dox
G-CSF	3.2 ± 0.8	4.7 ± 1.2	4.9 ± 1.5	48.4 ± 23.7*
GM-CSF	0	0	0	78.8 ± 26.3*
IL-12 (p40)	33.5 ± 5.9	38.1 ± 7.3	38.7 ± 3.8	119.8 ± 24.5*
KC	19.8 ± 3.1	16.2 ± 2.6	26.1 ± 4.0	64.8 ± 15.0*
MCP-1	1.1 ± 1.1	14.2 ± 3.9	4.2 ± 2.0	27.9 ± 6.5*
RANTES	6.0 ± 3.1	11.3 ± 1.7	9.4 ± 1.4	45.5 ± 16.0*

CA-IKKβ transgene expression leads to airway hyperresponsiveness. We addressed the impact of NF-κB activation in airway epithelium on AHR, a cardinal feature of allergic airways disease. CA-IKKβ mice were given Dox for 1 week before the assessment of various parameters of airway mechanics at baseline or after ascending doses of inhaled methacholine (Mch) (201). Results in Figure 5 demonstrate that CA-IKKβ–transgenic mice that received Dox for 1 week displayed no differences in baseline (PBS) mechanics when evaluating Newtonian resistance (Rn), airflow heterogeneity/tissue elastance (G), or airway closure/elastance (H) in comparison with WT mice that received Dox, or CA-IKKβ mice not administered Dox. However, after induction of the CA-IKKβ transgene for 1 week, increases in the parameters Rn and G were apparent at doses of 3.125 and 12.5 mg/ml of Mch in comparison

with the control groups, whereas no differences were observed in H, indicating that hyperresponsiveness was localized to the central airways, consistent with the location of expression of the transgene and activation of NF-κB.

Airway epithelial NF-κB activation enhances ovalbumin-induced allergic airways disease. A role for NF-κB in eliciting the inflammatory response to allergen challenge has been demonstrated in mice incapable of activating NF-κB in the epithelium of the proximal airways (196, 197). Therefore, we sought to determine the contribution of airway epithelial NF-κB to the development of allergic airways disease during challenge with OVA. CA-IKKβ and WT littermate control animals were immunized with OVA on Day 1 and, after the second OVA sensitization (Day 7), Dox was administered until harvest (Day 18). In mice subjected to OVA sensitization and challenge, total cell counts were not further increased in CA-IKKβ transgene–expressing mice (Figure 6A). However CA-IKKβ mice that received Dox displayed an increase in the proportion of neutrophils in BAL compared with WT mice (Figure 6A). Interestingly, CA-IKKβ mice receiving Dox demonstrated a marked enhancement in CCL20 mRNA expression in lung homogenates (Figure 6B) and an increase in proinflammatory and Th2 cytokines, KC, MIP-1β, and IL-17 in the BAL fluid as compared with their WT control littermates, or CA-IKKβ littermates that did not receive Dox (Table 3). Evaluation of respiratory mechanics demonstrated that CA-IKKβ–transgenic mice receiving Dox and subjected to OVA sensitization and challenge (OVA/OVA) displayed increases in the parameter, G, in response to Mch compared with the other experimental groups that also were subjected to OVA/OVA (Figure 6C). Parameters Rn and H were not different between the experimental groups subjected to OVA/OVA (data not shown), suggesting that CA-IKKβ expression enhanced AHR in mice with allergic airways disease by promoting narrowing of smaller airways, or by increasing tissue resistance (206).

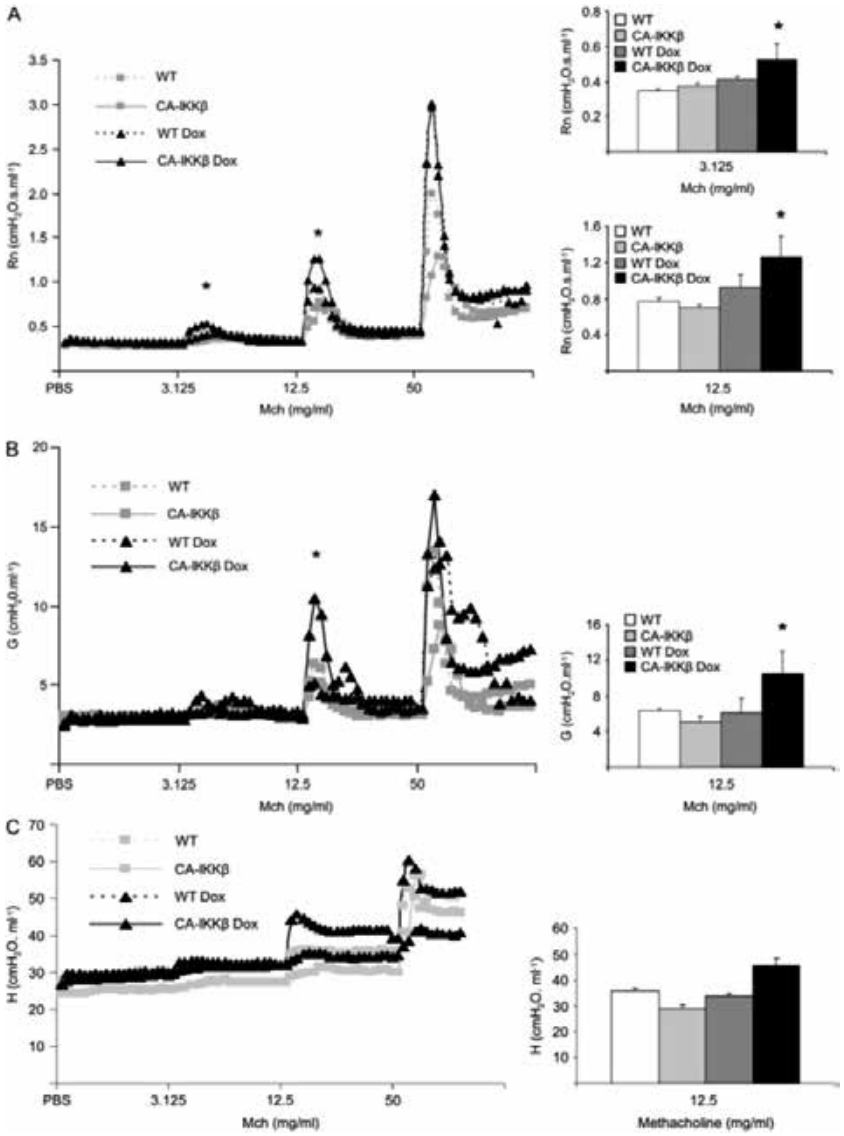

Figure 5. *CA-IKKβ transgene expression results in airway hyperresponsiveness (AHR).* (*A*) Wild-type (WT) and CA-IKKβ mice were given doxycycline (Dox) for 7 days, after which AHR was measured using the forced oscillation technique, as described in Methods. Shown are respiratory mechanics for the parameters: (*A*) Rn, Newtonian resistance, a measure of airway resistance; (*B*) G, a measure of airflow heterogeneity or tissue elastance; and (*C*) H, airway closure/elastance. Individual tracings are shown after nebulization of phosphate-buffered saline (PBS) as a control in addition to ascending doses of methacholine (Mch). *Right*: mean group averages of parameters Rn, G, and H at the indicated dose of Mch. $*P \leqslant 0.05$, analysis of variance. Results represent data obtained from three mice/group from line 50. A separate experiment performed with CA-IKKβ mice from line 33 (three mice/group) revealed similar trends that were statistically significant (data not shown).

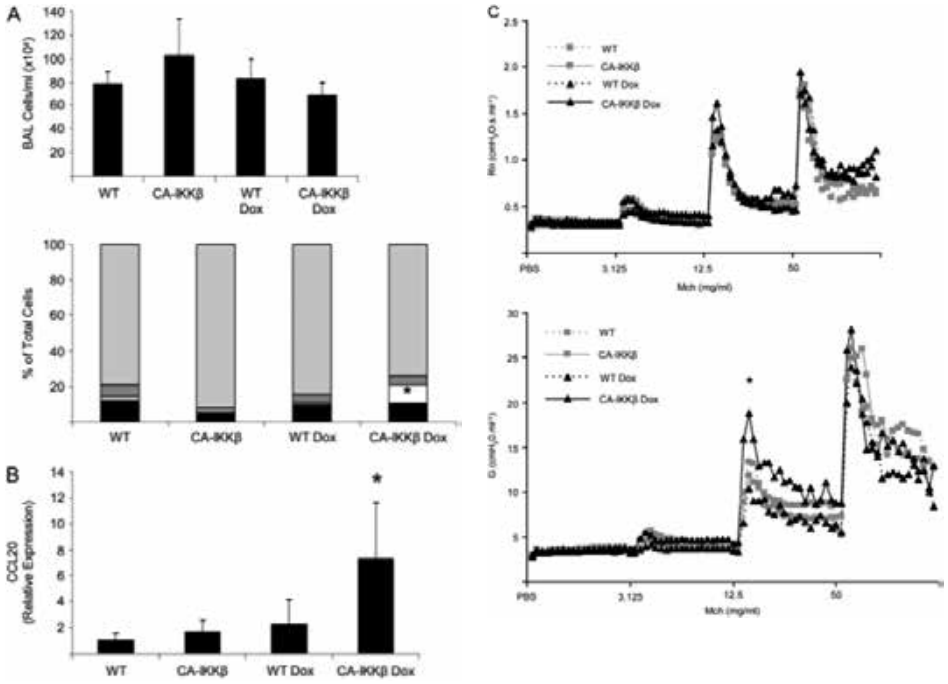

Figure 6. *Enhancement of inflammatory mediators and airway hyperresponsiveness (AHR) in CA-IKKβ mice (line 33) subjected to sensitization and challenge with ovalbumin (OVA).* Wild-type (WT) or CA-IKKβ mice were immunized with OVA on Days 1 and 7. On Day 7, doxycycline (Dox) administration was started and continued until mice were killed on Day 18. All mice were subjected to aerosolized OVA on Days 14, 15, and 16. (A) Total cell counts and differentials from the bronchoalveolar lavage (BAL) fluid of CA-IKKβ mice (CA-IKKβ, n = 7; CA-IKKβ Dox, n = 8; WT, n = 7; WT Dox, n = 8) subjected to immunization and challenge with OVA. *P ⩽ 0.05, ANOVA. Light gray bars, eosinophils; dark gray bars, lymphocytes; open bars, neutrophils; solid bars, macrophages. (B) Increased mRNA expression of CCL20 in lung homogenates. (C) Representative tracings for the variables Rn and G are shown after nebulization of phosphate-buffered saline (PBS) as a control in addition to ascending doses of methacholine (Mch).

Table 3. Increased levels of pro-inflammatory cytokines in the BAL fluid of CA-IKKβ mice subjected to sensitization and challenge with Ova (CA-IKKβ n=7, CA-IKKβ dox n=6; WT n=5, WT dox n=5).

	WT	WT Dox	CA-IKKβ	CA-IKKβ Dox
IL-17	47.2 ± 12.0	38.2 ± 7.5	35.3 ± 10.8	64.0 ± 12.8*
IL-4	185.9 ± 69.3	108.9 ± 28.0	127.2 ± 42	240.9 ± 52.0*
KC	304.5 ± 28.3	336.3 ± 62.5	337.8 ± 43.3	847.2 ± 219.1*
MIP-1β	53.5 ± 14.1	17.3 ± 4.4	42.9 ± 14.6	101.2 ± 23.9*

* denotes p ≤ 0.05, ANOVA. Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-10, IL-12p40, IL-12p70, IL-13, IL-1α, IL-1β, IL-2, IL-3, IL-5, IL-6, IL-9, MCP-1, MIP-1α, RANTES, and TNF-α were not different between any of the experimental groups (data not shown). *= significance (p<0.05) when compared to wild type and CA-IKKβ mice not receiving Dox.

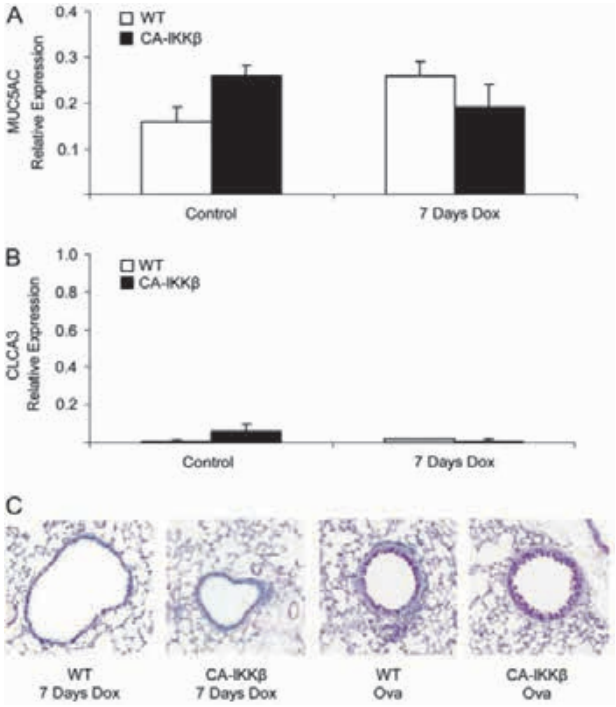

Figure 7. *CA-IKKβ expression has no effect on mucus metaplasia in naive or ovalbumin (OVA)-treated mice.* (*A*) Assessment of *MUC5AC* and (*B*) *CLCA3* gene expression in lung homogenates of CA-IKKβ mice that were fed doxycycline (Dox) for 1 week (line 33) (results were obtained from the after numbers of mice: CA-IKKβ, n = 6; CA-IKKβ Dox, n = 6; wild-type [WT], n = 3; WT Dox, n = 3). (*C*) Evaluation of periodic acid Schiff reactivity in lung tissue of CA-IKKβ that received Dox for 1 week or fed normal chow; OVA groups on the *right* represent WT or CA-IKKβ mice that received Dox and were immunized and challenged with OVA, as described in Figure 6. Original magnification, ×200.

Increased response of CA-IKKβ mice to allergen challenge is not due to mucus metaplasia. Increased mucus production is considered to be a major cause of airflow obstruction in asthma, and mucus metaplasia is believed to contribute to AHR in mouse models of the disease. Therefore, we evaluated mucus metaplasia in CA-IKKβ expressing mice. Results in Figure 7 demonstrate that no increases in mRNA expression of Muc5AC (Fig. 7A), or CLCA3 (Fig. 7B) occurred in lung homogenates of CA-IKKβ expressing mice, consistent with the lack of Periodic Acid Schiff (PAS) reactivity in CA-IKKβ mice receiving Dox (Fig. 7C). In contrast, CA-IKKβ expressing mice subjected to sensitization and challenge with OVA displayed mucus metaplasia, based upon PAS reactivity to a comparable extent as transgenic littermate control animals exposed to OVA. These findings suggest that the increases in AHR observed following expression of the CA-IKKβ transgene (Figures 5 and 6C) occurred independently of mucus metaplasia.

Thickening of airway smooth muscle in CA-IKKβ expressing mice. Closer evaluation of parameters of AHR revealed that in CA-IKKβ expressing mice the time to peak Mch responses occurred faster, and that parameters Rn and G remained elevated (Figure 5B), whereas in other groups these parameters returned to baseline. These patterns of responsiveness suggest potential alterations in smooth muscle in CA-IKKβ expressing mice. We therefore evaluated airway smooth muscle (ASM) using an antibody directed against α-SMA. Results in Figure 8 demonstrate marked thickening of the smooth muscle layer, and the appearance of muscle bundles in CA-IKKβ transgenic mice that received Dox, whereas no changes in smooth muscle were apparent in the control groups (Figure 8A). Blinded scoring of α-SMA immunoreactivity confirmed that thickening of the smooth muscle layer occurred in both the large airwaysas well as in small bronchioles (Figure 8B).

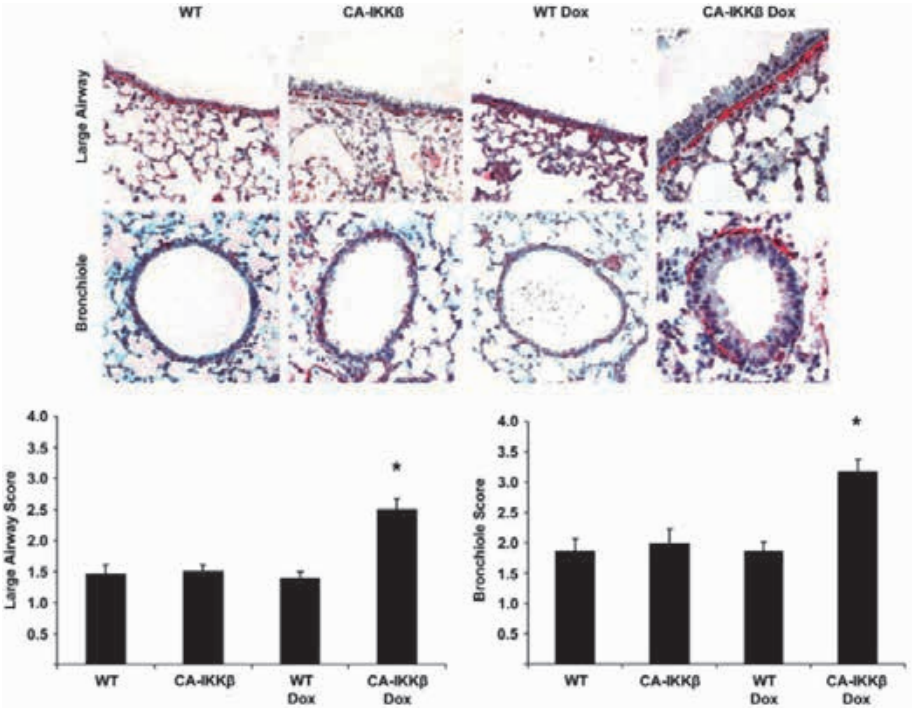

Figure 8. *CA-IKKβ expression causes thickening of the airway smooth muscle (ASM) layer.* Wild-type (WT) or CA-IKKβ–transgenic mice were kept in the absence or doxycycline (Dox) or fed Dox for 1 week, at which time mice were killed, and paraffin-embedded lung sections were prepared for evaluation of α-smooth muscle actin (SMA) reactivity or staining with hematoxylin and eosin for histopathologic evaluation. *Red* reflects α-SMA reactivity. Slides were evaluated by light microscopy. Original magnification, ×200. (*B*) Quantification of ASM thickening in CA-IKKβ–expressing mice. Images were scored on a scale from 1 to 4 (1 representing the least reactivity, 4 representing the most α-SMA reactivity), according to the methodologies described in Methods section. Shown are mean scores (+SEM). * $P < 0.05$, analysis of variance.

Discussion

The pathogenesis of asthma is characterized by elevated levels of Th2 cytokines, eosinophilic inflammation, mucus metaplasia, and AHR. Recently, a role for airway epithelium in asthma pathogenesis has emerged, and epithelial cells are now considered an integral part of the innate immune system based upon their ability to sense and respond to a diverse array of inhaled stimuli with the activation of signaling pathways that control the production of oxidants, mucus, proinflammatory mediators, and proteins involved in airway remodeling (207). Previous studies that used transgenic or conditional ablation approaches to specifically inhibit NF-κB in airway or distal epithelium firmly established a causal role for this transcription factor in inflammatory responses to LPS or inhaled antigen (196, 197, 200, 208). Although inhibition of NF-κB in airway epithelium was sufficient to block the majority of the inflammatory response to antigen, antigen-induced AHR was not attenuated in this model, suggesting that NF-κB activation in airways may control only certain parameters of allergic airway disease (196). Moreover, the aforementioned studies corroborated the importance of NF-κB in the inflammatory process, but did not definitively discern whether NF-κB activation in these cells is sufficient to drive and promote allergic inflammation or AHR. To directly address this issue, we created transgenic mice wherein a CA-IKKβ was expressed in the airway epithelium, in a Dox-inducible manner, in order to activate the canonical pathway of NF-κB in the airway epithelium.

It is of relevance to highlight that Cheng and colleagues (209) recently created a transgenic mouse expressing the same CA-IKKβ construct in airway epithelium using almost identical strategies. Those investigators reported neutrophilic inflammation, pulmonary edema, and lung injury. Furthermore, chronic expression of the transgene led to mortality (209). Results from the present study confirm the increased production of proinflammatory mediators, such as MIP-2 and KC, and neutrophilic inflammation that were reported in CA-IKKβ–expressing mice (209). However, in contrast to the previous study, continued presence of Dox for 7 days and Dox feeding up to 1 month did not cause mortality in either line of CA-IKKβ–transgenic mice that we generated, suggesting that the levels of IKKβ transgene expression that are achieved with the Dox exposure regimen in our study were lower than those reported in the previous study. Alternatively, differences in outcomes may also be due to differences in genetic backgrounds of mice, or the strategies used to create the CA-IKKβ–expressing transgenic mice. An intriguing finding of the current study that was not reported previously is that CA-IKKβ expression was sufficient to cause increases in AHR in otherwise naive mice. The pattern of the mechanical response to Mch is reminiscent of the response observed in the AJ strain of mice, where AHR is related to increases in ASM cell contraction (210). CA-IKKβ–driven AHR occurred in the absence of marked elevations in Th2 cytokines,

eosinophila, or mucus metaplasia. We identified here that CA-IKKβ expression in airways causes thickening of ASM. These results are of interest for a number of reasons. First, the transgene is expressed in mice in a C57BL\6 background, which is notably resistant to antigen-induced AHR compared with BALB/c mice, which are routinely used to measure antigen-induced AHR (211). It is therefore possible that CA-IKKβ–triggered AHR may be more robust when mice are backcrossed onto the BALB/c background, an endeavor that was beyond the scope of the present study.

Increases in AHR in naive CA-IKKβ–transgenic mice, or CA-IKKβ–transgenic mice subjected to OVA sensitization and challenge, occurred in association with a substantial presence of neutrophils (Figures 3 and 6), whereas we did not observe any eosinophils or changes in eosinophilia in response to antigen in CA-IKKβ–expressing mice. In general, eosinophils have been linked to AHR and remodeling (212), although some controversy exists regarding the exact roles of eosinophils in the disease process (213, 214). The role of the neutrophil in the pathogenesis of allergic airway disease remains enigmatic. Neutrophils are recovered in the BAL fluid of patients with severe asthma (215) and neutrophilic inflammation in experimental models has, in fact, been linked to AHR. For example, LPS administration, which triggers airway neutrophilia, also induces changes in respiratory mechanics and increases in AHR (216-218). Therefore, the marked presence of neutrophils might contribute to increases in AHR in CA-IKKβ–expressing mice. Neutrophils are capable of generating a number of mediators, such as reactive oxygen and nitrogen species, matrix metalloproteinases, and elastase, which have been linked to increases in AHR directly, or indirectly after stimulation of ASM cells or airway epithelial cells (219-222).

Mucus cell metaplasia is a feature of allergic airway disease that is considered to be a contributing component to airway obstruction and AHR (223, 224). As is demonstrated in Figure 7, CA-IKKβ–expressing mice did not express increased levels of *MUC5AC* or *CLCA3* mRNA in lung tissue, nor did they display reactivity for PAS. This is in contrast to mice that were immunized and challenged with OVA, which revealed marked reactivity for those parameters. The lack of mucus metaplasia in CA-IKKβ–expressing mice is in line with the absence of marked increases in IL-13, the major Th2 cytokine that is linked to mucus metaplasia (225, 226). However, the lack of mucus metaplasia is surprising in light of previous studies demonstrating that neutrophil elastase induced mucin production and hypersecretion in human bronchial epithelial cells (221, 222) and caused mucus metaplasia in mouse lungs (227). It is plausible that mucus metaplasia occurs in CA-IKKβ–expressing mice beyond the timeframe of investigation within the current study, which may contribute to potentially chronic abnormalities in respiratory mechanics, although these possibilities remain to be formally tested.

Perhaps the most striking histopathologic change in lungs of CA-IKKβ–expressing mice is the thickening of the smooth muscle layer of large airways, which may be a contributing factor to the intrinsic increases in AHR observed in those mice. These new findings, which demonstrate a functional link between epithelial NF-κB activation to thickness of smooth muscle, are in contrast to a previous study demonstrating that conditional ablation of IKKβ in airway epithelium did not affect smooth muscle thickness in response to sensitization and challenge with OVA (197). This discrepancy may stem from the differences in experimental regimens, which used opposing strategies to evaluate the function of IKKβ. Furthermore, the OVA exposure regimen used in previous work might have resulted in production of mediators that masked the effect of epithelial NF-κB on smooth muscle thickness. An aberrant phenotype of ASM cells has been speculated to be sufficient to explain the pathology of asthma (228-230), and a role for smooth muscle hypertrophy and hyperplasia in asthma has been suggested (231). The current study did not investigate whether smooth muscle hypertrophy or proliferation occurred in CA-IKKβ–expressing mice, nor did we unravel whether the ASM exhibited an activated phenotype. Furthermore, the identity of mediators produced after CA-IKKβ expression within the airway epithelium, or intermediary structural or inflammatory cells that are responsible for thickening of the smooth muscle layer, remain to be determined.

In conclusion, we demonstrate here that activation of the canonical NF-κB pathway in airway epithelium is sufficient to drive airway inflammation, characterized by neutrophils and lymphocytes, thickening of the ASM layer, and AHR. Epithelial NF-κB activation also enhanced OVA-induced proinflammatory mediator production and AHR. Together, these findings demonstrate that activation of the canonical NF-κB pathway in airway epithelium, in the absence of other agonists, is sufficient to drive select features associated with human asthma. These findings further highlight the functional significance of changes in epithelial cell homeostasis or activation in chronic inflammatory lung diseases.

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CHAPTER 3

Distinct functions of airway epithelial nuclear factor-kB activity regulate nitrogen dioxide–induced acute lung injury

Jennifer L. Ather, John F. Alcorn, Amy L. Brown, Amy S. Guala, Benjamin T. Suratt, Yvonne M. W. Janssen-Heininger, and Matthew E. Poynter

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ABSTRACT

Reactive oxidants such as nitrogen dioxide (NO_2) injure the pulmonary epithelium, causing airway damage and inflammation. We previously demonstrated that nuclear factor-κ B (NF-κB) activation within airway epithelial cells occurs in response to NO_2 inhalation, and is critical for lipopolysaccharide-induced or antigen-induced inflammatory responses. Here, we investigated whether manipulation of NF-κB activity in lung epithelium affected severe lung injuries induced by NO_2 inhalation. Wild-type C57BL/6J, CC10-IκBα$_{SR}$ transgenic mice with repressed airway epithelial NF-κB function, or transgenic mice expressing a doxycycline-inducible, constitutively active I κ B kinase β (CC10-rTet-$_{CA}$IKKβ) with augmented NF-κB function in airway epithelium, were exposed to toxic levels of 25 ppm or 50 ppm NO_2 for 6 hours a day for 1 or 3 days. In wild-type mice, NO_2 caused the activation of NF-κB in airway epithelium after 6 hours, and after 3 days resulted in severe acute lung injury, characterized by neutrophilia, peribronchiolar lesions, and increased protein, lactate dehydrogenase, and inflammatory cytokines. Compared with wild-type mice, neutrophilic inflammation and elastase activity, lung injury, and several proinflammatory cytokines were significantly suppressed in CC10-IκBα$_{SR}$ mice exposed to 25 or 50 ppm NO_2. Paradoxically, CC10-rTet-$_{CA}$IKKβ mice that received doxycycline showed no further increase in NO_2-induced lung injury compared with wild-type mice exposed to NO_2, instead displaying significant reductions in histologic parameters of lung injury, despite elevations in several proinflammatory cytokines. These intriguing findings demonstrate distinct functions of airway epithelial NF-κB activities in oxidant-induced severe acute lung injury, and suggest that although airway epithelial NF-κB activities modulate NO_2-induced pulmonary inflammation, additional NF-κB–regulated functions confer partial protection from lung injury.

Introduction

Acute lung injury (ALI) is induced by a variety of insults, including endotoxin, acid aspiration, complement activation, hyperoxia, and oxidant gases such as nitrogen dioxide (NO_2) and ozone, and it is characterized by the increased presence of neutrophils, platelets, fibrin, edema, and epithelial and endothelial damage and cell death (1). Nitrogen dioxide, as a byproduct of combustion, is a toxic gas present in ambient air that can cause respiratory symptoms at low doses, and severe respiratory distress or death at higher concentrations (2). In addition to exogenous sources, NO_2 can also be produced endogenously, as a byproduct of inflammatory cell activity (3). Inhaled NO_2 is absorbed along the respiratory tract (4), and the stable endproduct of NO_2 reactivity, nitrotyrosine, is present in the lungs of patients with asthma, chronic obstructive pulmonary disease (COPD), and other pulmonary diseases (5). Studies using lung epithelial cells demonstrated proinflammatory activities and cell death in response to NO_2. For instance, human bronchial epithelial cells exposed to NO_2 secrete increased levels of IL-8, IL-1β, TNF-α, granulocyte/macrophage colony–stimulating factor (GM-CSF), and nitric oxide (6, 7). Nitrogen dioxide also promotes the selective death of proliferating or migrating epithelial cells via a mechanism involving the Fas-dependent activation of c-Jun-*N*-terminal kinase (6, 8-10).

In recent years, the role of the airway epithelium in the functional response to diverse stimuli has become an area of substantial research, and airway epithelial cells are now appreciated to be among the primary responders to respiratory insults produced by bacteria, viruses, and oxidant stress (5, 6, 11, 12). Activation of the transcription factor nuclear factor-κ B (NF-κB) within the airway epithelium augments the production of proinflammatory cytokines, leading to an inflammatory response in the lung (11, 12). The activation of NF-κB also exerts antiapoptotic affects, because this transcription factor positively regulates the expression of prosurvival genes (13, 14). Notably, NF-κB–dependent survival factors, including growth arrest and DNA damage induced gene 45 (GADD45) and manganese superoxide dismutase (MnSOD) are important in down-regulating the activity of c-Jun-N-terminal kinase, and in protecting against oxidant-induced cell death (15).

Airway epithelial NF-κB participates in the control of processes that include both inflammation and protection from cell death. However, the outcome of modulation of NF-κB within airway epithelium in the pathophysiology of severe ALI is unclear. Protection from inflammation and enhanced cell death or damage are both plausible outcomes of inhibiting NF-κB activities in these cells *in vivo*. The goal of the present study was to determine whether positive or negative modulation of NF-κB activity in the airway epithelium affects the extent of NO_2-induced lung injury.

Materials and Methods

Mice

Transgenic mice (CC10-IκBα$_{SR}$) with repressed NF-κB function in airway epithelium (11) were backcrossed with wild-type C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) for more than 10 generations. Bi-transgenic mice (CC10-rTet-$_{CA}$IKKβ) (16) expressing $_{CA}$IKKβ in the bronchiolar epithelium after the administration of 6 g/kg doxycycline (Dox) in chow (TestDiet, Richmond, IN) were backcrossed with C57BL/6J mice for at least five generations. Wild-type mice were age-matched and sex-matched transgene-negative littermates. Mice were maintained on a 12-hour light/dark cycle, and were provided food and water *ad libitum*. All animal studies were approved by the University of Vermont Institutional Animal Care and Use Committee.

Exposure to NO_2.

Mice were exposed to high efficiency particulate air (HEPA)-filtered air or to NO_2 (17) at doses of 25 ppm or 50 ppm for 6 hours a day for 3 consecutive days, to induce severe ALI. Lower doses of NO_2 or less exposure time induced only modest levels of lung injury and inflammatory response (5). As indicated, mice were provided Dox chow *ad libitum* for 3 days before the 3-day exposure to NO_2, and were maintained on Dox chow until being killed on Day 7.

Bronchoalveolar Lavage and Lung Processing.

Lungs were lavaged with a single instillation and recovery of 1 ml ice-cold PBS (Gibco, Carlsbad, CA). Lavage samples were centrifuged at 400 × *g* for 6 minutes, and the supernatant was separated from the cell pellet. Cells were resuspended in 200 µl PBS, counted, spun onto glass slides with a cytospin, and stained with hematoxylin and eosin for the differential counting of more than 200 cells/slide. Cell-free bronchoalveolar lavage (BAL) fluid was flash-frozen in liquid N_2 for cytokine analysis. Left lung lobes were inflated with 4% paraformaldehyde in PBS, and immersed overnight in the same solution before being transferred to 70% ethanol for 24 hours. Lungs were then embedded in paraffin blocks, cut into 5-µm sections, mounted onto slides, and stained with hematoxylin and eosin. The right lung lobes were flash-frozen for RNA and protein analysis.

Protein, Cytokine, and Enzymatic Activity Profiling from BAL Fluid.

Cytokine levels were assessed using a Bio-Plex 23-plex panel (Bio-Rad, Hercules, CA). Protein levels were measured using the Bradford assay (Bio-Rad). Lactate dehydrogenase activity was determined using the LDH Detection Assay Kit (Promega, Madison, WI). Neutrophil elastase activity was measured using the EnzCheck Elastase Assay kit (Molecular Probes, Eugene OR).

Assessment of Localization of Antibody Recognizing p65 in Lung Tissue.
Mice were exposed to room air or 25 ppm NO_2 for 6 hours once, and killed 1 hour later. Lungs were instilled with and placed into Tissue-Tek OCT Compound (Sakura Finetek, Inc., Torrance, CA), and frozen in liquid nitrogen–chilled isopentane, and 5-μm frozen sections were cut and placed onto glass slides. Slides were fixed for 30 minutes with 4% paraformaldehyde in PBS, washed, and permeabilized for 20 minutes with 1% Triton X-100 in PBS, and blocked with 1% bovine serum albumin in PBS for 1 hour. Slides were then incubated overnight at 4°C with antibody (Ab) recognizing p65 (RelA) (5 μg/ml, SC-372; Santa Cruz Biotechnology, Santa Cruz, CA) in 1% BSA/PBS. after three washes in 1% BSA/PBS, slides were incubated for 1 hour with goat anti-rabbit Alexa 568–labeled secondary Ab (Molecular Probes) in PBS, and counterstained with a 1:8,000 dilution of SYTOX Green (Molecular Probes) in PBS to label DNA. Slides were washed with PBS, rinsed with H_2O, and coverslipped using Aqua PolyMount (Polysciences, Inc., Warrington, PA). Sections were scanned using a Bio-Rad MRC 1024 confocal scanning laser microscope system and a ×40 objective.

Histologic Scoring Method.
To measure overall injury of lung sections, the total numbers of bronchioles with a length:diameter ratio of less than 2:1 were counted per slide and then assessed for percent injured, as defined by the appearance of disrupted airway wall or alveolar thickening. This was determined to be the "percent foci" per section. Subsequently, 10 airways per slide were scored by two independent researchers blinded to the identity of samples, and graded on a scale of 1–3 for intensity of injury, with 1 as the least injured, and 3 as the most severely injured. Parameters of airway injury included airway epithelial thickening, peribronchiolar lesions, and the presence of inflammatory cells.

Immunoblotting.
Twenty micrograms of protein from lung homogenates prepared in 1× PBS were run on a 15% acrylamide gel, and transferred to a nitrocellulose membrane. Lysates were blotted for IKKβ (Cell Signaling Technology, Danvers, MA). Membranes were stripped and reprobed with β-actin as a loading control.

Statistical Analyses.
All statistical analyses were performed using Excel software (Microsoft, Seattle, WA) with one-way ANOVA, followed by the Tukey test for multiple comparisons. Values of $P < 0.05$ were considered statistically significant. Data are presented as mean value ± SEM.

Results

Nitrogen Dioxide–Induced RelA Nuclear Translocation in Airway Epithelium Is Attenuated in CC10-IκBα$_{SR}$ Mice. We previously reported that brief exposure of mice to 10 ppm NO_2 induces airway epithelial NF-κB activation, as characterized by the presence of nuclear RelA accumulation (17). To determine whether higher doses and longer durations of inhaled NO_2 induce airway epithelial NF-κB activation, lung sections were examined by RelA immunofluoresence after a single 6-hour, 25-ppm exposure to NO_2. Neither wild-type nor transgenic mice expressing IκBα$_{SR}$ in airway epithelium displayed activation of NF-κB after exposure to control air (Figure 1). However, NO_2 induced the nuclear translocation of RelA in epithelial cells lining the airways of wild-type mice. In contrast, CC10-IκBα$_{SR}$ mice demonstrated abrogated RelA nuclear translocation, confirming that the transgene inhibits the activation of NF-κB in response to NO_2 inhalation. These data demonstrate that higher doses and longer durations of NO_2 activate NF-κB in the airway epithelium, and that this NF-κB activation is inhibited in mice expressing IκBα$_{SR}$ in airway epithelium, thus suggesting a potential role for NF-κB in NO_2-induced severe lung injury.

Nitrogen Dioxide–Induced Airway Inflammation Requires Epithelial NF-κB Activation. Exposure to NO_2 (25 or 50 ppm) for 6 hours a day for 3 days induced a significant recruitment of inflammatory cells to the lavageable airspaces (Figure 2A). Although the influx was predominantly monocytic, there was a significant increase of neutrophils in response to either dose of NO_2 (Figure 2B). The CC10-IκBα$_{SR}$ mice lacking airway epithelial NF-κB activation showed significantly decreased BAL neutrophil numbers in response to NO_2 exposure, and monocytic cell accumulation was also attenuated. These data indicate that NF-κB in the airway epithelium is required for NO_2-induced neutrophil and monocyte recruitment into the airways. We next evaluated the activity of neutrophil elastase, a 29-kD serine protease stored in neutrophil granules that can degrade extracellular matrix, plasma proteins, and protease inhibitors, and activate matrix metalloproteases (18). Moreover, NO_2-induced neutrophil protease production was attenuated in CC10-IκBα$_{SR}$ mice exposed to 25 ppm NO_2 (Figure 2C), consistent with the decreased neutrophil numbers.

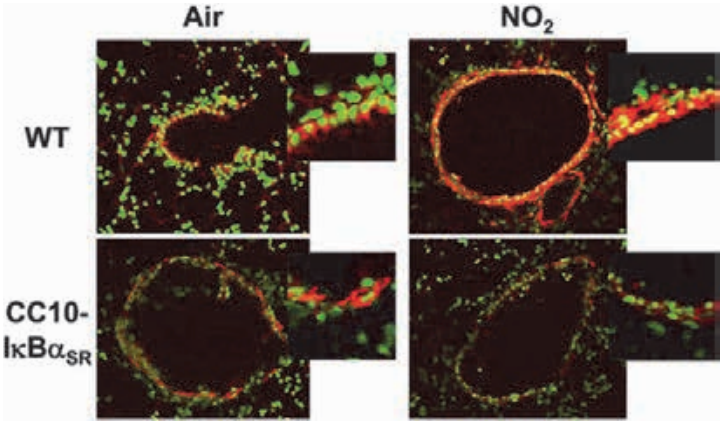

Figure 1. *Localization of NF-κB activation in lung sections from wild-type and CC10-IκBα$_{SR}$ transgenic mice exposed to NO$_2$.* The CC10-IκBα$_{SR}$ and wild-type (*WT*) littermate control animals were exposed to room air or 25 ppm NO_2 for 6 hours, and killed 1 hour later. Lung sections were evaluated for nuclear translocation of RelA, using immunofluorescence and confocal laser scanning microscopy. Nuclei were visualized with Sytox (*green*), and RelA was visualized using a Cy3-conjugated secondary antibody (*red*). Nuclear localization is indicated by the overlap of fluorophores (*yellow*). Original magnification of large images is ×200, and is representative of patterns observed in at least four mice per group. For a better illustration of the differences in nuclear NF-κB localization, airway epithelial cells from the original images were magnified using a 2.5 optical zoom and are shown at upper right (*insets*).

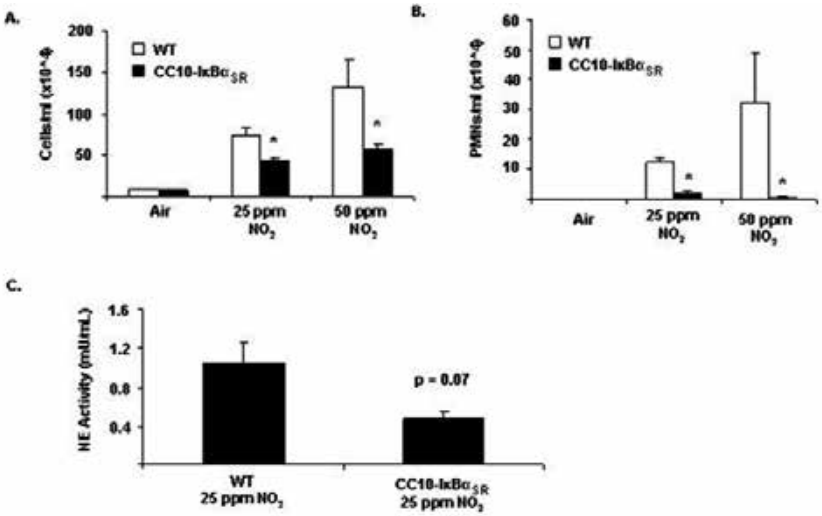

Figure 2. *Assessment of airway inflammation and neutrophil elastase activity in wild-type (WT) and CC10-IκBα$_{SR}$ mice exposed to NO$_2$.* Total (*A*) and neutrophil (*B*) cell counts in BAL fluid from WT and CC10-IκBα$_{SR}$ mice are shown after 25 ppm and 50 ppm exposure to NO_2 (6 hours per day for 3 days). (*C*) Neutrophil elastase (*NE*) activity in BAL of WT and CC10-IκBα$_{SR}$ mice exposed to 25 ppm NO_2 for 6 hours a day for 3 days (*$P < 0.05$ compared with WT mice; air, $n = 6$ mice; 25 ppm NO_2, $n = 8$ mice; 50 ppm NO_2, $n = 5$ mice).

Nitrogen Dioxide–Induced Lung Injury Is Attenuated in CC10-IκBα$_{SR}$ Mice. To quantitate the extent of lung injury in mice exposed to NO_2, we assessed levels of total protein and lactate dehydrogenase activity in BAL fluid. Exposure to NO_2 caused increases in protein levels to similar extents in wild-type and CC10-IκBα$_{SR}$ mice (Figure 3A). In contrast, increases in lactate dehydrogenase activity in BAL fluid in response to 25 ppm NO_2 were somewhat attenuated in CC10-IκBα$_{SR}$ mice compared with wild-type mice. However, these differences were not evident in response to 50 ppm NO_2 (Figure 3B).

To further assess the extent of ALI, histologic analyses of lung sections from mice exposed to NO_2 were performed. Wild-type mice displayed infiltrations of inflammatory cells, peribronchiolar lesions (Figure 4A), and shedding of the epithelial lining, which was extensive in response to 50 ppm NO_2 (Figure 4B). The extent of NO_2-induced injury was attenuated in CC10-IκBα$_{SR}$ mice compared with wild-type groups, and both the extent (Figure 4C) and severity (Figure 4D) of injury were attenuated in transgenic mice in response to both NO_2 doses. However, in mice exposed to 50 ppm NO_2, considerable epithelial shedding was apparent in the large airways, and this effect was not abrogated in CC10-IκBα$_{SR}$ mice (Figure 4B). Taken together, these results demonstrate a causal role for the activation of NF-κB in airway epithelium in the pathogenesis of NO_2-induced severe ALI.

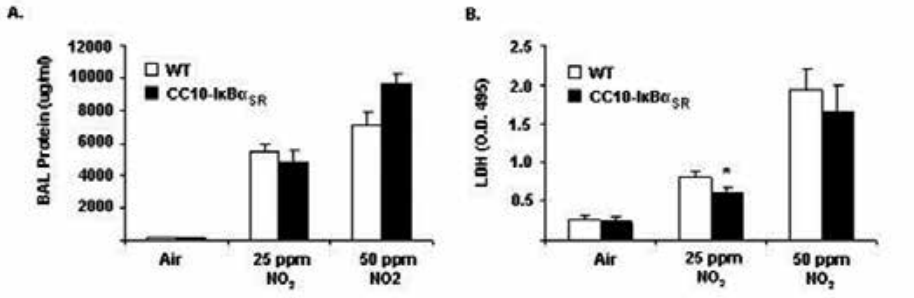

Figure 3. *Evaluation of protein (A) and lactate dehydrogenase (LDH, B) in BAL from wild-type (WT) or CC10-IκBα$_{SR}$ mice exposed for 6 hours/day to 25 ppm NO_2 for 3 days* (*P < 0.05 compared with WT mice; air, n = 6 mice; 25 ppm NO_2, n = 8 mice; 50 ppm NO_2, n = 5 mice).

Nitrogen Dioxide Exposure Induces Inflammatory Chemokines in an NF-κB–Dependent Manner. Nuclear factor–κB transcriptionally activates a myriad of chemokines and cytokines that participate in leukocyte recruitment and activation. Analyses of BAL fluid demonstrated that exposure to NO_2 increased concentrations of IL-6, IL-9, IL-12 (p40), granulocyte colony–stimulating factor (G-CSF), keratinocyte-derive chemoattractant (KC), macrophage chemotactic protein (MCP)-1, and regulated upon activation normal T-cell expressed and presumably secreted

(RANTES) RANTES in wild-type mice (Table 1). The expressions of RANTES, MIP-1β, macrophage inflammatory protein (MIP)-1α, MIP-2, and MCP-1 were also visualized at the RNA level, using RNase protection assays (as shown in Figure E1 in the online supplement), demonstrating elevated expression in response to NO_2. As expected, CC10-IκBα$_{SR}$ mice demonstrated significantly abrogated concentrations of these chemokines and cytokines, compared with wild-type mice (Table 1).

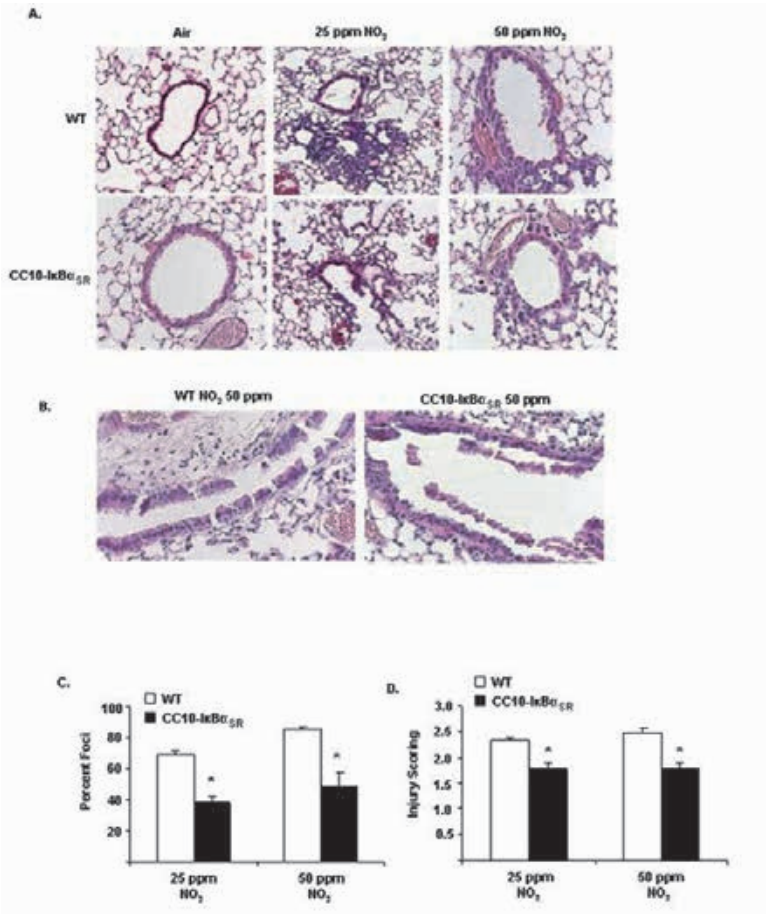

Figure 4. *Evaluation of histopathology in wild-type (WT) and CC10-IκBα$_{SR}$ mice exposed for 6 hours/day to 25 ppm or 50 ppm of NO_2 for 3 days.* (A) Hematoxylin-and-eosin staining of 5-μm sections of mouse lungs. (B) Hematoxylin-and-eosin staining of large airways from mice exposed to 50 ppm NO_2. All magnifications are ×200, and are representative of patterns observed in each group. Scoring of lesions by assessment of the percentage of airways involved per section (C) and the intensity of foci (D) was performed as described in Materials and Methods (*$P < 0.05$ compared with WT mice; 25 ppm NO_2, n = 8 mice/group; 50 ppm NO_2, n = 5 mice/group).

Table I. BAL cytokines (pg/ml) of WT vs. CC10-IκBαSR mice exposed to NO_2.

	Air		NO_2	
	WT	CC10-IκBα	WT	CC10-IκBα
IL-6	0	0.2 ± 0.1	8.2 ± 2.9*	0.9 ± 0.6**
IL-9	1.5 ± 0.9	4.9 ± 1.0	4.2 ± 0.8*	2.1 ± 0.4**
IL-12 (p40)	38.4 ± 12.7	22.8 ± 1.8	114.2 ± 21.5*	86. 2 ± 22.1**
G-CSF	0.7 ± 0.4	0.7 ± 0.2	14.7 ± 4.3*	7.0 ± 2.4**
KC	0.6 ± 0.1	0.2 ± 0.1	7.3 ± 1.2*	1.2 ± 0.1**
MCP-1	0	0.5 ± 0.5	31.4 ± 8.2*	14.5 ± 2.6**
RANTES	0.1 ± 0.04	0.1 ± 0.05	0.5 ± 0.1*	0.2 ± 0.1**

*indicates a significant increase in response to NO_2

**indicates a significant decrease compared to WT littermates exposed to NO2

No detectable levels of: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-10, Il-12(p70), IL-13, IL-17, GM-CSF, MIP-1α, MIP-1β Eotaxin, IFNγ, TNF-α. Cytokines measured in BAL by Bioplex Assay in wild type littermate and CC10-IκBα$_{SR}$ mice after 25 ppm NO_2 (6h a day for 3 days). Significance determined by $p < 0.05$. n=5.

Nuclear Factor–κB Activation Before Exposure to NO_2 Modulates Neutrophil Accumulation in the Airways. Based on our observations that inhibition of NF-κB in the airway epithelium decreases NO_2-induced inflammation and lung injury, we postulated that enhanced activation of NF-κB in the epithelium before exposure to NO_2 would worsen inflammation and injury. We reported that CC10-rTet-$_{CA}$IKKβ bitransgenic mice demonstrate an activation of airway epithelial NF-κB when receiving Dox chow, resulting in robust airway neutrophilia, increases in inflammatory cytokines, and airway hyperresponsiveness (16). In the present study, we confirmed the expression of IKKβ in lung homogenates from wild-type littermate controls, and augmented expression in Dox-fed CC10-$_{CA}$IKKβ mice. The results depicted in Figure 5A demonstrate marked increases in the expression of IKKβ protein in $_{CA}$IKKβ transgenic mice that received Dox compared with wild-type littermate controls, and that increases in IKKβ protein levels were not affected after exposure to NO_2. Furthermore, CC10-rTet-$_{CA}$IKKβ mice receiving doxycycline demonstrated an increase in neutrophils in BAL fluid, whereas wild-type littermates or CC10-rTet-$_{CA}$IKKβ mice that received regular food had neutrophil concentrations in BAL fluid similar to those of control animals (Figure 5B). As shown in Figure 2, the inhalation of 25 ppm NO_2 for 6 hours/day for 3 days also resulted in increases in airway neutrophilia. However, the extent of NO_2-induced neutrophilia (10,000 cells/ml) was markedly less than for the airway neutrophilia observed in $_{CA}$IKKβ-expressing mice (Figure 5B). Surprisingly, in mice expressing the $_{CA}$IKKβ transgene, subsequent

exposure to NO_2 resulted in no significant differences ($P = 0.15$) in airway neutrophilia, compared with $_{CA}$IKKβ-expressing mice that were maintained in room air (Figure 5B). Although $_{CA}$IKKβ mice on doxycycline showed enhanced neutrophilia in response to NO_2 compared with non-Dox control animals, the magnitude of neutrophilia was not additive between transgene expression and NO_2 exposure.

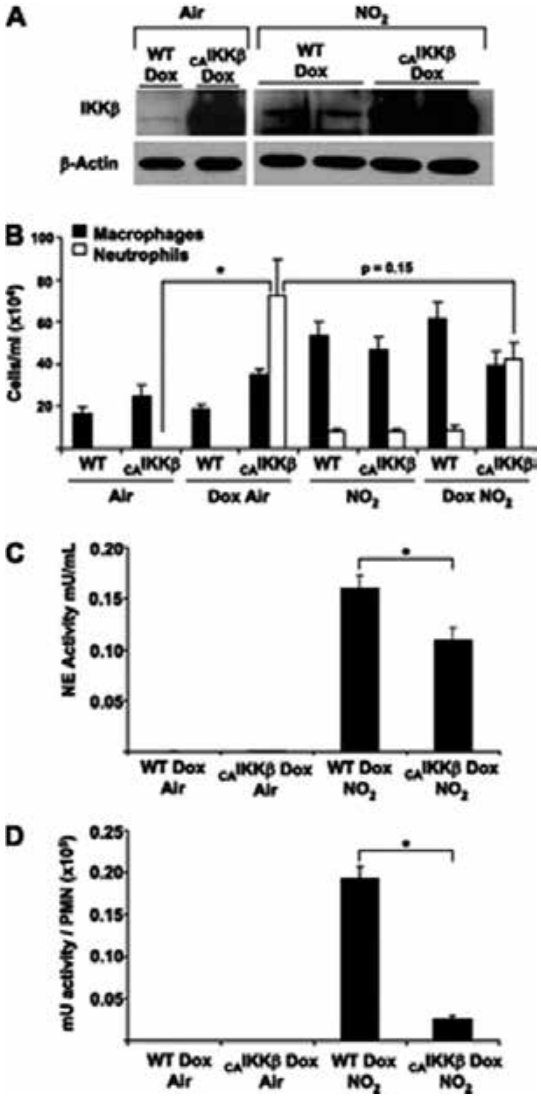

Figure 5. Assessment of inflammation and inflammatory mediators in BAL fluid of (WT) or $_{CA}$IKKβ-expressing mice exposed to room air or 25 ppm of NO_2 for 3 days. (A) CC10-$_{CA}$IKKβ mice or WT littermates received Dox food for 7 days. The NO_2 exposure groups received Dox food for 3 days before and during the exposure regimen of 6 hours per day of 25 ppm NO_2 for 3 days. All mice were killed on Day 7, and whole-lung homogenates were prepared for evaluation of IKKβ content (top) and β-actin (bottom). Representative blots are shown. (B) Cell counts in BAL from WT and $_{CA}$IKKβ-expressing mice exposed to air or 25 ppm NO_2 exposure (*P < 0.05 compared with WT group; air, n = 5 mice/group; Dox air, n = 8 mice/group; NO_2, n = 6 mice/group; Dox NO_2, n = 8 mice/group). Expression of neutrophil elastase (NE) activity in BAL fluid (C) or presented as a ratio of milliunits (mU) per 100,000 polymorphonuclear cells (PMNs) (D), from WT and $_{CA}$IKKβ-expressing mice receiving Dox and exposed to air or 25 ppm NO_2.

Interestingly, although concentrations of neutrophil elastase activity in the BAL fluid of Dox-fed and air-exposed wild-type and CC10-$_{CA}$IKKβ mice were undetectable, when the neutrophil elastase activity in the BAL fluid of wild-type and $_{CA}$IKKβ mice receiving Dox and exposed to NO_2 was measured, a significant reduction occurred in the amount of elastase activity in the $_{CA}$IKKβ mice (Figure 5C). When this reduced activity is expressed relative to the number of neutrophils present in BAL fluid, although the $_{CA}$IKKβ mice display enhanced neutrophilia, they exhibit severely decreased neutrophil elastase activity (Figure 5D). Therefore, the large numbers of neutrophils in $_{CA}$IKKβ mice on Dox do not likely contribute to the injury, because they are not inherently active with respect to neutrophil elastase activity, especially in comparison to that induced by inhalation of NO_2.

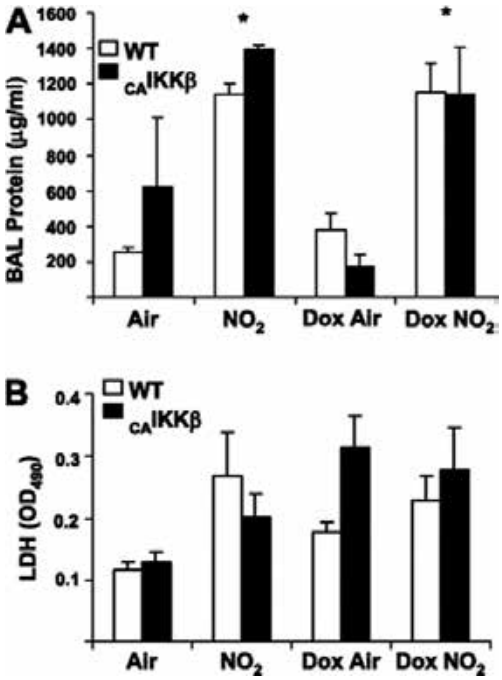

Figure 6. Evaluation of protein (A) and lactate dehydrogenase (LDH, B) in BAL from wild-type (WT) or CC10-$_{CA}$IKKβ transgenic mice exposed for 6 hours/day to 25 ppm NO_2 for 3 days.
* $P < 0.05$ compared with WT groups; n = 4 mice/group). Mice received regular or doxycycline food (Dox) for 3 days before beginning exposure to NO_2. All groups were killed on Day 7 (*$P < 0.05$ compared with WT littermate groups; air, n = 8 mice/group; Dox, n = 8 mice/group; NO_2, n = 6 mice/group; Dox NO_2, n = 8 mice/group).

Effects of NF-κB Activation in Airway Epithelium on NO_2-Induced Protein and Lactate Dehydrogenase in BAL. To determine whether enhanced NF-κB activity would affect the extent of lung injury in response to NO_2, protein content and lactate dehydrogenase activity in BAL fluid were analyzed. As shown in Figure 6, increases in total BAL protein (Figure 6A) and lactate dehydrogenase (LDH) (Figure 6B) were not augmented by previous activation of NF-κB ($_{CA}$IKKβ-expressing mice).

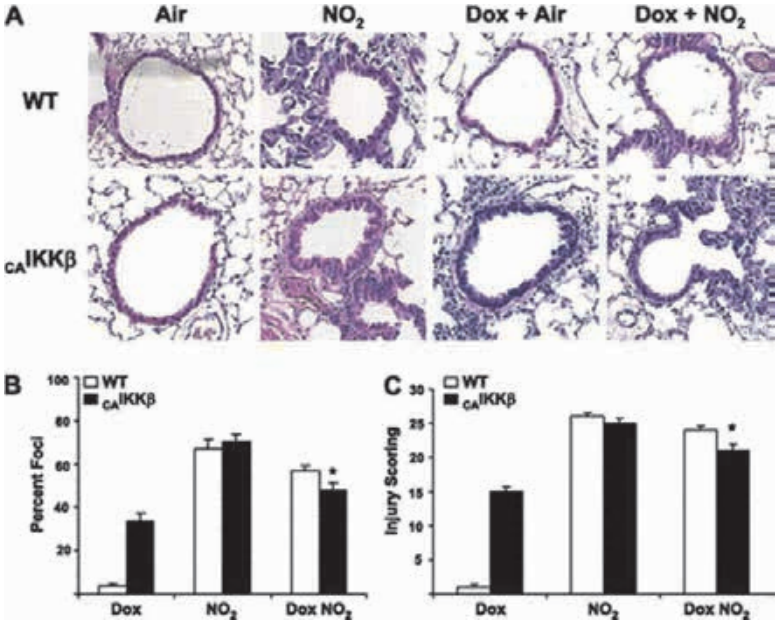

Figure 7. *Evaluation of histopathology from wild-type (WT) and CC10-$_{CA}$IKKβ transgenic mice exposed to room air or 6 hours/day of 25 ppm of NO_2 for 3 days.*
Mice received regular or doxycycline food (Dox) for 3 days before beginning exposure to NO_2. All groups were killed on Day 7. (A) Hematoxylin-and-eosin staining of 5-μm sections of mouse lungs. All magnifications are ×200, and are representative of patterns observed in each group. Scoring of lesions was performed by assessment of percentage of airways involved per section evaluated (B) and the intensity of foci (C), as described in Materials and Methods (*P < 0.05 compared with WT littermate groups; Dox, n = 8 mice/group; NO_2, n = 6 mice/group; Dox NO_2, n = 8 mice/group).

Nuclear Factor–κB Activation in Airway Epithelium Diminishes Lung Injury in Response to NO_2. We next evaluated lung histopathology in $_{CA}$IKKβ-expressing or wild-type mice exposed to room air or 25 ppm NO_2 for 3 days In agreement with our previous observations, $_{CA}$IKKβ mice receiving Dox exhibited robust leukocyte recruitment and evidence of thickening of the epithelial lining (16). In response to NO_2, all groups demonstrated leukocyte infiltrates, epithelial wall thickening, and perivascular lesions (Figure 7A). The $_{CA}$IKKβ mice that received Dox and were exposed to NO_2 displayed somewhat attenuated lesions compared with NO_2-exposed littermate controls or $_{CA}$IKKβ mice receiving regular chow (Figure 7A). Blinded scoring of these lung sections revealed a small but statistically significant reduction in both the percentage of airways involved (Figure 7B) and the intensity of injury (Figure 7C) in $_{CA}$IKKβ-expressing mice exposed to NO_2, compared with wild-type control animals. Measurements of prosurvival and antioxidant genes known to be regulated by NF-κB showed no difference between transgenic and wild-type mice receiving Dox and exposed to NO_2 (Figure E2). Furthermore, the abundance

of the cleaved (active) form of the proapoptotic protein caspase-3 was not different in the lungs of wild-type or CC10-$_{CA}$IKKβ mice exposed to NO_2 (Figure E3).

Table 2: BAL cytokines of WT vs. CA-IKKβ mice exposed to air or NO2, in the presence of absence of Dox.

	Air		NO2		Dox Air		Dox NO2	
	WT	CA-IKKβ	WT	CA-IKKβ	WT	CA-IKKβ	WT	CA-IKKβ
IL-5	0.3 ± 0.3	1.4 ± 0.07	18.2 ± 2.5*	12.0 ± 2.5*	3.4 ± 2.1	4.3 ± 1.8	12.9 ± 3.0	2.8 ± 0.7
IL-6	6.7 ± 6.7	21.6 ± 1.8	29.9 ± 1.5*	29.9 ± 0.6*	16.3 ± 0.9	30.7 ± 4.2**	25.5 ± 31	29.9 ± 1.5
IL-9	21.9 ± 3.5	14.9 ± 5.9	148.4 ± 56.0*	141.6 ± 43.6*	32.0 ± 4.3	21.4 ± 2.6	60.6 ± 30.0	31.2 ± 8.3
IL-10	15.3 ± 4.7	18.0 ± 7.4	102.8 ± 35.0*	100.0 ± 23.2*	23.5 ± 1.4	23.1 ± 5.0	51.6 ± 14.4	24.5 ± 5.0
IL-12(p40)	33.5 ± 6.0	38.7 ± 3.8	98.1 ± 3.4*	84.3 ± 9.4*	38.1 ± 7.3	119.8 ± 24.5**	79.8 ± 10.1	166.8 ± 21.8^
G-CSF	3.2 ± 0.8	4.9 ± 1.5	28.8 ± 3.5*	34.9 ± 0.9*	4.5 ± 1.2	48.4 ± 23.7**	26.2 ± 10.0	37.3 ± 4.0
KC	19.8 ± 3.1	26.1 ± 4.0	34.8 ± 5.4*	40.7 ± 3.0*	16.2 ± 2.6	64.8 ± 15.0**	22.3 ± 3.9	55.4 ± 7.5
MCP-1	1.1 ± 1.1	4.0 ± 2.0	32.7 ± 5.8*	32.2 ± 2.6*	14.2 ± 3.9	27.9 ± 6.5**	15.4 ± 5.4	53.2 ± 11.0^
MIP-1β	0.0 ± 0.0	0.0 ± 0.0	8.9 ± 1.0*	15.2 ± 2.6*	0.0 ± 0.0	11.8 ± 4.7**	5.2 ± 0.2	10.8 ± 2.3
RANTES	9.0 ± 1.3	9.4 ± 1.4	21.6 ± 1.6*	24.5 ± 1.7*	11.3 ± 1.7	45.5 ± 16.0**	20.2 ± 2.0	43.2 ± 4.6
TNF-α	8.1 ± 4.1	7.8 ± 3.9	14.2 ± 1.5	23.9 ± 1.0	9.5 ± 3.9	12.9 ± 1.8	6.6 ± 2.3	13.9 ± 2.1

*indicates a significant increase in response to NO2
**indicates a significant increase in CA-IKKβ mice compared to WT littermates receiving Dox.
^ indicates a significant increase in CA-IKKβ mice receiving Dox and exposed to NO2 compared to CA-IKKβ mice receiving Dox exposed to air.
No detectable levels of: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-12(p70), IL-13, IL-17, GM-CSF, MIP-1α, Eotaxin, IFNγ. Cytokines measured in BAL by Bioplex Assay in wild type littermate and CA-IKKβ mice that were exposed to air with or without Dox or exposed to after 25 ppm NO_2 (6h a day for 3 days) with or without Dox. Significance determined by $p < 0.05$. n=4.

Nuclear Factor–κB Activation in Airway Epithelium Modulates Concentrations of Inflammatory Cytokines in Response to NO_2. We next evaluated the impact of airway epithelial NF-κB activation on NO_2-induced inflammatory mediators in BAL fluid. In line with the results described in Table 1, exposure to NO_2 increased the levels of multiple proinflammatory mediators. Table 2 demonstrates robust increases in the levels of IL-5, IL-6, IL-9, IL-10, IL-12 (p40), G-CSF, KC, MCP-1, MIP-1β, and RANTES in BAL fluid from mice exposed to NO_2. Activation of NF-κB in the airway epithelium alone caused significant expression of IL-12 (p40), G-CSF, KC, MCP-1, MIP-1β, and RANTES. In addition, concentrations of IL-12 (p40) and MCP-1 were significantly enhanced when NF-κB was activated in transgenic mice before exposure to NO_2, compared with their respective control groups (Table 2). Further-

more, administration of Dox to wild-type or $_{CA}$IKKβ mice attenuated the increases in IL-9 and IL-10 concentrations that occurred in response to NO_2 exposure. The increases in proinflammatory mediators that follow the induction of $_{CA}$IKKβ expression explain the recruitment of leukocytes, but are paradoxical in light of the diminished lung injury measured in these mice.

Discussion

Because it functions as a regulator of both proinflammatory and prosurvival factors, as well as processes modulating proliferation and cell fate, establishing the role of NF-κB in severe ALI is complex. Nonetheless, the inhibition of NF-κB is considered a means of alleviating inflammation in diseases such allergic asthma (19) and other lung diseases, including ALI (20).

As previous studies demonstrated (5), high doses of NO_2 exposure result in a robust inflammatory response that includes leukocyte influx, increased cytokine expression, and tissue injury characterized by airway epithelial thickening and perivascular lesions. We demonstrate here that this inflammation is mediated by the activities of airway epithelial NF-κB, because RelA is present in the nuclei of airway epithelia after the first 6 hours of NO_2 exposure, and the inflammatory response to sustained NO_2 exposure is abrogated in CC10-IκBα$_{SR}$ mice. The lack of canonical NF-κB activation in these mice also interferes with the induction of the NF-κB–regulated inflammatory chemokines KC, MCP-1, and IL-12 (p40), and subsequently decreases the number of inflammatory leukocytes. This correlation between the lack of neutrophils and the reduction in neutrophil elastase suggests that a causal role may exist for neutrophils in oxidant-induced, NF-κB–dependent airway injury.

The reduction in airway epithelial inflammation and injury in CC10-IκBα$_{SR}$ mice *in vivo* is consistent with previous studies. Patients with neutropenia demonstrated a reduced severity of ALI (21), and patients show an increased severity of ALI as neutrophil numbers recover (22). In addition, neutrophils were also implicated in airway remodeling, and this was suggested to occur via neutrophil production of matrix metalloproteases and elastase (18). Furthermore, normal human bronchial epithelial cells exposed to NO_2 show an enhanced adhesion to neutrophils, and in turn an increased rate of epithelial cell death (23). Finally, the depletion of neutrophils in animal models reduces the activation of NF-κB observed in ALI (24). Consistent with these findings, our studies demonstrate reduced neutrophil numbers and subsequently lower neutrophil elastase activity in CC10-IκBα$_{SR}$ mice, corresponding with the evidence of decreased lung injury. While attacking pathogens, neutrophils activate a respiratory burst and undergo apoptosis, releasing a number of factors that are indiscriminate in their damaging effects. There-

fore, apoptotic neutrophils must be cleared from the site of inflammation in a timely manner, and thus are rapidly scavenged by macrophages. Dysregulated neutrophil apoptosis was suggested to contribute to lingering inflammatory conditions (25). This concept is supported by the fact that BAL fluid from patients with ALI often contains antiapoptotic factors such as GM-CSF and G-CSF (26). Therefore, the expression of prosurvival factors downstream of airway epithelial NF-κB activation may plausibly prolong the survival of the neutrophils themselves, leading to an overaccumulation at the site of injury and subsequent chronic inflammation (27).

Intriguingly, in our study, airway epithelial $_{CA}$IKKβ-expressing mice recruited an overwhelming number of neutrophils, yet showed significantly less severe injury than wild-type mice exposed to NO_2, despite the augmented expression of some proinflammatory mediators. These findings raise the question of whether these recruited neutrophils are inactive in the absence of other stimuli. Previous studies in which mice expressed the neutrophil chemokines KC and MIP-2 in the lung demonstrated that the subsequent neutrophil influx does not cause injury or vascular leakage. These studies indicated that although KC and other CXC chemokines are potent recruiters of neutrophils, additional mediators are required for their full activation and degranulation (28). Indeed (as shown by our measurement of neutrophil elastase activity), despite the increased population of neutrophils seen in $_{CA}$IKKβ mice receiving Dox and exposed to NO_2, the lavageable airspaces appear to possess a marked reduction in elastase activity, suggesting that these cells are not as active as those from wild-type mice under the same conditions.

The influx of neutrophils and macrophages in response to NO_2 is clearly explained by the host of well-known chemotactic factors present in BAL fluid, and particularly KC and MCP-1. In addition, mice exposed to NO_2 show significant increases in concentrations of IL-12 (p40) in BAL fluid, which appears to be strongly NF-κB–driven, because it is enhanced in $_{CA}$IKKβ mice after exposure to NO_2. Interleukin-12 (p40) was found in the BAL fluid of patients with asthma (29) and patients with fibrosis associated with silica exposure (30), suggesting another link for airway epithelial NF-κB in asthma and airway remodeling beyond those we (11, 16, 31, 32) and others (12, 33-36) described using mouse models of disease.

The relationship between pulmonary inflammation and tissue damage is not uniformly direct. Leukocyte infiltration does not necessarily correlate with protein increase, and the influx of neutrophils and monocytes can occur independently of vascular leakage (37). Studies in intestinal epithelium showed that inhibiting caspases can prevent barrier dysfunction (38). However, the expression of prosurvival and antioxidant genes that may be enhanced by $_{CA}$IKKβ mice receiving Dox was not significantly increased. Furthermore, the measurement of apoptosis (cleaved caspase-3; Figure E3) in NO_2-exposed lungs showed no differences be-

tween wild-type and $_{CA}$IKKβ mice, leading us to believe that, in this case, the $_{CA}$IKKβ mice do not exert a prosurvival, antiapoptotic effect in the epithelium itself.

Although further studies are required to elucidate the signaling mechanism responsible for NO_2-induced injury and the underlying mechanism of neutrophil activity modulation, the recruited activated neutrophils clearly play a role in damaging airway epithelium and lung tissue, and that this is, in part, NF-κB–dependent. Our work further indicates that although NF-κB activation is sufficient to recruit neutrophils to the lung, additional factors are required for their activation, degranulation, and subsequent tissue injury. Perhaps the most intriguing outcome in our study is the finding that airway epithelial NF-κB activation in the absence of other overt stimuli causes an injury and an inflammatory response qualitatively similar to that induced by NO_2 exposure, and that the effects of NO_2 inhalation are not augmented but are somewhat diminished by previous airway epithelial NF-κB activation. Collectively, these novel findings indicate that a therapeutic modulation of NF-κB in lung disease needs to take into account the diverse functions of this potent transcription factor.

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CHAPTER 4

Airway epithelial NF-κB activation promotes allergic sensitization to an innocuous inhaled antigen.

Jennifer L. Ather, Samantha R. Hodgkins, Yvonne M. W. Janssen-Heininger, and Matthew E. Poynter

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ABSTRACT

Activation of NF-κB in airway epithelium is observed in allergic asthma and is induced by inhalation of numerous infectious and reactive substances. Many of the substances that activate NF-κB in the airway epithelium are also capable of acting as adjuvants to elicit antigen-specific sensitization to concomitantly inhaled protein, thereby circumventing the inherent bias of the lung to promote tolerance to innocuous antigens. We have used a transgenic mouse inducibly expressing a constitutively active mutant of the inhibitor of nuclear factor κB (IκB) kinase β ($_{CA}$IKKβ) that activates NF-κB only in nonciliated airway epithelial cells to test whether activation of this intracellular signaling pathway in this specific cell type is sufficient to establish a pulmonary environment permissive to the development of allergic sensitization to inhaled protein. When airway epithelial $_{CA}$IKKβ was transiently expressed in antigen-naive mice only during initial inhalation of ovalbumin, the mice became allergically sensitized to the antigen. As a consequence, subsequent inhalation of ovalbumin alone led to an allergic asthma–like response that included airway hyperresponsiveness to methacholine, eosinophilia, mucus expression, elevated serum levels of antigen-specific IgE and IgG1, and splenic $CD4^+$ T cells that secreted T helper type 2 and type 17 cytokines in response to *in vitro* antigen restimulation. Furthermore, $CD11c^+$ cells in the mediastinal lymph nodes (MLN) of $_{CA}$IKKβ-expressing mice displayed significantly elevated levels of activation markers. These data implicate airway epithelial NF-κB activation as a critical modulator of the adaptive immune response to inhaled antigens via the secretion of soluble mediators that affect the capacity of $CD11c^+$ cells to undergo maturation and promote antigen-specific allergic responses.

Introduction

Allergic asthma affects approximately 300 million people worldwide, with the incidence continuing to increase steadily in industrialized and developing countries (1). A multifaceted syndrome, allergic asthma is defined by chronic inflammation, bronchoconstriction, mucus metaplasia, airway hyperresponsiveness, T helper (Th) 2 phenotype skewing, and elevated levels of serum IgE (2, 3). Individuals with severe asthma also suffer from increases in airway smooth muscle proliferation and a predisposition to airway remodeling, leading to further permanent long-term damage (4, 5). Current treatment relies on inhaled corticosteroids to control the disorder, which do not prevent asthmatic attacks and do not work effectively for certain patients, such as those with neutrophilic (rather than eosinophilic) infiltrates, those with pre-exisiting inflammatory conditions, and those with certain glucocorticoid receptor polymorphisms (3, 6). Recent studies also suggest that activated Th17-skewed cells are contributors to steroid-resistant and severe allergic asthma (reviewed in Ref. (7)).

Allergic asthma is widely recognized as an inflammatory disorder, and recent studies have implicated the transcription factor, NF-κB, considered to be a critical modulator of inflammation in the pathogenesis of lung disease (8-10). Indeed, prolonged and robust airway epithelial NF-κB activation is observed in patients with asthma (11). In previous studies, we have demonstrated that lack of airway epithelial NF-κB activation in a mouse model of allergic asthma results in a diminished inflammatory phenotype; specifically, abrogation of cellular influx, mucus production, production of Th2 cytokines, IL-5 and IL-13, and antigen-specific serum antibodies (12). Th2 cells, which secrete primarily IL-4, -5, and -13, play a key role in allergic asthma by promoting class switching to IgE by B cells, and supporting the accumulation and survival of eosinophils and mast cells, in addition to inducing goblet cell hyperplasia and altering airway smooth muscle constriction and hyperresponsiveness (13, 14).

The airway epithelium, in addition to being the first line of defense against inhaled pathogens, has recently been shown to play an immunomodulatory role in the lung (11). In addition to robust NF-κB activity and the release of proinflammatory cytokines and chemokines, the epithelium also secretes factors that directly influence dendritic cells, the major antigen-presenting cell (APC) in the immune system (13, 15, 16). Epithelial cells recruit dendritic cells to the lung via secretion of the chemokine, CCL20 (macrophage inflammatory protein [MIP]-3α), which is the only cytokine known to interact with the CCR6 receptor expressed on immature dendritic cells (11). Furthermore, airway epithelial cells can also secrete thymic stromal lymphopoeitin (TSLP), granulocyte/macrophage colony–stimulating factor (GM-CSF), and IL-6 in response to antigen, factors that influence

the maturation of dendritic cells and polarize $CD4^+$ T cells toward a Th2 phenotype (11, 13, 17, 18).

Under normal circumstances, inhalation of an innocuous antigen, such as an environmental allergen, is perceived and responded to by the immune system in a manner that promotes antigen-specific immunologic tolerance. In experimental models, this immunologic tolerance is prevented by the use of adjuvants (e.g., aluminum hydroxide, cytokines, microbial products, cholera toxin, etc.), which prime the immune system to respond to an otherwise innocuous antigen. We have previously demonstrated that the oxidant gas, nitrogen dioxide, can promote the sensitization of mice to an innocuous inhaled antigen, and that this sensitization occurs in conjunction with activation of airway epithelial NF-κB (19).

Using a transgenic mouse model in which mice express a doxycycline (Dox)-inducible, constitutively active form of the inhibitor of NF-κB (IκB) kinase β ($_{CA}$IKKβ) specifically in the airway epithelium, we have previously published that airway epithelial activation of NF-κB during antigen challenge exacerbates a conventional alum/ovalbumin (OVA) model of allergic airways disease. This exacerbation promotes neutrophilia, as well as increasing the magnitude of eosinophilia, enhancing lung heterogeneity in response to methacholine, and augmenting bronchoalveolar lavage (BAL) cytokine levels of IL-17, IL-4, keratinocyte-derived chemokine (KC), and MIP-1β (10). Given these results, we sought to determine the effects of airway epithelial NF-κB activation alone during the antigen sensitization phase.

We therefore investigated whether activation of airway epithelial NF-κB is sufficient to promote allergic sensitization to an innocuous inhaled antigen, independent of an exogenously administered adjuvant, and, if so, whether the effect is at least partially due to the activation of pulmonary $CD11c^+$ APCs. Our results demonstrate that transient activation of NF-κB in the airway epithelium is sufficient to initiate events that promote allergic sensitization to an inhaled antigen. These data implicate airway epithelial NF-κB activity as an important facilitator of allergic sensitization that may be a common mediator involved in the capacity of several infectious and environmental agents to predispose susceptible individuals to developing atopic responses to inhaled antigens.

Materials and Methods

Mice.

CC10-rtTA × TetOP-$_{CA}$IKKβ bitransgenic mice (10, 20) on the C57BL/6 background express $_{CA}$IKKβ in bronchiolar epithelium after administration of 6 g/kg Dox in chow (TestDiet, Richmond, IN). Wild-type mice were age- and sex-matched transgene-negative littermates. Mice were housed in an Association for Assess-

ment and Accreditation of Laboratory Animal Care-approved facility, maintained on a 12-hour light/dark cycle, and were provided food and water *ad libitum*. All animal studies were approved by the University of Vermont Institutional Animal Care and Use committee.

Model of Airway Epithelial NF-κB–Promoted Allergic Sensitization.
Mice received Dox chow for 48 hours before 30 min inhalation of 1% OVA (grade V; Sigma-Aldrich, St. Louis, MO) on Day 2, after which they returned to normal food. On Day 7, mice were provided Dox chow for another 48-hour period, followed by a second 1% OVA inhalation on Day 9, after which they were again returned to normal food, on which they were maintained for the remainder of the study. After 1 week, on Days 14, 15, and 16, mice inhaled 1% OVA for 30 minutes each day. Mice were analyzed on Day 18, 48 hours after the last antigen challenge inhalation.

Pulmonary Function Assessment to Measure Airway Hyperresponsiveness.
Mice were anesthetized and mechanically ventilated for the assessment of pulmonary function using the forced oscillation technique, as previously described (12, 21). Airway resistance (R_N), tissue damping (G), and tissue stiffness (H) (22) were calculated at baseline and after challenge with 25 mg/ml aerosolized methacholine (Sigma-Aldrich) in saline, an appropriate concentration for submucosal responses based on our previously published dose response studies (19, 20). The percentage change from baseline (ΔR_N, ΔG, and ΔH), and the peak change in each parameter is reported.

Bronchoalveolar Lavage and Lung Processing.
Lungs were lavaged with a single instillation and recovery of 1 ml ice-cold PBS containing protease inhibitor cocktail (Sigma-Aldrich) and processed for cytospin counting as previously described (19). Right lung lobes were flash frozen for RNA and protein analysis. Left lung lobes were processed for hematoxylin and eosin staining as previously described (19). Photomicrographs of airways with a length-to-width ratio of 0.5:2.0 were provided for assessment by two independent individuals blinded to the identity of the specimens, and representative pictures were selected for presentation.

Immunoblotting.
Protein (25 μg) from lung homogenates prepared in 1× PBS were run on a 4–20% Tris-Glycine gel and transferred to a nitrocellulose membrane. Lysates were probed for IKKβ (Cell Signaling Technology, Danvers, MA) and reprobed with β-actin as a loading control as previously described (10).

Cytokine Profiling, Protein Assessment, and Lactate Dehydrogenase Measurement from BAL Fluid.
Cytokine levels were assessed using a Bioplex 23-plex panel (Bio-Rad, Hercules, CA), as previously described (23). Total protein levels were measured using the Bradford Assay (Bio-Rad), and lactate hehydrogenase (LDH) activity was measured using the LDH Detection Assay Kit (Promega, Madison, WI).

Serum Collection and Ig Analysis.
After mice were killed, blood was collected via cardiac puncture into serum separator tubes (Becton Dickinson, Franklin Lanes, NJ), centrifuged, and serum was kept frozen at −80°C. Antigen-specific and total Ig ELISAs were performed as previously described (19).

Quantitative RT-PCR.
Total RNA was isolated from lungs using the PrepEase RNA Isolation Kit (USB Corp., Cleveland, OH) and reverse transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative RT-PCR was performed using SYBR Green PCR Supermix (Bio-Rad) and intron-spanning primers designed for mouse GM-CSF, IL-12p19 (IL-23), serum amyloid A (SAA)3, TSLP, IL-6, IL-23, and IL-25. IQ Supermix (Bio-Rad) and TaqMan AODs (Applied Biosystems Inc, Foster City, CA) were used for GAPDH, CCL20, ZO-1, E-cadherin, and Muc5AC. The level of gene expression was normalized to GAPDH levels and relative gene expression was calculated using the ΔΔCt method, as previously described (19).

Preparation, Stimulation, and Analysis of Single-Cell Mediastinal Lymph Node and $CD4^+$ Lymphocyte Suspensions.
Splenic tissue was processed for the isolation of $CD4^+$ T cells from experimental mice and APCs from naive C57BL/6 mice, as previously described (24). $CD4^+$ T cells (4×10^6 cells/ml) were activated with 100 μg/ml OVA in the presence of syngeneic APCs (4×10^6 cells/ml in 48-well plates). After 96 hours of stimulation, supernatants were collected and analyzed by Bio-Plex (Bio-Rad).

Flow Cytometric Analysis of Mediastinal Lymph Node Cells.
Mediastinal lymph node (MLN) cells were dissociated by mechanical disruption, filtered through a 40-μm nylon mesh membrane and stained with the following antibodies: CD45-PO, CD11c-PETR, F4/80-Alexa 647, CD86-Alexa 647 (all from Caltag, Carlsbad, CA); I-A/I-E-PerCP/Cy5.5 (BioLegend, San Diego, CA); and CD11b-APCcy7 and glucocorticoid receptor–1–PE (BD Pharmingen, San Diego, CA). Cells (1×10^6) were first blocked with Fc block (anti-CD16/CD32) (2.5 μg/ml; BD Pharmingen) for 30 minutes at 4°C, washed in FACS buffer (Dulbecco's phosphate buffered saline [DPBS]; CellGro, Manassas, VA) DPBS with 5% FBS (Invitrogen, Carlsbad, CA), and then stained for 30 minutes at 4°C in 100 μl of antibody solution

at the optimal concentration. After staining, all cells were washed and fixed in DPBS with 5% FBS and 1% paraformaldhehyde. Cells were analyzed on a Becton Dickinson LSR II FACS flow cytometer equipped to distinguish as many as seven fluorophores 1–3 days after staining. Dead cells were excluded from analysis by forward scatter (FSC) and side scatter (SSC) gating, and $CD45^{+}$, $F4/80^{neg}$, $FITC^{low}$ cells were gated for further analysis of major histocompatability complex class II (MHCII), CD11b, and CD11c staining.

Statistical Analysis.
Data were analyzed by two-tailed unpaired *t* test using GraphPad Prism 4 for Windows (GraphPad Software, Inc., La Jolla, CA). A *P* value less than 0.05 was considered statistically significant.

Results

Mice Allergically Sensitized to OVA via Airway Epithelial NF-κB Activation and Antigen Inhalation Exhibit Hyperresponsiveness to Inhaled Methacholine after Antigen Challenge. Transgenic ($_{CA}$IKKβ) and wild-type littermate mice received Dox for two 48-hour periods, each of which were followed by a 30-minute exposure to 1% aerosolized OVA. A week later, mice were challenged with aerosolized 1% OVA on 3 consecutive days and analyzed 48 hours after the final exposure (Figure 1A). Western blot analysis shows that IKKβ can be detected at the protein level after 48 hours of Dox chow. However, when analyzed just before inhaled antigen challenge on Day 14, the IKKβ protein is undetectable, illustrating that, after 5 days on normal chow, transgene activation returns to the undetectable levels observed before Dox administration (Figure 1B). All mice exhibited normal

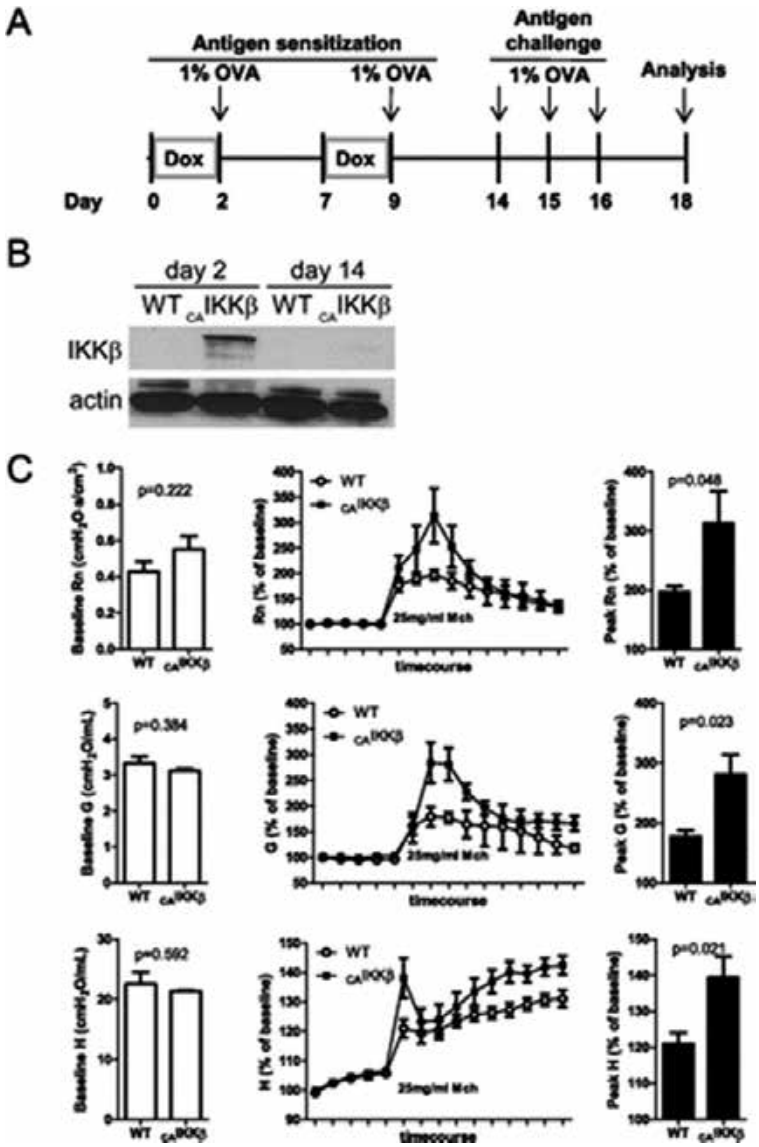

Figure 1. *Airway epithelial NF-κB activation promoted antigen sensitization and airway hyperresponsiveness to methacholine.*

(*A*) A timeline of exposure regimens to promote antigen sensitization for constitutively active inhibitor of NF-κB (IκB) kinase β ($_{CA}$IKKβ) transgenic and wild-type (WT) littermate mice. All 1% ovalbumin (OVA) inhalations were 30 minutes long. (*B*) Western blot of lung homogenates for expression of the $_{CA}$IKKβ transgene at Days 2 and 14. Note that endogenous IKKβ is below the detection limit of these Western blot procedures. (*C*) Respiratory mechanics assessment of methacholine responsiveness using the forced oscillation technique in mice on Day 18 at baseline before administration of methacholine (*left*), during the methacholine challenge regimen (*middle*), and at the peak response to 25 mg/ml methacholine (*right*) (n = 5 mice/group).

pulmonary physiology at baseline, as illustrated in the first five points of the time course before the inhalation of methacholine, but the antigen-challenged $_{CA}$IKKβ mice were hyperresponsive to methacholine in comparison with their wild-type counterparts (Figure 1C). Methacholine responsiveness, as measured by the parameters R_N, G, and H, were significantly increased in $_{CA}$IKKβ mice as a consequence of antigen challenge (Figure 1C, insets). Because methacholine hyperresponsiveness is indicative of an allergic asthma-like phenotype, we further characterized inflammatory and immunologic consequences in the $_{CA}$IKKβ mice.

Airway Epithelial NF-κB Activation in Combination with OVA Inhalation Promotes an Allergic Pattern of Pulmonary Inflammation, Mucus Metaplasia, and a Th2-Skewed Phenotype in Response to Challenge with Inhaled OVA. Lungs were lavaged at harvest and the BAL fluid was examined for cellular infiltration. Whereas wild-type mice subjected to the OVA inhalation regimen described in Figure 1A exhibited normal levels of macrophages and a small number of eosinophils, $_{CA}$IKKβ mice displayed substantial and significant increase in eosinophils and lymphocytes (Figure 2A), as is observed in conventional allergic asthma models in which alum is used as an adjuvant. In addition, we also measured significant increases in airway neutrophils after allergen challenge (Figure 2A). Airway epithelial NF-κB activation also promoted mucus metaplasia in response to inhaled antigen, as well as augmented gene expression of *Muc5ac*, a product of mucus-producing cells (Figure 2B). Furthermore, when serum from the mice was examined by ELISA, $_{CA}$IKKβ mice displayed significant increases in Th2 cytokine–promoted total and antigen-specific IgE, as well as antigen-specific IgG1, with no significant changes in IgG2c, a Th1-driven Ig (Figure 2C).

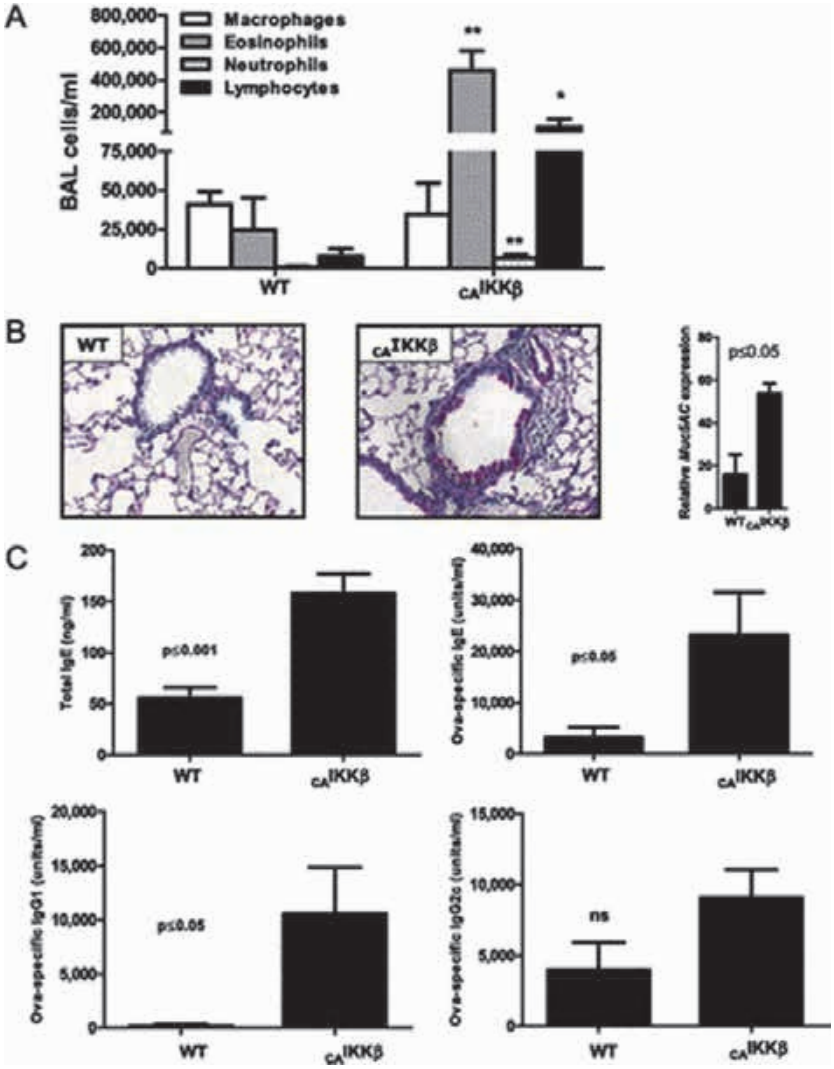

Figure 2. *Airway epithelial activation at the time of first encounter with antigen induces an influx of inflammatory cells subsequent to inhaled allergen challenge.*

CAIKKβ and WT littermate mice were exposed as depticed in Figure 1A. (A) On Day 18, differential cell counts were assessed from the bronchoalveolar lavage (BAL). Periodic acid–Schiff reactivity was assessed from histologic lung specimens visualized at 200× (B), and expression of the mucus gene Muc5AC was measured by quantitative RT-PCR and displayed relative to the levels in naive mice. (C) Serum Ig levels were measured by ELISA. Standard curves for OVA-specific IgE and IgG1 were generated from alum/OVA-sensitized BALB/cJ mouse serum, whereas nitrogen dioxide/OVA-sensitized (19) C57BL/6J serum was used to generate standards for OVA-specific IgG2c. For comparisons, values for the most concentrated standards were set to 10,000 U/ml (n = 6 mice/group). Data are representative of studies performed twice. *P ≤0.05; **P ≤0.01.

$CD4^+$ T Cells from Transgenic Mice Restimulated with Antigen Secrete Th2 Cytokines and IL-17. $CD4^+$ T cells purified from the spleen at Day 18 were stimulated with OVA by APCs from naive C57BL/6J mice. Cell supernatants analyzed by Bio-Plex at 96 hours displayed a distinctive Th2 profile, with significant increases in IL-4, -5, -9, -10, and -13 (Figure 3), and no significant changes in the Th1-driven cytokine IFN-γ. Interestingly, $_{CA}$IKKβ transgenic mice also showed substantial increases in IL-17, a cytokine not abundantly produced by $CD4^+$ T cells in alum/OVA-sensitized mice.

Airway Epithelial NF-κB Expression Promotes the Activation of Pulmonary Dendritic Cells. As a potential mechanism to explain the capacity of airway epithelial NF-κB activation to promote allergic sensitization to an innocuous inhaled antigen, we measured several parameters related to pulmonary dendritic cell activation. RNA from whole-lung homogenates was analyzed by quantitative RT-PCR from $_{CA}$IKKβ and wild-type littermate mice after 48 hours of Dox chow (Figure 4A). Expression of several genes implicated in dendritic cell recruitment and differentiation, including *Ccl20*, *Gmcsf*, *Il12p19* (*IL-23*), and *Saa3* were significantly increased in $_{CA}$IKKβ mice on Dox compared with wild-type littermates (Figure 4B). However, several other Th2- or Th17-promoting genes, which are capable of being expressed by epithelium, including IL-6, -33, -25, and TSLP (25), were not elevated in the lungs of $_{CA}$IKKβ mice on Dox (Figure 4B). Furthermore, mediastinal lymph nodes from these mice contained significantly more cells with a surface phenotype indicative of mature dendritic cells, along with increases in cell surface expression of MHCII and CD11b. as analyzed by flow cytometry (Figure 4C).

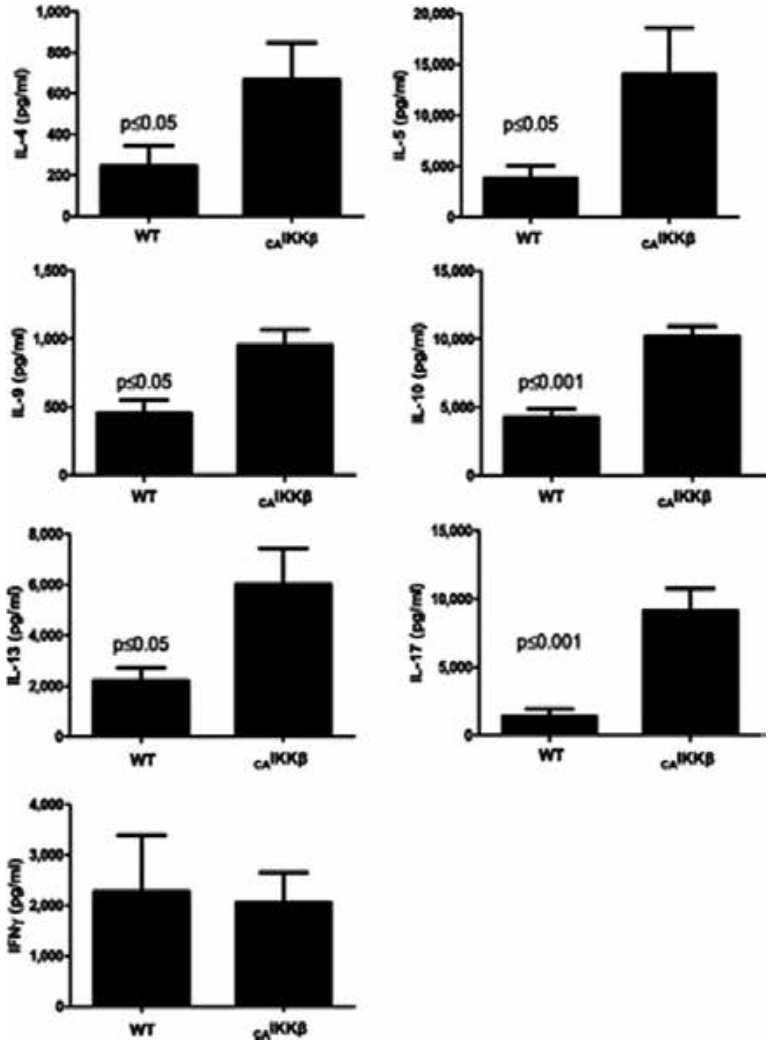

Figure 3. *$_{CA}$IKKβ transgene expression promotes an antigen-specific T helper (Th) 2– and Th17-skewed CD4$^+$ T-cell phenotype.*
Splenic CD4$^+$ T cells were incubated with antigen-presenting cells (APCs) and OVA for 96 hours, after which cytokines in cell culture supernatants were measured by Bio-Plex (n = 6 mice/group). Data are representative of studies performed twice.

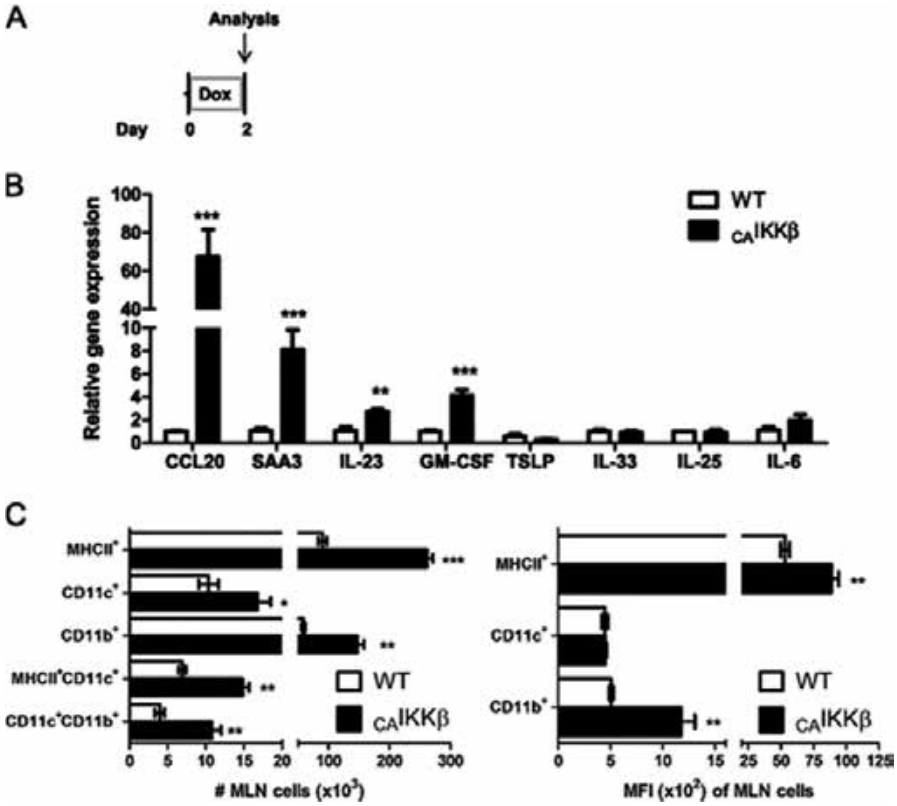

Figure 4. *Airway epithelial NF-κB expression modulates pulmonary CD11c⁺ APCs.*
(*A*) $_{CA}$IKKβ and WT littermates fed doxycycline (Dox) chow for 48 hours and harvested immediately were (*B*) assessed by quantitative RT-PCR from whole lung (n = 4 mice/group). (*C*) Mediastinal lymph node cells were stained and analyzed by flow cytometry for APC markers. Total cell numbers in each subset were calculated (*left*) and median fluorescence intensity (MFI) was calculated for each of the cell markers used (*right*) (n = 3 mice/group). *$P \le 0.05$; **$P \le 0.01$; ***$P \le 0.001$.

Airway Epithelial NF-κB Activation Does Not Compromise Epithelial Barrier Integrity. Recent studies have suggested that dendritic cells can be primed by antigen alone if there is sufficient disruption of the epithelial barrier comprised of the adherens junction complex (26). Therefore, we measured BAL total protein and lactate dehydrogenase (Figure 5A), and found no significant differences between $_{CA}$IKKβ mice and wild-type control animals, both of which were fed Dox for 48 hours. In addition, we measured gene expression of zona occludens (ZO)–1 and E-cadherin, two constituents of epithelial adherens junctions, and found no reduction in expression in $_{CA}$IKKβ mice compared with wild-type control mice (Figure 5B). Together, these data indicate that the epithelial barrier remains intact subsequent to airway epithelial NF-κB activation.

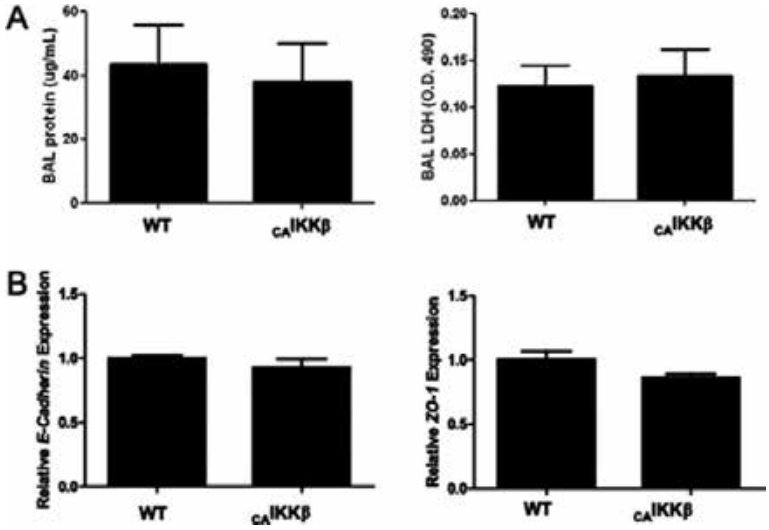

Figure 5. *Airway epithelial NF-κB expression does not affect epithelial barrier integrity.* $_{CA}$IKKβ and WT littermates fed Dox chow for 48 hours and harvested immediately. (*A*) BAL fluid was collected and total protein by Bradford assay (*left*) and lactate dehydrogenase activity (*right*) was recorded. (*B*) Whole-lung homogenates were assessed by quantitative RT-PCR for epithelial adherens junction markers *zona occludens* (*ZO*)*–1* (*left*) and *E-cadherin* (*right*) (n = 4 mice per group). Data are representative of studies performed twice.

Discussion

Once considered to primarily serve a barrier function in the lung, airway epithelium is now widely recognized for its immunomodulatory capabilities. This has allowed for a better understanding of how allergic sensitization in the lung-draining lymph node may occur subsequent to inhalation of an antigen in the context of an appropriate immunostimulatory milieu. Indeed, the data presented herein are the first to demonstrate that airway epithelial intracellular signaling through the NF-κB pathway is sufficient to promote allergic sensitization to an innocuous inhaled antigen. Many immunostimulatory factors are expressed by airway epithelial cells subsequent to inhalational exposure to microbes or environmental antigens (11). Several of these immunostimulatory factors (8-10) are transcriptionally regulated by NF-κB and were measured in our model of inducible airway epithelial NF-κB activation. The $_{CA}$IKKβ mice expressed a robust Th2 and Th17 cytokine profile as a consequence of antigen inhalation, creating the proper environment for dendritic cells to drive a mixed Th2 and Th17 effector response instead of developing inhalational tolerance. It is possible that viral and bacterial infections can act in the same manner: as an “NF-κB adjuvant,” creating an inflammatory environment that al-

lows for otherwise harmless inhaled antigens to become immunogenic. Similarly, several agents that are potent activators of NF-κB are also capable of promoting allergic sensitization to innocuous inhaled proteins. These include both microbial products and environmental agents, such as endotoxin (27), silica (28), residual oil fly ash (29), diesel exhaust particles (30), ultrafine particles (31), cigarette smoke (32), ozone (33), and nitrogen dioxide (19), suggesting that airway epithelial NF-κB activation may be a common mechanism through which several agents promote antigen-specific allergic sensitization.

The presence of a mixed Th2 and Th17 response is not new; recent studies have revealed that inhalational methods of allergic sensitization (as opposed to the traditional intraperitoneal alum/OVA model of sensitization) generate a modest Th2 profile and a more robust Th17 response (34, 35). Our model of sensitization also relies on inflammatory events that originate in the lung; therefore, it is not surprising that we have seen similar mixed T-cell phenotypes, with a Th2 profile that is modest compared with that seen in the traditional alum/OVA model. It is important to note that recent studies have implicated inhalational models of allergic sensitization to be more physiologically relevant than the traditional intraperitoneal alum/OVA model. For instance, the alum/OVA model can induce eosinophilia so severe that it makes up 80% of the total BAL cells, whereas, in patients with asthma, eosinophils make up 5% at most (34). Our previous alum/OVA sensitization regimen achieved eosinophilia on the order of 80–90% of total BAL cells (10), in contrast to the more modest 60% seen in our current model. Likewise, inhalational models, such as LPS/OVA achieve a similar moderate eosinophilia (27, 31, 36).

Recent studies have shown that airway epithelial cells exert control over dendritic cells, the primary APC instructing adaptive immune responses, which are therefore a key player in allergic disease (11, 17). Under normal conditions, dendritic cells that encounter a harmless antigen do not up-regulate the expression of costimulatory molecules and do not initiate an inflammatory T-cell effector response; instead, they promote a tolerogenic response accompanied by the activation of T regulatory cells (2). This tolerance is assisted by incompletely matured dendritic cells, including plasmacytoid dendritic cells, which suppress T cell effector generation. In the case of encounters with microbial products or environmental agents, airway epithelial cells secrete TSLP, which acts directly upon dendritic cells to up-regulate the costimulatory molecules, OX40L, CD40, and CD80, which facilitate Th2 polarization (17). In addition to TSLP, it has been reported that epithelial cells are capable of promoting Th2 polarization by secretion of IL-33, -25, and -6 (18, 25, 37). Furthermore, airway epithelial cells secrete factors that induce the differentiation of monocytes into dendritic cells and promote their maturation, namely, GM-CSF (11). GM-CSF has been shown to demonstrate adjuvant-like properties, and concomitant exposure of GM-CSF and OVA (38) or GM-CSF and Derp1

(39), can sensitize mice to a Th2 allergic response. Although our mouse model did not demonstrate any significant differences in TSLP expression (data not shown), $_{CA}$IKKβ mice did transcriptionally up-regulate GM-CSF. Further studies focused on neutralization of GM-CSF in our sensitization model may further elucidate the critical role of this cytokine in polarizing pulmonary CD11c^{+} cells, but were beyond the scope of the current study. In this study, we have demonstrated that airway epithelial NF-κB is sufficient to promote an inflammatory milieu that favors allergic sensitization and modulation of pulmonary dendritic cell activation.

It is important to note, however, that, although our model of inducible airway epithelial NF-κB activation induces cytokine production and exerts an effect upon other surrounding cells, transgene activation is transient and resolved before antigen challenge. Recently, there has been an emergence of reports supporting a role for antigens in directly effecting dendritic cells (26). Herbert and colleagues (40) demonstrated that house dust mite challenge causes a disruption in epithelial tight junctions, allowing access to dendritic cells within the airway wall. It has likewise been shown that the lungs of patients with asthma demonstrate poorly developed tight junctions, as evidenced by poor staining of ZO-1 (41). We examined epithelial integrity by measuring BAL protein and LDH that would suggest epithelial leakage or airway cell death. However, we observed no changes in either after 48 hours of Dox administration. In addition, gene expression of *ZO-1* and *E-cadherin* remained unchanged. This leads us to suspect that the changes in dendritic cell maturity found in the MLN of $_{CA}$IKKβ mice are not due to increased interaction with environmental antigen as a consequence of compromised epithelial barrier integrity. Certainly, if mice were provided Dox chow *ad libitum* for a more protracted period of time than we employed, and epithelial NF-κB activation was sustained, we would likely have revealed substantial compromise of the epithelial barrier integrity. However, at the time points relevant to our study, airway epithelial NF-κB activation itself was not sufficient to induce fluid leak or alter expression of tight junction–expressing genes. Instead, airway epithelial NF-κB activation did itself promote the expression of cytokines implicated in dendritic cell and Th cell recruitment and activation.

The analysis of gene induction in our transgenic mice after 48 hours of Dox chow revealed an increase in both CCL20, a potent dendritic cell chemokine, and GM-CSF, which differentiates monocytes into a dendritic cell phenotype. $_{CA}$IKKβ mice also expressed increased levels of the IL-12p19 subunit, which helps to form the active IL-23 heterodimer, a cytokine that recent studies have implicated as a precursor to the Th17-skewed T-cell response (42, 43). SAA3, an acute-phase protein expressed outside the liver, the gene expression of which is up-regulated in the $_{CA}$IKKβ mice, has been shown to cause blood monocytes to preferentially secrete IL-23 instead of IL-12p40 (42), thus possibly contributing to the generation of a Th17-involving antigen-specific allergic response. In addition, SAA3 signals

through Toll-like receptor 2 (44, 45), a pattern recognition receptor that we have demonstrated to be essential for nitrogen dioxide–promoted allergic sensitization (19).

In a healthy lung, tolerance is the prevailing response after initial exposure to innocuous inhaled antigen (46), and requires the collaboration of several cell types and soluble mediators. However, when the signals for APC activation and Th cell polarization are already present as a consequence of lung inflammation, an otherwise harmless antigen can initiate an allergic response, mimicking the manner in which an adjuvant allows for immune sensitization. Although we have used inducible airway epithelial NF-κB activation as an effective model to illustrate this point, it is clear that other signaling pathways and cell types could mediate this effect as well. Therefore, airway epithelial NF-κB activity appears to be a central mediator in the inflammatory and immune response to infections and environmental insults that modulates allergic sensitization and the severity of subsequent allergic airway disease.

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CHAPTER 5

Serum Amyloid A Activates the NLRP3 Inflammasome and Promotes Th17 Allergic Asthma in Mice

Jennifer L. Ather, Karina Ckless, Rebecca Martin, Kathryn L. Foley, Benjamin T. Suratt, Jonathan E. Boyson, Katherine A. Fitzgerald, Richard A. Flavell, Stephanie C. Eisenbarth, and Matthew E. Poynter

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ABSTRACT

IL-1β is a cytokine critical to several inflammatory diseases in which pathogenic Th17 responses are implicated. Activation of the NLRP3 inflammasome by microbial and environmental stimuli can enable the caspase-1–dependent processing and secretion of IL-1β. The acute-phase protein serum amyloid A (SAA) is highly induced during inflammatory responses, wherein it participates in systemic modulation of innate and adaptive immune responses. Elevated levels of IL-1β, SAA, and IL-17 are present in subjects with severe allergic asthma, yet the mechanistic relationship among these mediators has yet to be identified. In this study, we demonstrate that *Saa3* is expressed in the lungs of mice exposed to several mixed Th2/Th17-polarizing allergic sensitization regimens. SAA instillation into the lungs elicits robust TLR2-, MyD88-, and IL-1–dependent pulmonary neutrophilic inflammation. Furthermore, SAA drives production of IL-1α, IL-1β, IL-6, IL-23, and PGE_2, causes dendritic cell (DC) maturation, and requires TLR2, MyD88, and the NLRP3 inflammasome for secretion of IL-1β by DCs and macrophages. $CD4^+$ T cells polyclonally stimulated in the presence of conditioned media from SAA-exposed DCs produced IL-17, and the capacity of polyclonally stimulated splenocytes to secrete IL-17 is dependent upon IL-1, TLR2, and the NLRP3 inflammasome. Additionally, in a model of allergic airway inflammation, administration of SAA to the lungs functions as an adjuvant to sensitize mice to inhaled OVA, resulting in leukocyte influx after Ag challenge and a predominance of IL-17 production from restimulated splenocytes that is dependent upon IL-1R signaling.

Introduction

The IL-1 family of cytokines is critical to the host response to infection, playing a variety of functions not only in the acute-phase response from the liver, but also in alterations in metabolism, induction of fever, and lymphocyte activation (1). Overproduction of IL-1β, in particular, is thought to be responsible for a variety of autoinflammatory syndromes such as familial Mediterranean fever and Muckle-Wells syndrome and is also a contributing factor in osteoarthritis, rheumatoid arthritis, gout, multiple sclerosis (experimental autoimmune encephalomyelitis), colitis, diabetes, and Alzheimer's disease (2-9). Setting IL-1β apart from other acute-phase cytokines such as IL-6 and TNF-α is the requirement for processing from an inactive proform to an active secreted form by caspase-1 cleavage, which itself is activated by the assembly of a cytoplasmic inflammasome complex. The NLRP3 inflammasome is not only critical for IL-1β release in response to a variety of stimuli, but has also been implicated in several of the same autoimmune and autoinflammatory disorders in which IL-1β plays a causative role. Key to these models of inappropriate adaptive immune responsiveness is the development of a Th17-biased phenotype, and recent research has highlighted the importance of IL-1β in Th17 development (10, 11). IL-1β, in conjunction with IL-6 and TGF-β, is critical in humans for the development of the Th17 lineage. Not only do Th17 cells upregulate mRNA expression of the IL-1R compared with Th1 and Th2 cells, but lack of this receptor on polyclonally stimulated T cells results in significant reduction of IL-17A, IL-17F, IL-21, and IL-22 production (10, 12).

Long known to be a biomarker of inflammation in a multitude of diseases (13), serum amyloid A (SAA) is also a critical mediator of disease pathogenesis. SAA can stimulate cells via TLR2 to elicit a robust signaling cascade in human monocytes (14) and mouse macrophages (15), whereas it can also signal through the formyl-peptide receptor (FPR)-like 1/FPR2 to promote neutrophil chemotaxis and activation (16-18). In addition, SAA induces expression of matrix metalloproteinases and collagenases that are pivotal in tissue remodeling after injury (19). SAA can also promote the development of Th17 responses, which has been demonstrated to be an important mechanism by which segmented filamentous bacteria induce intestinal disease (20). However, the importance of SAA on modulating adaptive immune responses in other tissues and beyond the scope of infection has not yet been demonstrated. Most well known is the role of SAA fibril deposition in severe conditions such as amyloidosis (21). It has recently been demonstrated that β-amyloid fibrils in Alzheimer's disease (22) and islet amyloid polypeptide in type 2 diabetes (2) signal through the NLRP3 inflammasome and drive caspase-1–dependent cleavage of IL-1β.

Asthma is conventionally considered to be a Th2-driven disease associated with wheezing, airway hyperresponsiveness, IgE, eosinophilia, and mucus meta-

plasia. However, in a substantial percentage of patients, asthma presents as nonatopic, instead manifesting as a neutrophilic and steroid-resistant phenotype that results in increased severity and morbidity of disease (23, 24). Severe allergic asthma is associated with elevated levels of several mediators, including SAA (25-27), IL-1β (28), and IL-17 (29-36), although a mechanistic link among these molecules has not yet been established. IL-1β and IL-17A have been demonstrated to upregulate expression of the mucin gene *Muc5ac* (37), and IL-1β also acts via cyclooxygenase-2 and PGE_2 production to desensitize airway smooth muscle cells to β-adrenergic agonists (38).

Mouse models of allergic asthma have classically exploited the Th2-promoting adjuvant aluminum hydroxide (Alum), delivered as an emulsion with Ag via i.p. injection (39). However, allergic asthma models are evolving to encompass inhalational methods of sensitization to aeroantigens, which promote a mixed Th2/Th17 allergic airway disease phenotype (40). Although the Th17 response in mouse models of allergic airway disease is associated with neutrophilia, tissue destruction, and steroid unresponsiveness, little is known about the endogenous mediators that are critical to this response. Recent reports have implicated IL-1β, IL-6, and IL-23 in the initiation and expansion of IL-17–producing T cells, three cytokines that are highly induced by SAA (14, 41).

In this study, we report that multiple models of respiratory system exposure that promote mixed Th2/Th17 responses also induce pulmonary *Saa3* expression. SAA signals through TLR2 to induce inflammatory mediators and through the Nlrp3 inflammasome to induce IL-1β secretion. In addition, SAA induces dendritic cells (DCs) to undergo maturation and produce soluble mediators, including IL-1α, IL-1β, IL-6, PGE_2, and IL-23, that function in an IL-1–dependent manner to promote $CD4^+$ T cells to secrete IL-17A upon stimulation. Finally, SAA sensitizes mice to a mixed Th2/Th17 allergic airway disease via an IL-1R–dependent mechanism. Together, these data implicate pulmonary SAA as a proinflammatory mediator capable of promoting Ag-specific pulmonary Th17 responses through the activities of the cytokine mediator IL-1.

Materials and Methods

Mice.

C57BL/6 and IL-1Rα$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). TLR2$^{-/-}$ (42), TLR4$^{-/-}$ (43), MyD88$^{-/-}$ (44), NLRP3$^{-/-}$ (45), ASC$^{-/-}$ (45), caspase-1$^{-/-}$ (46), and CC10-rtTA × TetOP-CAIKKβ bitransgenic mice on the C57BL/6 background (47), which express a constitutively active IκB kinase β (CAIKKβ) in bronchiolar epithelium following administration of 6 g/kg doxycycline (Dox) in chow (TestDiet, Richmond, IN), and age- and sex-matched transgene-

negative littermates were also bred at the University of Vermont. Mice were housed in an American Association for the Accreditation of Laboratory Animal Care-approved facility, maintained on a 12-h light/dark cycle, and were provided food and water ad libitum. All animal studies were approved by the University of Vermont Institutional Animal Care and Use Committee.

Exposures.
For acute studies, mice were anesthetized with inhaled isoflurane and received either 50 µl sterile saline, 100 ng ultra-pure LPS (InvivoGen, San Diego, CA), or 10 µg apo-SAA (PeproTech, Rocky Hill, NJ) in 50 µl sterile saline by oropharyngeal aspiration (o.a.), and analyzed 24 h later. Mice were exposed to 15 ppm NO_2 or high-efficiency particulate air-filtered room air for 1 h and analyzed 24 h later as previously described (48). CAIKKβ mice and transgene-negative littermates were provided Dox-containing chow for 60 h prior to analysis. For treatment with anakinra (Biovitrum, Stockholm, Sweden), mice were administered 1 mg drug in 200 µl sterile saline by s.c. injection twice daily beginning 1 d before SAA exposure. For Ag-sensitization studies, mice received either saline or SAA (as above) once on day 0, followed by 30 min of nebulized 1% OVA, Fraction V (Sigma-Aldrich, St. Louis, MO), in saline on days 0, 1, and 2. Mice were then challenged with 30 min of nebulized 1% OVA on days 14, 15, and 16 and analyzed on day 18. Alum/OVA-treated mice were sensitized on day 0 with 100 µg OVA in Imject Alum (Thermo Scientific, Rockford, IL), challenged with 30 min of nebulized 1% OVA on days 14, 15, and 16, and analyzed on day 18.

Bronchoalveolar lavage collection and lung processing.
Lungs were lavaged with 1 ml DPBS (Sigma-Aldrich) from which cells were counted by hemocytometer, and differential analysis was performed by cytospin and H&E stain. After lavage, lungs were flash frozen in liquid nitrogen for RNA analysis.

Macrophages.
For the isolation of primary macrophages, C57BL/6, TLR2$^{-/-}$, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice were administered 1 ml 4% thioglycollate by i.p. injection. Ninety-six hours later, mice were euthanized, and peritoneal lavage was performed to collect peritoneal exudate cells. Cells were plated in RPMI 1640 supplemented with 10% FBS, penicillin and streptomycin, l-glutamine, and 2-ME and challenged for 16 h with apo-SAA followed where indicated by 30 min of 5 mM ATP or 8 h of 500 µg/ml Imject Alum (Thermo Scientific). C57BL/6, NLRP3$^{-/-}$, ASC$^{-/-}$, and caspase-1$^{-/-}$ transformed macrophage cell lines (49) were maintained in vitro in RPMI 1640 with 10% FBS, penicillin and streptomycin, l-glutamine, and 2-ME. Following stimulation, cell-free supernatants were flash frozen for later analysis.

Cytokine analysis.
Cytokines from bronchoalveolar lavage (BAL) and cell supernatants were analyzed by ELISA for IL-1β and TNF-α (BD Biosciences, San Jose, CA), as well as IL-23 (R&D Systems, Minneapolis, MN). PGE_2 was assessed by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI). Customized Bio-Plex (Bio-Rad, Hercules, CA) and Milliplex assays (Millipore, Billerica, MA) were used to measure IL-5, IL-13, and IL-17, as well as IL-1α, IL-1β, TNF-α, IL-6, GM-CSF, G-CSF, keratinocyte-derived chemokine, MIP-1α, MIP-1β, MCP-1, IL-12p40, and IL-12p70.

Quantitative RT-PCR.
Total RNA was extracted from frozen whole lungs or transformed macrophages using the PrepEase RNA Isolation kit (USB, Cleveland, OH) and reversed transcribed to cDNA using the iScript kit from Bio-Rad. Primers were designed for mouse *Saa1*, *Saa2*, *Saa3*, and *Il1b*, and RT-PCR was performed using SYBR Green Supermix (Bio-Rad) and normalized to *Gapdh* or *Actb* using the $\Delta\Delta C_T$ method, as previously described (47).

Splenocyte restimulation.
Splenocytes from experimental mice and C57BL/6 control mice were isolated using Lymphocyte Separation Media (MP Biomedicals, Solon, OH) as previously described (48). A total of 4×10^6 cells/ml were cultured in RPMI 1640 supplemented with 10% FBS, penicillin/streptomycin, l-glutamine, and 2-ME and were activated with 100 μg/ml OVA in 48-well plates. Following 96 h of stimulation, supernatants were collected for analysis by Milliplex (Millipore).

Bone marrow-derived DCs.
Bone marrow was flushed from the femurs and tibiae and cultured on 24-well plates at 1×10^6 cells/well (1 ml/well) in RPMI 1640 containing 10% serum and 10% conditioned media from X63-GMCSF myeloma cells transfected with murine GM-CSF cDNA (kindly provided by Dr. Brent Berwin, Dartmouth College). Media was replaced on days 2 and 4, and the adherent and lightly adherent bone marrow-derived DCs (BMDCs), predominantly $CD11b^+CD11c^+$ by FACS, were collected on day 6. BMDCs were treated with SAA for 16 h. For flow cytometry, BMDCs were detached using versene and gentle scraping, washed in FACS buffer (DPBS with 5% FBS and 0.1% sodium azide), and 1×10^6 cells were incubated with Fc block (2.5 μg/ml anti-CD16/CD32) (BD Pharmingen) for 30 min at 4°C, washed in FACS buffer, and then stained for 30 min at 4°C in 100 μl Ab solution at the optimal concentration. Cells were stained with: anti-CD80–PE (BD Pharmingen), anti-CD86–Alexa 647 (Caltag Laboratories, Carlsbad, CA), anti-MHC class II-PerCP/Cy5.5 (BD Pharmingen), and biotinylated anti-OX40L (BD Pharmingen). Biotinylated Abs were detected using streptavidin-PE (BD Pharmingen). Following staining, all cells

were washed and fixed in DPBS with 5% FBS and 1% paraformaldehyde. Cells were analyzed on an LSR II FACS flow cytometer (BD Biosciences) equipped to distinguish as many as seven fluorophores 1–3 d following staining. Dead cells were excluded from analysis by forward light scatter and side scatter gating. Data were analyzed using FlowJo (Tree Star, Ashland, OR).

SAA contaminant analysis.
BMDCs were treated with SAA for 16 h in the presence or absence of polymyxin B (Sigma-Aldrich) at 25 and 1 μg/ml. Proteinase K (Sigma-Aldrich) at 25 μg/ml and 1 μg/ml (or absent) was incubated with apo-SAA at 37°C for 1 h, heated to 100°C for 5 min to deactivate the enzyme, and allowed to cool to room temperature before addition to cells. Cells were treated for 16 h, and cell-free supernatants were flash frozen prior to further analysis.

$CD4^+$ T cell culture with BMDC-conditioned media.
Cell-free conditioned media from unstimulated or 24 h SAA-exposed BMDCs were incubated with 1×10^6 splenic $CD4^+$ T cells from naive mice that were stimulated in the presence of 5 μg/ml immobilized anti-CD3 and 1 μg/ml soluble anti-CD28 for 96 h. Alternatively, $CD4^+$ T cells were polyclonally stimulated in media alone or in the presence of 1 μg/ml apo-SAA.

Splenocyte cultures.
Splenocytes from C57BL/6, $TLR2^{-/-}$, $NLRP3^{-/-}$, $ASC^{-/-}$, and $caspase\text{-}1^{-/-}$ mice were cultured at 4×10^6 cells/ml in RPMI 1640 containing 10% FBS, penicillin/streptomycin, l-glutamine, and 2-ME on plates coated with 5 μg/ml anti-CD3 and treated with 1 μg/ml soluble anti-CD28, 10 ng/ml anakinra, and 1 μg/ml SAA. Cell-free supernatants were analyzed 96 h after stimulation.

Statistics.
Data were analyzed by two-tailed unpaired *t* test or one-way ANOVA and Bonferroni post hoc test using GraphPad Prism 4 for Windows (GraphPad). A p value <0.05 was considered statistically significant.

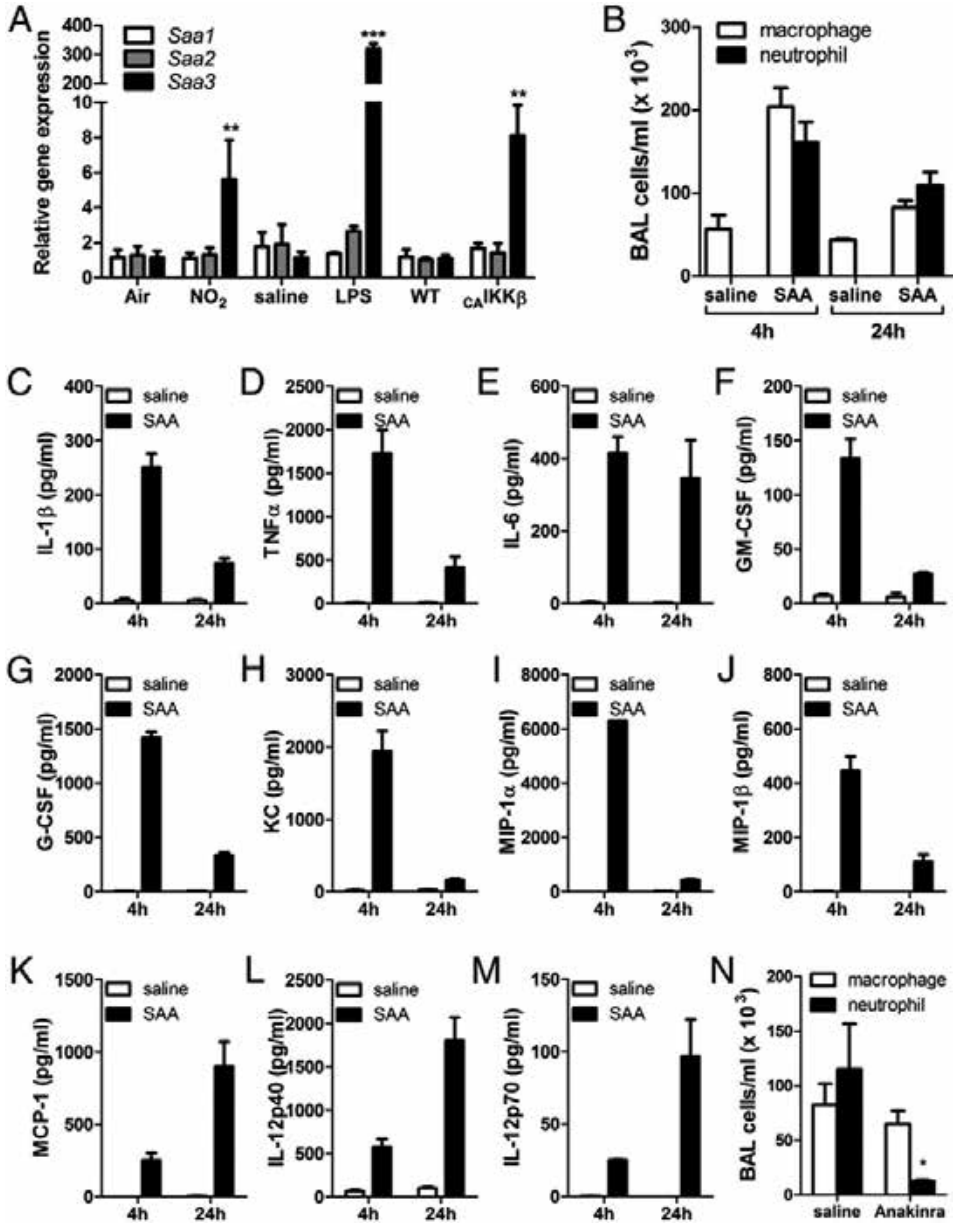
Results

SAA3 expression in mouse lung. Our studies have employed multiple mechanisms of allergic sensitization, including NO_2 exposure, oropharyngeal administration of LPS, and airway epithelial-specific NF-κB activation, each of which can promote mixed Th2/Th17 responses (40, 47, 48). Following exposure to these stimuli, lungs of C57BL/6 mice exhibited a preferential mRNA induction of *Saa3* over *Saa1* or *Saa2* (Fig. 1*A*). Mice exposed to 15 ppm NO_2 for 1 h and analyzed 24 h later showed

a 6-fold induction of *Saa3* in the lung, very similar to the response in transgenic mice that inducibly express CAIKKβ in the airway epithelium following 48 h of Dox administration (Fig. 1*A*). Oropharyngeal administration of 100 ng LPS, a low dose used in models of inhalational allergic sensitization (40, 50), induced high mRNA levels of *Saa3* 24 h postchallenge (Fig. 1*A*). These results demonstrate expression of *Saa3* in the lung under conditions that facilitate mixed Th2/Th17 polarization.

SAA elicits robust IL-1–dependent pulmonary inflammation. Recombinant human apo-SAA (with functional similarity to mouse SAA3) is available commercially from PeproTech and reported to contain <1 EU/μg endotoxin. Our own *Limulus* amebocyte lysate assay confirmed that this was indeed true (data not shown). To determine the effects of SAA in the lung, mice were administered 10 μg apo-SAA or saline by o.a. and analyzed at 4 and 24 h. In contrast to saline, SAA induced robust airway neutrophilia (Fig. 1*B*) and the production of inflammatory cytokines, as measured from BAL fluid (Fig. 1*C*–*M*). Because IL-1β was present at elevated concentrations in the BAL fluid (Fig. 1*C*), we administered an IL-1R antagonist (anakinra) or saline to mice prior to apo-SAA aspiration. In the anakinra-treated mice, airway neutrophilia was significantly reduced compared with apo-SAA–exposed mice treated with saline vehicle (Fig. 1*N*). These results implicate an important function of IL-1 in SAA-promoted inflammation.

Figure 1. SAA is expressed in the lungs during mixed Th2/Th17 allergic sensitization regimens and induces pulmonary inflammation upon inhalational exposure.
Quantitative PCR of whole lung for SAA isoforms in mice exposed to NO_2, LPS, or in which NF-κB has been activated in the airway epithelium (A). C57BL/6 mice were administered 10 μg SAA by o.a. and analyzed 4 and 24 h later. BAL total cell counts were performed by hemocytometer, and differential analysis was by cytospin (B). BAL fluid was analyzed by Milliplex assay (Millipore) for IL-1β (C), TNF-α (D), IL-6 (E), GM-CSF (F), G-CSF (G), keratinocyte-derived chemokine (H), MIP-1α (I), MIP-1β (J), MCP-1 (K), IL-12p40 (L), and IL-12p70 (M). Data are representative of three independent experiments. C57BL/6 mice were administered saline or 1 mg anakinra (n = 3 per group) by s.c. injection twice daily beginning one day prior to o.a. of 10 μg SAA. At 24 h, BAL total cell counts were performed by hemocytometer and differential analysis was by cytospin (N). *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ compared with control exposures (A) or saline controls (B–N).


A
Relative gene expression
Saa1
Saa2
Saa3
Air
NO2
saline
LPS
WT
caIKKβ
B
BAL cells/ml (x 10^3)
macrophage
neutrophil
saline
SAA
4h
24h
C
IL-1β (pg/ml)
D
TNFα (pg/ml)
E
IL-6 (pg/ml)
F
GM-CSF (pg/ml)
G
G-CSF (pg/ml)
H
KC (pg/ml)
I
MIP-1α (pg/ml)
J
MIP-1β (pg/ml)
K
MCP-1 (pg/ml)
L
IL-12p40 (pg/ml)
M
IL-12p70 (pg/ml)
N
BAL cells/ml (x 10^3)
Anakinra

SAA activates DCs that promote IL-17A production from CD4⁺ T cells. To determine the effects of SAA on APCs, which are critical for initiation of CD4⁺ T cell responses, BMDCs from C57BL/6 mice were challenged with apo-SAA and analyzed 16 h later. We observed increases in surface markers of DC maturation, including CD80, CD86, MHC class II, and OX40L (Fig. 2*A*), upon exposure of BMDCs to apo-SAA. In addition, BMDCs secreted IL-1α, IL-1β, IL-6, and IL-23 (Fig. 2*B*), cytokines that participate in Th17 polarization and maintenance (10, 23, 51). Furthermore, treatment with SAA caused BMDCs to secrete a significant amount of PGE_2 (Fig. 2*B*), which has recently been shown to induce DCs to preferentially secrete IL-23 and thus contribute to the development of a Th17 response (52, 53). DCs exposed to apo-SAA also secreted small amounts of the Th1-polarizing cytokine, IL-12p70, and substantial amounts of proinflammatory TNF-α (Fig. 2*B*). When the cell-free supernatants from these SAA-treated BMDCs were provided to polyclonally stimulated naive CD4⁺ T cells, a significant production of IL-17 was induced compared with very small amounts of IFN-γ and IL-4 (Fig. 2*C*). Importantly, treatment of the polyclonally stimulated naive CD4⁺ T cells with SAA resulted in no significant production of any of these cytokines (Fig. 2*D*), indicating that the effect of SAA is directly on DCs, which in turn drive the polarization of the CD4⁺ T cells through the secretion of soluble mediators, including IL-1β.

SAA-induced IL-1β secretion requires TLR2 and the NLRP3 inflammasome. Because IL-1β has been implicated as a Th17-polarizing and -priming factor, we determined cell receptors required for SAA-induced secretion of this cytokine. To ensure that the effects of SAA required the recombinant SAA protein and were not due to endotoxin contamination, we cultured BMDCs for 16 h with apo-SAA or LPS that had been treated with proteinase K or left untreated. All samples were then boiled to inactivate the proteinase K. Under these conditions, we observed a dose-dependent reduction in the response to apo-SAA, but no effect on the LPS-induced IL-1β response (Fig. 3*A*). In contrast, exposure of BMDCs to LPS in the presence of polymyxin B completely blocked IL-1β production, whereas the presence of polymyxin B during apo-SAA treatment still allowed for substantial amounts of IL-1β to be produced (Fig. 3*B*). Having demonstrated the requirement for SAA protein and the minimal contribution of contaminating endotoxin in the effects of apo-SAA, we exposed peritoneal exudate macrophages from TLR2$^{-/-}$, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice to SAA for 16 h. In these cells, IL-1β secretion is primarily dependent upon TLR2 and MyD88, both in the absence or presence of ATP or aluminum crystals (Fig. 3*C*), potent inducers of IL-1β secretion. TLR4$^{-/-}$ peritoneal exudate cells showed no significant reduction in their ability to secrete IL-1β following SAA treatment, further ruling out the contribution of endotoxin contamination of apo-SAA to its biological effects in these experimental systems. The response to SAA was also examined in transformed macrophage cell lines from wild-type, NLRP3$^{-/-}$,

ASC$^{-/-}$, and caspase-1$^{-/-}$ mice. Whereas apo-SAA–induced levels of TNF-α, an inflammasome-independent cytokine, were highly induced in all cells (Fig. 3*D*), complete abrogation of IL-1β secretion was observed in response to apo-SAA in NLRP3-, ASC-, and caspase-1–deficient macrophages (Fig. 3*E*). Indicative of the roles these molecules play in processing the pro–IL-1β protein, levels of *Il1b* message expression were robustly induced in the wild-type, NLRP3-, ASC-, and caspase-1–deficient macrophages (Fig. 3*F*).

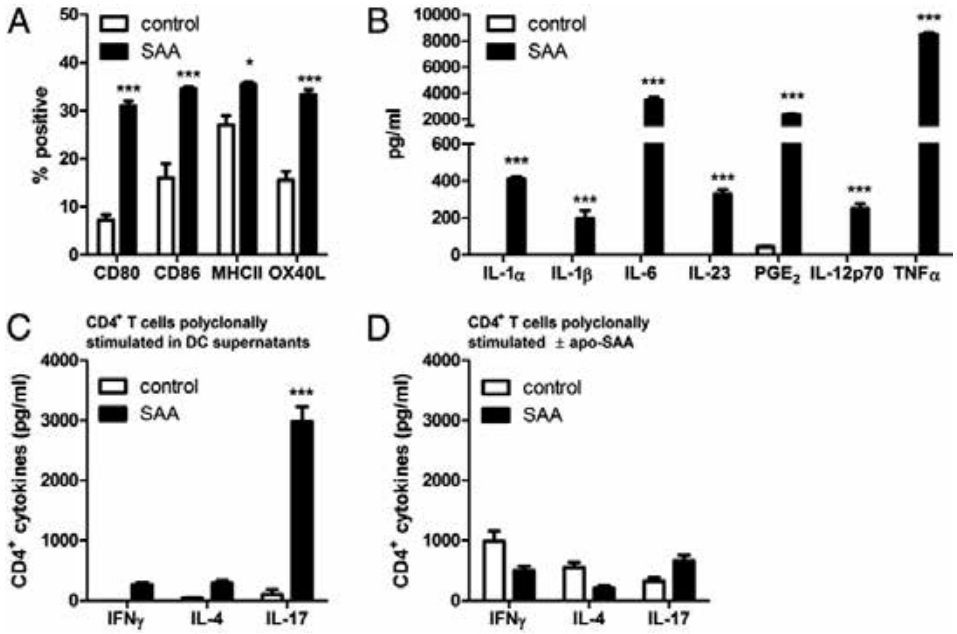


Figure 2. *SAA elicits inflammatory mediator production and DC maturation in vitro.*
BMDCs were treated with 1 μg/ml SAA for 16 h and analyzed for surface markers of maturation (*A*) and secretion of Th17-polarizing mediators (*B*). The conditioned media from control or SAA-exposed BMDCs (*C*) or fresh media with or without SAA (*D*) was transferred to CD4$^+$ T cells that were polyclonally stimulated with anti-CD3 and anti-CD28. After 96 h, IFN-γ, IL-4, and IL-17A were measured in supernatants. Data are representative of three independent experiments. *$p < 0.05$, ***$p < 0.001$ compared with control exposures.

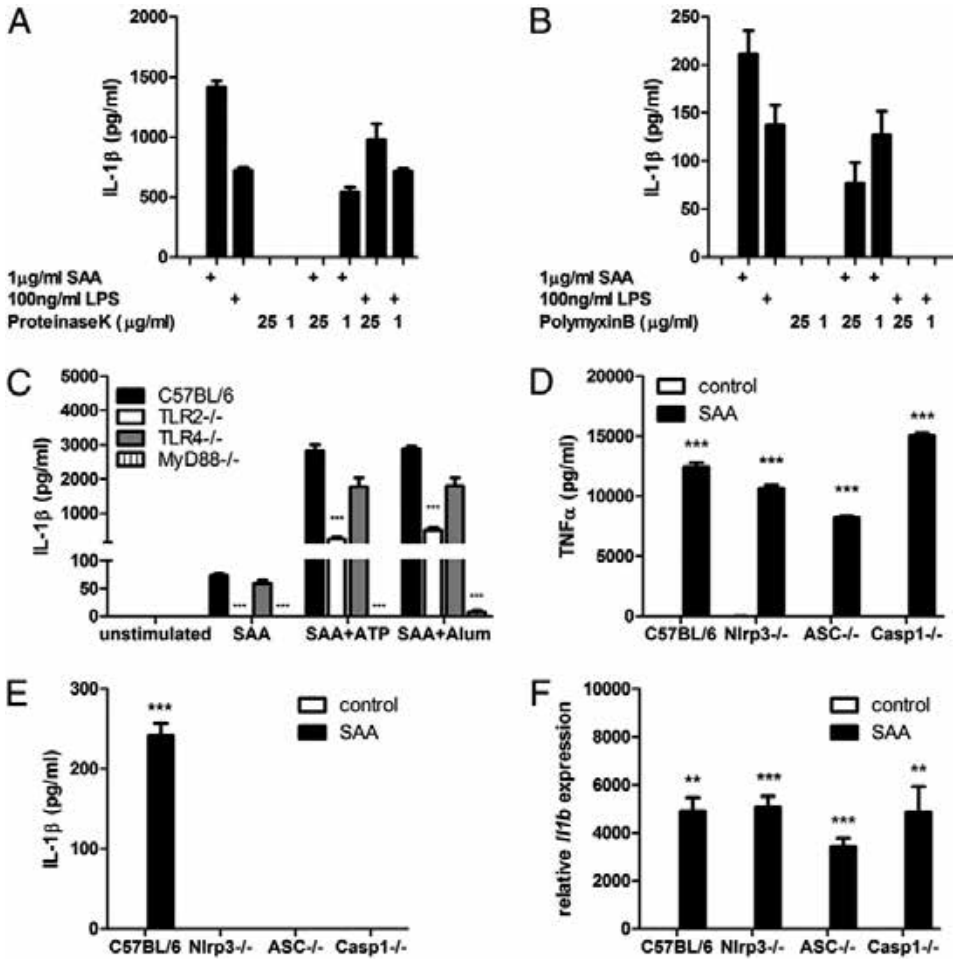


Figure 3. *SAA-induced IL-1β production requires TLR2, MyD88, and the NLRP3 inflammasome.*
Cell-free supernatants from BMDCs treated with SAA or LPS in the presence or absence of proteinase K (*A*) or polymyxin B (*B*) were analyzed by ELISA after 24 h. Peritoneal exudate macrophages from wild-type (C57BL/6), TLR2$^{-/-}$, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice were unstimulated or primed overnight with 1 μg/ml SAA alone or followed by 30 min of 5 mM ATP or 8 h of 500 μg/ml Alum, and supernatants were analyzed for IL-1β secretion (*C*). Transformed macrophages from wild-type (C57BL/6), Nlrp3$^{-/-}$, ASC$^{-/-}$, and caspase-1$^{-/-}$ mice were treated for 24 h with 1 μg/ml SAA, and supernatants were analyzed for TNF-α (*D*) and IL-1β (*E*) secretion. *Il1b* expression was measured from wild-type (C57BL/6), Nlrp3$^{-/-}$, ASC$^{-/-}$, and caspase-1$^{-/-}$ transformed macrophages that had been untreated or exposed for 4 h to 1 μg/ml SAA (*F*). $^{**}p < 0.005$, $^{***}p < 0.001$ compared with wild-type (*C*) or control exposures (*D*–*F*).

SAA-induced pulmonary inflammation requires TLR2 and involves the NLRP3 inflammasome. Having demonstrated the important functions of TLR2, NLRP3, and caspase-1 for SAA-induced IL-1β secretion in vitro, we next performed additional pulmonary SAA exposures to determine the contribution of these molecules to inflammation in vivo. Oropharyngeal administration of 10 μg apo-SAA elicited a robust influx of neutrophils into the lung after 24 h in C57BL/6 mice, a response that was diminished in TLR2$^{-/-}$, but not NLRP3$^{-/-}$ or caspase-1$^{-/-}$, mice (Fig. 4*A*). Measurement of *Il1b* gene expression in the lung following SAA aspiration revealed increased mRNA abundance in wild-type, NLRP3$^{-/-}$, and caspase-1$^{-/-}$ mice, but not in TLR2$^{-/-}$ mice (Fig. 4*B*). Analysis of the BAL fluid from these mice demonstrated that TLR2 signaling was also required for IL-1β, G-CSF, IL-6, and MCP-1 production, whereas NLRP3 and caspase-1 were necessary only for the production of IL-1β (Fig. 4*C*–*F*). The NLRP3$^{-/-}$ and caspase-1$^{-/-}$ mice also displayed reduced levels of IL-6 and MCP-1, two cytokines that can be induced by IL-1β (54, 55) and are perhaps diminished as a consequence of lost IL-1β signaling.

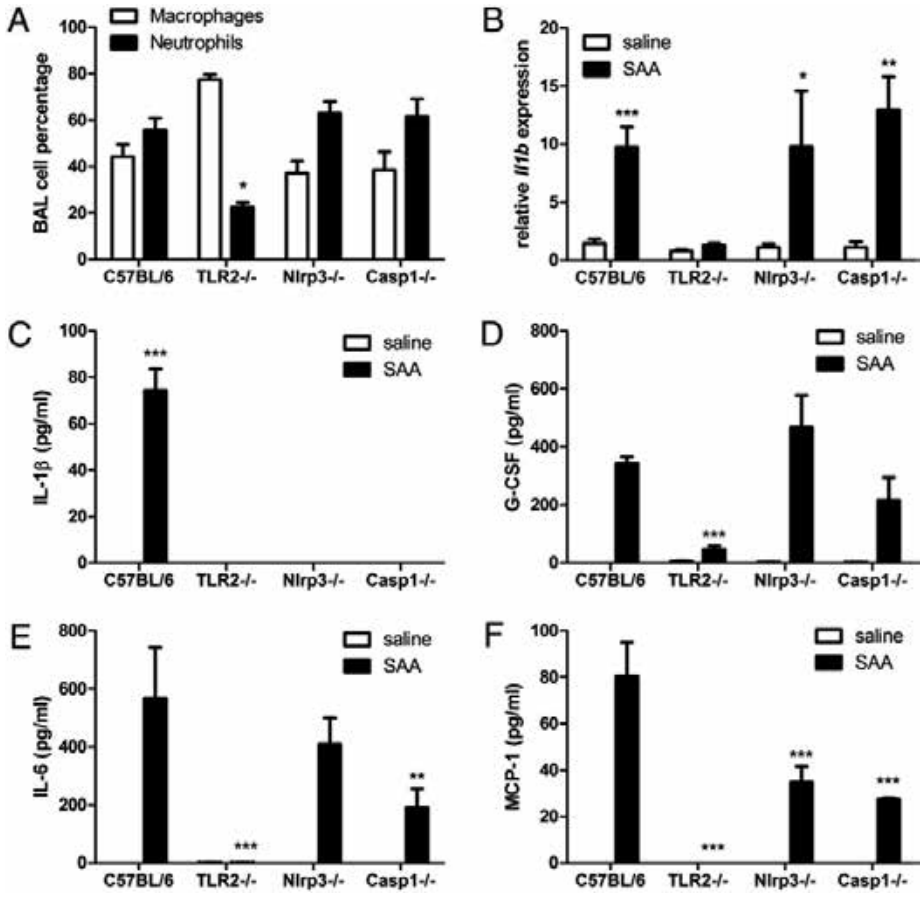


Figure 4. *SAA-induced pulmonary inflammation requires TLR2 and involves the NLRP3 inflammasome.* Wild-type (C57BL/6), TLR2$^{-/-}$, Nlrp3$^{-/-}$, and caspase-1$^{-/-}$ mice were administered 10 μg SAA by o.a. and analyzed 24 h later. BAL total cell counts were performed by hemocytometer and differential analysis was by cytospin (*A*). *Il1b* expression was measured from whole lung by quantitative RT-PCR (*B*). BAL fluid was analyzed by Milliplex assay (Millipore) for IL-1β (*C*), G-CSF (*D*), IL-6 (*E*), and MCP-1 (*F*). Data are representative of three independent experiments. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ compared with wild-type (*A*) or saline (*B–F*).

SAA-promoted allergic sensitization favors a Th17 response that requires IL-1Rα signaling. Having demonstrated evidence for DC maturation and Th17 polarization in response to apo-SAA in vitro, we sought to compare a conventional Th2 allergic sensitization protocol, a well-characterized Alum/OVA model (56, 57), with an experimental model of apo-SAA–promoted Ag sensitization (Fig. 5*A*). A very distinct profile of SAA gene expression occurred in the lung 24 h following Ag sensitization in the two models (Fig. 5*B*). Intraperitoneal injection of the adjuvant Alum elicited little SAA expression in the lung (Fig. 5*B*; 3.4-fold induction of *Saa1*, 6.9-fold induction of *Saa2*, and 3.0-fold induction of *Saa3*), but induced strong expres-

sion of *Saa1* (781-fold induction) and *Saa2* (199-fold induction) in the liver, with only 4.2-fold induction of *Saa3* (data not shown). This is consistent with the reported roles of liver SAA1 and SAA2 in the systemic inflammatory response. In contrast, pulmonary administration of apo-SAA by o.a. selectively induced mRNA expression of *Saa3* in the lungs (Fig. 5*B*), with no systemic (liver) production of any other isotypes (data not shown).

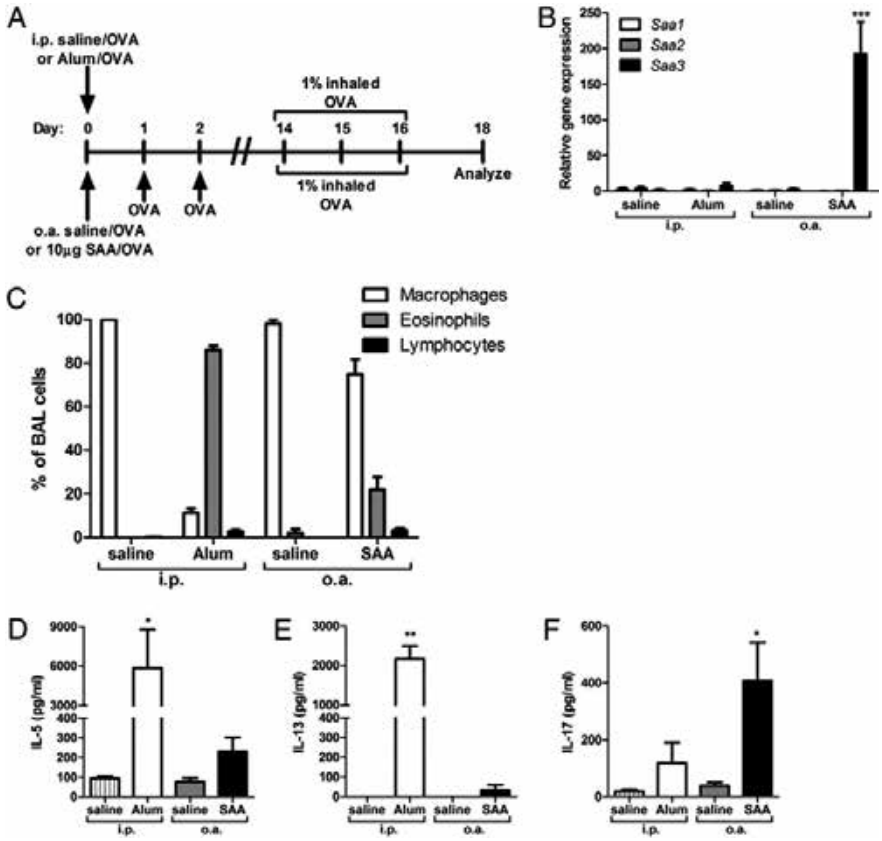


Figure 5. *SAA inhalation promotes Th17 allergic sensitization.*
Mice underwent Ag sensitization via o.a. with either saline and OVA (saline/OVA) or SAA and OVA (SAA/OVA) or via i.p. injection with either saline and OVA (saline/OVA) or Alum and OVA (Alum/OVA), according to the schema (*A*). *Saa1*, *Saa2*, and *Saa3* gene expression in whole lung was measured on day 1, 24 h after i.p. injection with saline or Alum or 24 h after o.a. administration of saline or SAA (*B*). On day 18, total cell counts from BAL fluid were performed by hemocytometer, and differential analysis was by cytospin (*C*). On day 18, splenocytes from i.p. saline, i.p. Alum, o.a. saline, and o.a. SAA mice were restimulated in vitro with OVA for 96 h, and IL-5 (*D*), IL-13 (*E*), and IL-17A (*F*) levels in culture media were measured. Data are representative of three independent experiments. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$ compared with saline controls (*B*, *D*–*F*).

The disparate induction of *Saa* expression in liver and lung led us to speculate that differences in route of sensitization and adjuvant used modulate local effects that may contribute to distinct responses in the Alum- and SAA-promoted allergic sensitization models. Therefore, we examined the BAL cell profiles from challenged mice subjected to the two models of Ag sensitization. Mice sensitized i.p. with Alum/OVA robustly recruited eosinophils into the lung compared with unsensitized saline/OVA controls, whereas the SAA/OVA sensitized mice showed eosinophilia on the order of 10–20%, which is more representative of that typically present in the BAL fluid in an asthmatic patient (Fig. 5*C*). Splenocytes from sensitized and control mice were cultured and restimulated with OVA for 96 h. The Alum/OVA mice, as expected, responded by producing copious amounts of the Th2 cytokines IL-5 and IL-13, whereas SAA/OVA mice produced modest but elevated levels of these cytokines compared with the saline control mice (Fig. 5*D*, 5*E*). However, SAA/OVA-sensitized mice, unlike Alum/OVA mice, displayed significant production of IL-17 in response to Ag (Fig. 5*F*), recapitulating our in vitro findings from Fig. 2*C* that the SAA-induced inflammatory response can polarize T cells to secrete primarily IL-17A.

As we have demonstrated, the capacity of Ag-restimulated splenocytes to produce IL-17 may rely on the microenvironmental cytokine milieu that is generated by DCs and other APCs in response to SAA. Because SAA promotes IL-1–dependent pulmonary inflammation, we repeated our SAA/OVA sensitization model (Fig. 5*A*) using IL-1Rα knockout mice to determine whether IL-1 signaling played a critical role in the $CD4^+$ T cell priming and polarization process. Following Ag challenge, the cellular BAL profile revealed that IL-1R$\alpha^{-/-}$ mice exposed to SAA/OVA recruited fewer eosinophils and lymphocytes to the lung than did wild-type mice (Fig. 6*A*). Furthermore, when splenocytes from these sensitized and challenged mice were restimulated in vitro with OVA, wild-type and knockouts showed a similar induction of IL-5 and IL-13, but the IL-17A production was absent in the IL-1R$\alpha^{-/-}$ mice (Fig. 6*B*–*D*). To show that an IL-1Rα ligand was required for SAA to induce IL-17A production and that the IL-17A deficiency seen in IL-1R$\alpha^{-/-}$ mice was not due to a developmental defect, splenocytes from wild-type mice were polyclonally stimulated in the presence of SAA, with or without anakinra. The addition of anakinra, even at a relatively low dose of 10 ng/ml, completely abrogated IL-17A production (Fig. 6*E*). Finally, to investigate the requirement for TLR2 and NLRP3 inflammasome components for SAA-promoted IL-17A production, splenocytes from C57BL/6, TLR2-/-, NLRP3-/-, ASC-/-, and caspase-1-/- mice were plated and polyclonally stimulated in the presence or absence of apoSAA. The TLR2-/- splenocytes showed a nearly complete inability of SAA to augment IL-17A production after 96 h of stimulation (Fig. 6*F*). IL-17 production was also impaired in splenocytes that lacked components of the NLRP3 inflammasome complex, although the abrogation was more moderate (Fig. 6*F*). Clearly, IL-1 sig-

naling plays a critical role in the induction of SAA-promoted allergic airway disease, specifically in mediating the production of IL-17A.

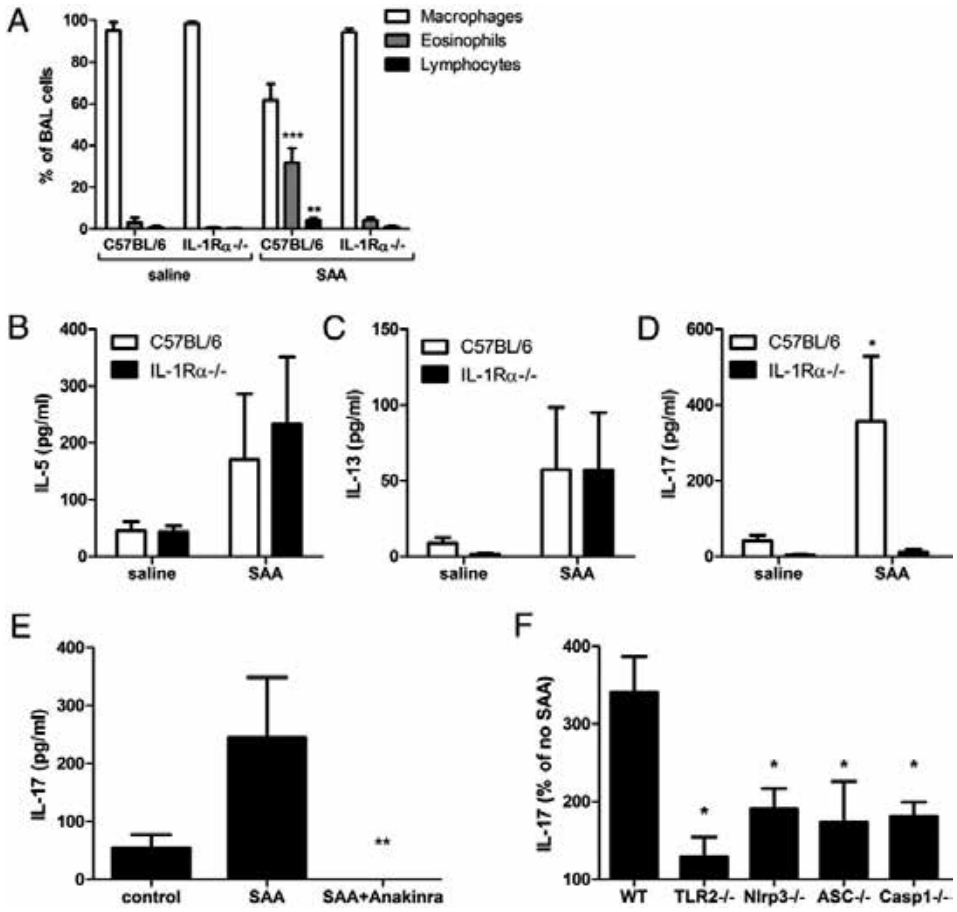


Figure 6. *SAA-promoted allergic sensitization and Th17 polarization require IL-1R.*
C57BL/6 and IL-1Rα$^{-/-}$ mice were Ag-sensitized with saline and OVA (saline) or SAA and OVA (SAA) by o.a., according to the timeline in Fig. 5*A*. On day 18, total and differential cell counts from BAL fluid were performed (*A*). Splenocytes were restimulated in vitro with OVA for 96 h, and IL-5 (*B*), IL-13 (*C*), and IL-17 (*D*) levels in culture media were measured. Splenocytes from C57BL/6 mice in the presence or absence of SAA and 10 ng/ml anakinra (*E*) and splenocytes from C57BL/6, TLR2$^{-/-}$, NLRP3$^{-/-}$, ASC$^{-/-}$, and caspase-1$^{-/-}$ (*F*) mice were polyclonally stimulated for 96 h with anti-CD3 and anti-CD28 in the presence or absence of 1 μg/ml SAA, and IL-17A was measured by ELISA (*F*). Data are representative of two independent experiments. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ compared with saline controls (*A*–*D*), SAA (*E*), or wild-type (*F*).

Discussion

Our results reported in this study implicate a causal role and a molecular mechanism for SAA in the pathogenesis of allergic asthma. Several of our previous studies have focused on inhalational Ag sensitization via exogenous insults, predominantly the pollutant NO_2. Inhalation of NO_2 activates airway epithelial NF-κB and promotes a mixed Th2/Th17 response (48, 58), which can be recapitulated through the activation of NF-κB in airway epithelium and inhalation of Ag (47). Within the lung, SAA3 is induced as a consequence of both NO_2 inhalation and airway epithelial NF-κB activation. SAA isotypes are differentially expressed in distinct tissues (13). SAA1 and -2 in mice are expressed predominantly in the liver, most commonly found bound to high-density lipoproteins in the circulation, and acutely upregulated in systemic disease states (13, 59). In contrast, SAA3 in mice has been shown to be expressed in a wide variety of cells and tissues, including leukocytes and epithelium, and has never been identified bound to high-density lipoproteins (60-62). In humans, SAA1 and -2 are expressed in the liver and in the lung and have been associated locally with the TLR2-dependent development of sarcoidosis (63). The rapid and robust induction of SAA in response to a panel of inhalational stimuli (Fig. 1*A*) indicates a possible role for SAA as a mediator in both allergic sensitization and during Ag challenge (exacerbation). Based on our results, we speculate that local production of SAA, rather than the particular isoform expressed, is capable of influencing local innate and adaptive immune responses. Therefore, whereas SAA3 is the predominant form of SAA expressed in mouse lungs, SAA1 or SAA2 in human lungs may exert effects similar to those we report in this study.

The inflammatory cytokine milieu surrounding naive DCs is key to their maturation and to the polarization of the $CD4^+$ T cell response, as we have previously demonstrated using a model of inducible airway epithelial NF-κB activation (47). In addition, the work of Ivanov et al. (20) has implicated SAA as an important mediator for Th17 polarization in the gut in response to colonization with segmented filamentous bacteria. We have shown a profound effect of apo-SAA on BMDCs that includes APC maturation and the secretion of the Th17-polarizing mediators IL-1α, IL-1β, IL-6, IL-23, and PGE_2. From the studies presented in this article using IL-1R antagonism with anakinra, it is clear that IL-1α and IL-1β are potential mediators induced by SAA that are capable of eliciting predominantly IL-17A production from naive, polyclonally stimulated $CD4^+$ T cells.

IL-1β requires cleavage via caspase-1 for proper secretion, which is facilitated as a consequence of inflammasome assembly and activation. The NLRP3 inflammasome has emerged as a critical cytosolic sensor for a number of endogenous mediators, including amyloid proteins (2, 22), that are capable of promoting IL-1β secretion. Our studies demonstrate that regulation of IL-1β production in response to SAA occurs as two distinct levels. At the transcriptional level, SAA-induced IL-1β

requires TLR2, a finding first reported in human ThP-1 cells by Cheng et al. (14). At the posttranslational level, use of transgenic mice that are deficient in proteins of the NLRP3 complex demonstrate a clear role for the NLRP3 inflammasome in SAA-induced IL-1β secretion. TLR2$^{-/-}$ mice exhibit severe impairment in the inflammatory response to oropharyngeal administration of apo-SAA, including a diminution in neutrophil recruitment and decreased secretion of inflammatory cytokines in the BAL. The remaining neutrophilia in these mice is likely due to the capacity of apo-SAA to stimulate neutrophil chemotaxis via FPRL1/FPR2 (17, 18), which remains intact in all of the mice we studied. Taken together, our in vitro and in vivo results indicate that proper production and secretion of IL-1β in response to SAA is regulated both at the transcriptional level by TLR2 and at the level of secretion by the NLRP3 inflammasome. Furthermore, they suggest that IL-1β not only participates in the inflammatory cascade, but also amplifies the response through the augmented production of select inflammatory cytokines. Nevertheless, our in vivo data using anakinra, an antagonist of the IL-1R that blocks the effects of both IL-1α and IL-1β, demonstrate a causal role for IL-1 in SAA-promoted pulmonary neutrophilia, whereas the in vivo data from the NLRP3- and caspase-1–deficient mice reveal very modest reductions in inflammatory cytokines (aside from IL-1β) and pulmonary neutrophilia following SAA aspiration. Taken together, these findings suggest that there may be an additional role for IL-1α release in response to SAA that requires further investigation.

Models of in vitro and in vivo Th17 polarization require the presence of IL-1, IL-23, and IL-6 (23, 64-67) and repression of Th1- and Th2-polarizing cytokines to establish a unique environment that challenge with apo-SAA appears to replicate. The Th2-polarizing Alum/OVA model has long been criticized for the magnitude of the response, whereas inhalational models of allergic sensitization (using different adjuvants such as LPS or cigarette smoke) tend to induce a modest eosinophilia that is more representative of human asthma (68). The more moderate response seen in our SAA/OVA sensitization model could represent a different aspect of the asthma syndrome, one that is less Th2 in nature and more Th17. It has recently been shown that IL-17 production promoted by IL-1β involves conversion of Foxp3^{+} T regulatory cells to retinoic acid-related orphan receptor γt-expressing Th17 cells (10, 11). In addition, in the human disease and in mouse models of severe allergic asthma, CD4^{+} T cell production of IL-17 can originate from a population of CRTh2^{+} effector cells/memory cells that are capable of secreting Th2 cytokines and coexpressing the transcription factors GATA3 and retinoic acid-related orphan receptor γt, a population that can be induced upon stimulation with proinflammatory cytokines, including IL-1β (69). Our studies have not identified the precursor CD4^{+} T cell population that develops the capacity to produce IL-17A in response to SAA, nor have we yet thoroughly investigated the spectrum of Th17-related cytokines produced by these IL-17A–producing CD4^{+} T cells. Regardless of

the mechanism of $CD4^+$ T cell conversion, the role that SAA may play in the process as an endogenous mediator has far-reaching implications for the pathogenesis of IL-17–producing $CD4^+$ T cells in severe allergic asthma.

The conclusions of the studies described in this study are 3-fold. First, it is clear that the acute-phase SAA proteins are more than simply biomarkers of disease severity. Instead, they function as biological mediators through the stimulation of TLR2 and the NLRP3 inflammasome to regulate pulmonary cytokine production and neutrophilia. Second, although the properties of SAA that enable activation of these pathways remain to be determined, the capacity for both to induce IL-1β gene expression and allow for IL-1β secretion make SAA distinct from other endogenous amyloid peptides and proteins that function solely in the later step of IL-1β release (2, 22). Third, SAA is sufficient to function as an adjuvant to promote allergy to an innocuous inhaled Ag in a manner that is dependent upon IL-1R signaling to stimulate the capacity of $CD4^+$ T cells to produce IL-17A. Whether SAA is necessary for Th17 development in response to inhalational Ag sensitization and at what threshold concentration endogenous SAA manifests TLR2/NLRP3 stimulation remain to be determined. These results are the first, to our knowledge, to link pulmonary SAA, IL-1β, and IL-17A in a manner that explains their interrelationships in allergic asthma and their mechanisms of action. Based upon our findings, it is evident that novel models of SAA/IL-1–mediated allergic airway disease may provide new insight into the endogenous mechanisms behind the inappropriate or maladaptive immune responses at play in the complex phenotypes of allergic asthma.

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CHAPTER 6

Airway epithelial NF-κB activation promotes the ability to overcome inhalational antigen tolerance

Jennifer L. Ather, Kathryn L. Foley, Benjamin T. Suratt, Jonathan E. Boyson, and Matthew E. Poynter

Under submission

ABSTRACT

We examined whether pre-existing inhalational antigen tolerance, important in the prevention of allergic asthma, could be overcome by airway epithelial NF-κB activation. Wildtype and transgenic mice capable of expressing constitutively active IκB kinase β (CAIKKβ) in airway epithelium were tolerized to inhaled ovalbumin (OVA). Twenty-eight days later, the transgene was expressed and all mice were exposed to inhaled OVA on day 30. Following OVA challenge on days 40-42, transgene-expressing CAIKKβ mice exhibited characteristic features of allergic airway disease, including eosinophilic airway inflammation and methacholine hyperresponsiveness. Increases in the $CD103^{+}$ and $CD11b^{HI}$ lung dendritic cell populations were present in CAIKKβ mice on day 31 and were capable of inducing Th2 cytokine and IL-17A secretion from naïve OT-II $CD4^{+}$ T cells in the presence of OVA. Finally, and BAL from CAIKKβ mice induced *in vitro* T_{REG}-polarized $CD4^{+}$ T cells to secrete Th2 and Th17 cytokines in the presence of dendritic cells, effects that required IL-4, IL-6, and IL-1. Our studies demonstrate that soluble mediators generated in response to airway epithelial NF-κB activation orchestrate the breaking of inhalational tolerance through effects on pulmonary dendritic cells as well as naive and T_{REG} $CD4^{+}$ T cells.

Introduction

Despite recent insights into the molecular mechanisms of allergic asthma, incidence of the disease continues to rise worldwide (1). Treatments for those afflicted with this multi-faceted syndrome are largely supportive and rely heavily on the use of inhaled corticosteroids and β-agonists to control symptoms of inflammation and bronchoconstriction (2). Recent studies and reviews have clarified the distinction between T_H2 asthma, characterized by eosinophilic lung infiltration, increased serum IgE, and T cell production of IL-4, IL-5, and IL-13, and T_H17 asthma, which is instead predominantly neutrophilic, non-atopic, and steroid-resistant (3). We and others have explored the roots of inhalational sensitization, wherein a naïve mouse is allergically-sensitized to the innocuous inhaled antigen, ovalbumin (OVA). However, less work has been conducted to address the situation in which a previously-tolerized individual develops allergic airway disease despite prior antigen exposure. We have adapted a mouse model system to experimentally assess this situation.

Under normal, non-inflammatory circumstances, mice that are exposed to an innocuous antigen develop immune tolerance to that antigen that persists despite subsequent attempts to sensitize, even when using highly immunogenic adjuvants (4, 5). This tolerogenic response relies upon the activity of $CD4^+CD25^+$ T regulatory cells (T_{REGS}), which in the absence of an adjuvant suppress naïve T cell proliferation and cytokine production in response to an innocuous antigen (6). Indeed, adoptive transfer of T_{REG} cells prior to sensitization confers resistance to the development of allergic asthma (7). Studies have implicated several key effectors of T_{REG}-mediated suppression, including indolamine-2,3-dioxygenase (IDO), IL-10, and TGF-β (6, 8-10). In addition, inflammatory stimuli such as bacterial lipopolysaccahride (LPS) and CpG DNA can overcome the T_{REG} response, possibly by inducing naïve $CD4^+$ T cells to become refractory to T_{REG} suppression (6). A critical role has also been implicated for dendritic cells (DCs) as producers of soluble factors that influence naïve T cells (9).

Dendritic cells bridge the gap between innate and adaptive immunity, sampling and processing antigen for presentation to $CD4^+$ T cells. Three DC populations have been described in the lung, each with distinct immune functions (11-13). Plasmacytoid DCs (pDCs) are poorly phagocytic, express high levels of IL-10 and IDO, and as illustrated through their selective depletion, are required for the generation of inhalational tolerance (14, 15). In contrast, $CD103^+$ DCs and $CD11b^{HI}$ myeloid DCs are implicated in inflammatory and allergic responses (11, 12). $CD103^+$ DCs reside in close proximity to the epithelium, where they express tight junction proteins and are the first to sample antigen from the airway lumen (13, 16). In contrast, $CD11b^{HI}$ DCs occupy the lung parenchyma (13, 17). In the absence of activating stimuli, such a pathogen- or damage-associated molecular patterns

(PAMPs, DAMPs) or inflammatory cytokines, DCs present harmless antigens to T cells, but do not provide the co-stimulatory signals necessary for the generation of strong $CD4^+$ T cell responses; instead, an anergic T cell (T_{REG}) population undergoes expansion (18, 19). However, under inflammatory conditions, $CD103^+$ DCs secrete cytokines capable of polarizing T_H2 and/or T_H17 responses and $CD11b^{HI}$ DCs strongly promote $CD4^+$ T cell proliferation (12, 20).

Activation of pulmonary DCs can be directly influenced by the surrounding cellular microenvironment, especially the pulmonary epithelium. In response to microbial products, physical insult, or injury, airway epithelial cells secrete cytokines such as CCL20, TNFα, IL-6, and GM-CSF, driving the recruitment, maturation, and activation of dendritic cells (21, 22). In particular, airway epithelial activation of the transcription factor nuclear factor kappa-B (NF-κB) serves as a critical regulator of allergic airway disease and is induced by many agonists that promote inflammatory cytokine production (22-24). Previously, we have reported that airway epithelial NF-κB activation in an inducible transgenic mouse model is sufficient to sensitize mice to an innocuous inhaled antigen (25). We therefore sought to determine whether airway epithelial NF-κB activation would allow for allergic sensitization in mice that had previously been tolerized to the antigen OVA and, if so, whether the "breaking" of inhalational tolerance in this manner would elicit epithelial-induced changes in the lung DC phenotype and influence T_{REG} cytokine production. Our results implicate airway epithelial NF-κB activity as a profound modulator of the sensitivity to overcome inhalational tolerance and develop allergic airway disease through the influence of soluble mediators on DCs and T_{REGS}.

Materials and Methods

Mice.

CC10-rtTA x TetOP-CAIKKβ bitransgenic mice on the C57BL/6 background (22), which express a constitutively active IκB kinase B (CAIKKβ) in bronchiolar epithelium following administration of 6g/kg doxycycline (Dox) in chow (TestDiet, Richmond, IN), were bred at the University of Vermont. C57BL/6 mice and OT-II TCR transgenic mice (C57BL/6-Tg(TcraTcrb)425Cbn) were purchased from Jackson Laboratories (Bar Harbor, ME) and bred at the University of Vermont. Mice were housed in an American Association for the Accreditation of Laboratory Animal Care (AALAC)-approved facility, maintained on a 12h light/dark cycle, and provided food and water ad libitum. All animal studies were approved by the University of Vermont Institutional Animal Care and Use Committee.

Inhalational tolerance model.
Transgenic CAIKKβ mice and wildtype littermates were exposed by oropharyngeal aspiration on Day 0, 1, and 2 to sterile saline (50 µl) as a negative control or 100 µg of low endotoxin (LE)-OVA (Hyglos, Germany) in sterile saline to induce inhalational tolerance. Mice were then administered Dox for 60 hours prior to 30 minutes of 1% OVA (Grade V, Sigma-Aldrich, St. Louis, MO) nebulization on day 30. Positive control C57BL/6 mice were unmanipulated until they received 20 µg OVA in 100 µl of 50% Imject Alum (Thermo Fisher Scientific, Rockford, IL) in one i.p. injection on Day 30. All mice were challenged on Day 40, 41, and 42 with 30 minutes of nebulized 1% OVA in sterile saline and mice were analyzed on Day 44, forty-eight hours after the final antigen challenge.

Bronchoalveolar lavage collection and lung processing.
Lungs were lavaged with 1 ml DPBS (Sigma-Aldrich) from which cells were counted by hemocytometer. Differential analysis was performed by cytospin and H&E stain.

Quantitative RT-PCR.
Total RNA was extracted from flash-frozen whole lungs using the PrepEase RNA Isolation kit (USB, Cleveland, OH) and reverse transcribed to cDNA using the iScript kit (Bio-Rad Laboratories, Hercules, CA). Primers were designed for mouse *Il13* and *Cxcl1*, while *Muc5ac* and *Gapdh* were analyzed using Taqman Assay on Demand (Life Technologies, Carlsbad, CA). PCR was performed using SYBR Green Supermix (Bio-Rad) or iQ Supermix (Bio-Rad) and normalized to *Gapdh* using the $\Delta\Delta C_T$ method, as previously described (25).
Splenocyte restimulation. Splenocytes from mice were isolated using Lymphocyte Separation Media (MP Biomedicals, Solon, OH) following mechanical disruption as previously described (26). A total of 4 x 10^6 cells/ml were cultured in RPMI-1640 supplemented with 10% FBS (Cell Generation, Fort Collins, CO), penicillin/streptomycin, L-glutamine, folic acid, and 2-ME and were activated with 100 µg/ml OVA in 48 well plates. Supernatants were collected for analysis after 96 hours of incubation at 37°C in 5% CO_2.

ELISA analysis.
Analysis of cytokine content from unconcentrated bronchoalveolar lavage fluid (BAL) and cell supernatants was performed using ELISA kits for TGF-β, IL-5, IL-10, IL-13, IL-17A, IFNγ (R&D Systems, Minneapolis, MN), IL-4 and TNFα (BD Biosciences, San Diego, CA. BAL was concentrated using Amicon Ultra centrifugal filters (Millipore, Billerica, MA), and concentrated samples were analyzed for cytokine content using custom Milliplex panels (Millipore) for SAA3, KC, IL-6, MIP-2, G-CSF,

GM-CSF, TNF-α, IL-1α, IL-1β, IP-10, TNF-α, IL-12p70, and MCP-1. Immunoglobulin ELISAs were performed as previously described (27).

Methacholine responsiveness.
Mice were anesthetized and mechanically ventilated using the forced oscillation technique to assess methacholine responsiveness, as previously described (23, 25). Airway resistance (R_N), tissue damping (G), and tissue resistance (H) were calculated at baseline and after challenge with three incremental doses of aerosolized methacholine (3.125, 12.5, and 25 mg/ml) in saline and peak values are presented as a percentage of the baseline value.

Lung single cell suspensions.
Whole lungs were dissociated to single cell suspension by mechanical disruption using a razor blade followed by incubation at 37°C with 0.5 mg/ml Liberase TM (Roche Applied Science, Indianapolis, IN) and 40 μg/ml DNase for a total of 30 minutes with 3x 20 strokes of trituration through a trochar needle using a 5ml syringe. Red blood cells were removed using Lymphocyte Separation Media and the lung suspension was filtered through a 40 μm nylon mesh membrane. Total cells were then counted by hemocytometer and were stained for flow cytometry, as described below.

Cell staining and flow cytometry.
Lung single cell suspensions were stained with the following antibodies: CD11c-PETR (Invitrogen, Camarillo, CA), CD11b-APC Cy7 (BD Pharmingen), I-A/I-E-PerCP/Cy5.5 (BioLegend, San Diego, CA), and CD103-APC (eBioscience, San Diego, CA). Dead cells were excluded by UV-Blue Live/Dead stain (Invitrogen). Briefly, 1×10^6 cells were first blocked with 2.5 μg/ml Fc block (anti-CD16/CD32, BD Pharmingen) for 30 minutes, washed in FACS buffer (DPBS with 5% FBS) and then stained for 30 minutes in 100 μl of antibody solution at the optimal concentration. Following staining, all cells were washed and fixed in DPBS with 5% FBS and 1% paraformaldhehyde. Cells were analyzed on the flow cytometer 1 day following staining using a Becton Dickinson LSR II FACS equipped to distinguish as many as 7 fluorophores. Dendritic cells were identified as the $CD11c^+$, FITC-$autofluorescence^{LO}$ population as described in (12). Macrophages were excluded as the $CD11c^+$, FITC-$autofluorecence^{HI}$ population.

Co-culture of $CD11c^+$ pulmonary dendritic cells and OT-II $CD4^+$ T cells.
$CD11c^+$ dendritic cells ($CD11c^+$, FITC-$autofluroescence^{LO}$, sorted by FACS) from the lung (1×10^6 cells/ml) were co-cultured with OT-II $CD4^+$ T cells (1×10^6 cells/ml) isolated by magnetic negative selection (Stem Cell Technologies, Vancouver, BC, Canada) and 100 μg/ml of whole OVA protein (Sigma) in a total volume of 200 μl/well in a flat-bottomed 96-well tissue culture plate. In some instances, 10 μg/ml

anti-IL-4 (R&D Systems), 10 μg/ml anti-IL-6 (R&D Systems), or 200 ng/ml anakinra (Biovitrum, Stockholm, Sweden) was added to the culture. After 96 hours, cell-free supernatants were collected and frozen at -20°C prior to analysis.

T_{REG} polarization:
$CD4^+$ $CD25^-$ T cells were isolated from naïve C57BL/6 spleen using magnetic negative selection (Stem Cell Technologies) and T_{REG} polarization was carried out as previously published (10). In brief, cells were cultured with plate-bound anti-CD3ε coated at (5 μg/ml) and soluble anti-CD28 (2 μg/ml) (BD Pharmingen) in the presence of rhTGF-β1 (5 ng/ml) and rhIL-2 (100 U/ml) (R&D Systems). On day 2, cells were split into RPMI-1640 complete with rhIL-2 (100 U/ml). Cells were harvested on day 7, washed in DPBS, and replated in either fresh media or cell-free BALF from wild type or CAIKKβ mice that had been administered Dox for 60 hours. Cells were cultured for an additional 72 hours, whereafter supernatants were collected and analyzed by ELISA. Intracellular Foxp3+ staining was performed using the Anti-Mouse/Rat Foxp3 Staining Set PE (eBioscience) according to manufacturer's instructions.

Statistical analyses.
Data were analyzed by two-tailed unpaired *t* test, one-way ANOVA or two-way ANOVA and Bonferroni post hoc test using GraphPad Prism 5 for Windows (GraphPad). A p value <0.05 was considered statistically significant.

Results

Airway epithelial NF-κB activation sensitizes mice to allergic airway disease despite previous tolerization. To examine the responses of mice tolerized to ovalbumin (OVA), we utilized two models. The first is a well-documented regimen for antigen-specific tolerance induction (Fig. 1A), which has been used to demonstrate that intraperitoneal (i.p.) injection of Alum/OVA cannot sensitize mice to allergic airway disease if they have already been exposed to inhaled OVA in the absence of an adjuvant (8). In our hands, inhallationally-tolerized mice that underwent a subsequent sensitization regimen via i.p. Alum/OVA did not develop airway eosinophilia after challenge, and neither did they upregulate OVA-specific serum IgE or inflammatory genes in the lung (supplemental figure 1). In addition, splenocytes isolated from these mice following antigen challenge did not secrete T_H2 cytokines upon restimulation, and instead predominantly produced IL-10 (supplemental figure 1). These results demonstrate our implementation of a robust inhalational tolerance induction model with which we could test the impact of airway epithelial NF-κB in breaking tolerance.

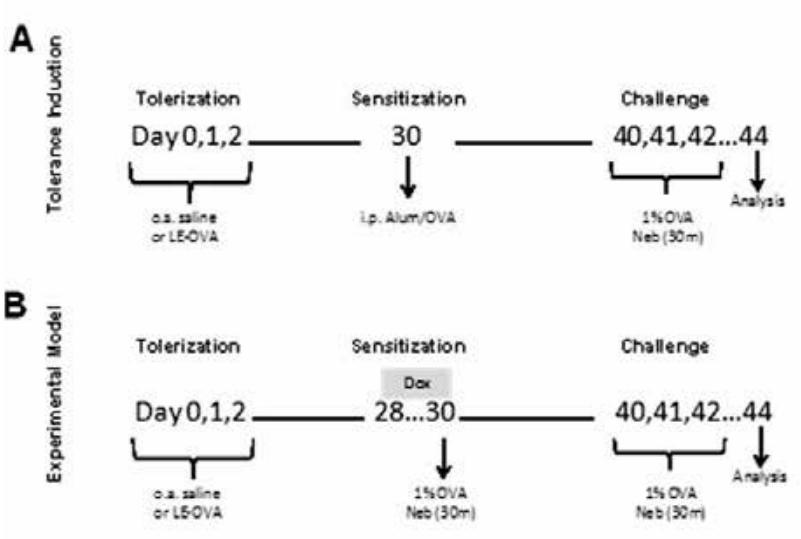
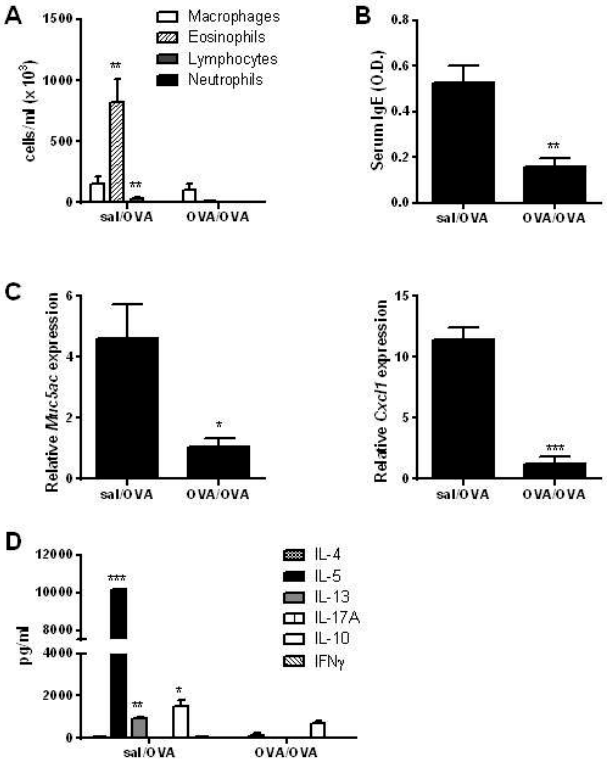


Figure 1. *Models for inducing and attempting to break inhalational tolerance.*
(A) C57BL/6 mice were exposed on days 0, 1, and 2 to either oropharyngeal aspiration (o.a.) of saline or 100 mg low-endotoxin (LE-OVA), followed by i.p. injection on Day 30 with Alum/OVA. All mice were challenged on Day 40, 41, and 42 with a 30 minute exposure of nebulized (Neb) 1% w/v OVA, and were analyzed 48 hours later on Day 44. (B) In contrast, wild type and CAIKKβ mice underwent the same tolerization and challenge regimen, but were administered Dox on days 28-30, followed by a single aerosolized exposure to 1% OVA for 30 minutes on day 30.

Our experimental model eschewed the i.p. Alum/OVA regimen for attempting to break inhalational tolerance in favor of a transgenic model of Dox-inducible airway epithelial NF-κB activation (22) (Fig. 1B). Despite having already been tolerized, transgenic CAIKKβ mice exposed to Dox became sensitized to OVA and induced robust airway eosinophilia and modest neutrophilia in response to OVA challenge (Fig. 2A, TG O/O+Dox). Similar results were obtained in mice that had not been previously tolerized (Fig 2A, TG sal/O+Dox), consistent with our earlier findings (25) . In contrast, wildtype littermates developed no symptoms of allergic airway disease. Mice that did not receive Dox also showed no development of allergic airway inflammation (Fig. 2A) and all subsequent data will include only mice that received Dox. In addition to cell accumulation in the lavageable airspaces, CAIKKβ mice secreted pro-fibrotic TGF-β into the bronchoalveolar lavage (Fig. 2B), whereas wildtype mice did not. CAIKKβ mice also expressed the inflammatory genes *Muc5ac* (a mucin gene), the T_H2 cytokine *Il13,* and *Cxcl1* (encoding the chemokine keratinocyte-derived chemoattractant, KC) (Figs. 2C-E) in the lung following challenge. Furthermore, upon antigen restimulation, splenocytes from previously

tolerized transgenic mice produced levels of strongly induced production of IL-5, IL-13, and IL-17A that were similar to those that produced by transgenic mice that had not undergone inhalational tolerance (Figs. 2F-H).



Supplemental Figure 1. *Mice tolerized with LE-OVA prior to Alum/OVA i.p. sensitization are resistant to the development of allergic asthma.* A) Differential cell counts from mice that were sensitized via Alum/OVA, without (sal/OVA) or with (OVA/OVA) tolerization (see Fig. 1A). Serum Ova-specific IgE was measured by ELISA (B), and gene expression was measured by quantitative PCR from whole lung cDNA (C). Splenocytes were restimulated for 96 hours with OVA and supernatants were analyzed by ELISA (D).

Airway epithelial NF-κB activation promotes antigen-induced methacholine hyperresponsivness irrespective of prior tolerization. We have previously reported that transient airway epithelial NF-κB activation is sufficient to allergically sensitize naïve mice to OVA and drive airway hyperresponsiveness (R_N) to inhaled methacholine following antigen challenge. To examine whether a similar effect is elicited in mice that had previously been tolerized with LE-OVA, mice underwent meth-

acholine challenge using forced oscillations. Although there were no differences in baseline levels of Newtonian airway resistance (R_N), tissue damping (G) or tissue stiffness (H) among any of the groups (Figs. 3A-C), CAIKKβ mice that had been previously tolerized demonstrated a comparable increase in R_N to their non-tolerized counterparts (Fig 3D), with no difference in G or H (Figs. 3E-F). These data further demonstrate the allergic airway disease phenotype of airway methacholine hyperresponsiveness that develops in CAIKKβ mice despite previous antigen-induced immunological tolerance.

Airway epithelial NF-κB activation drives myeloid dendritic cell maturation. To examine mechanisms that allow NF-κB activation specifically in the airway epithelium to promote adaptive immune responses and overcome inhalational tolerance, naïve dendritic cells were isolated from the spleens of C57BL/6 mice and treated with cell-free BAL collected from wild type or CAIKKβ mice following 60 hours of Dox administration. Dendritic cells were cultured for 24 hours and then stained for surface markers of maturation. Flow cytometric analysis showed that dendritic cells treated with BAL from CAIKKβ mice displayed elevated levels of surface MHC II (Fig 4A) and CD86 (Fig 4B). To examine specifically which pulmonary dendritic cells were affected by airway epithelial NF-κB activation in our tolerance model, we isolated lung cells immediately following tolerization (day 3) or immediately following sensitization of mice that had been previously tolerized (day 31) and stained for DC cell surface markers. As anticipated, no differences were measured between wild type and transgenic mice on day 3. Day 31 analysis showed a small but significant increase in CD11c positivity (Fig. 4C) which could reflect both dendritic cells and macrophages, and significant increases in MHC II (Fig. 4D) and CD103 (Fig. 4E). No differences were observed in CD11b (Fig. 4F). These lung cell suspensions were then gated on the $CD11c^+$ FITC-autoflourescence^{LO} population to specifically examine the two myeloid dendritic cell types known to be present in the lung. On day 31, there was an equal increase in the $CD11b^+$/MHC II^+ (Fig. 4G) and $CD103^+$/$MHCII^+$ (Fig. 4H) dendritic cell populations, both of which have been observed to be causal in development of allergic asthma (11).

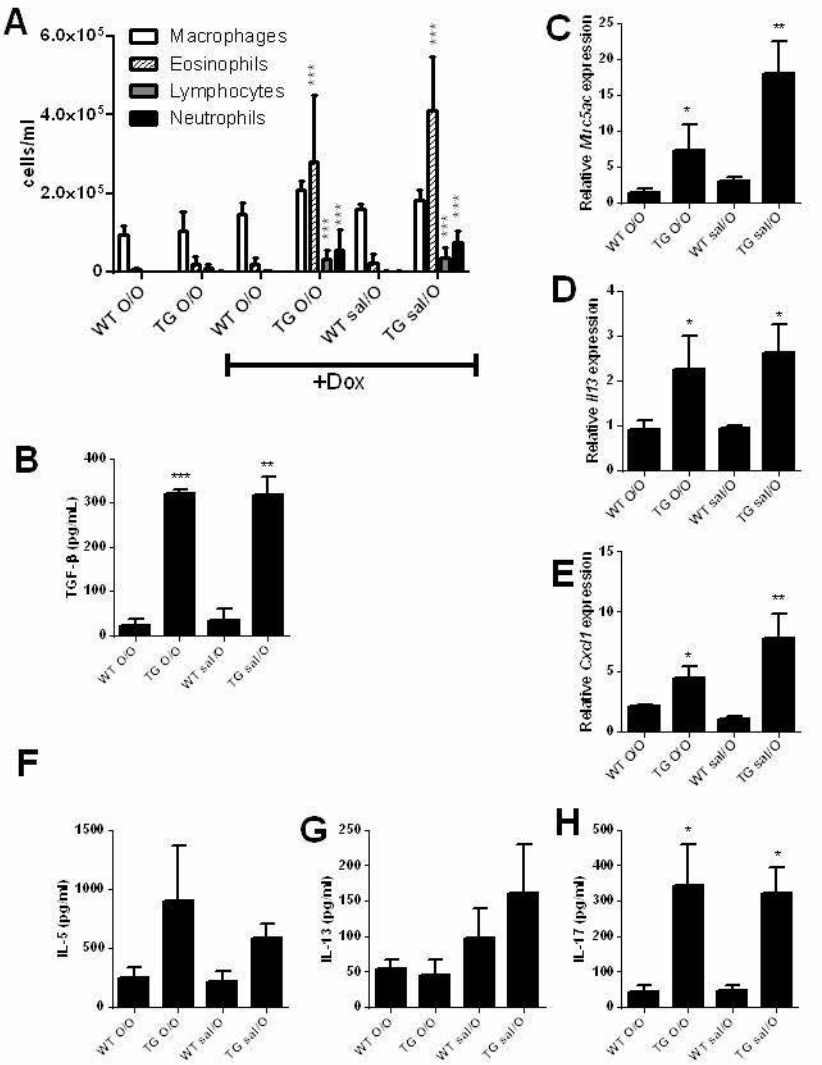


Figure 2. *Inducible airway epithelial NF-κB activation promotes antigen-induced sensitization to allergic airway disease, regardless of tolerized status.*

Differential BAL cell counts (A) of wild type (WT) or transgenic CAIKKβ (TG) mice that were sensitized via 60 hours of Dox chow administration followed by aerosolized OVA, with (O/O) or without (sal/O) previous OVA tolerization (see Fig. 1B). TGF-β was measured from BAL (B) and gene expression was measured by quantitative PCR from whole lung cDNA.(C-E). Splenocytes were harvested from mice 48h after challenge, and restimulated with OVA for 96 hours before IL-5, IL-13, and IL-17 from cell-free supernatants were analyzed by ELISA (F-H). n = 6-7 mice per/group. (*p=<0.05, **p=<0.01, ***p=<0.001).

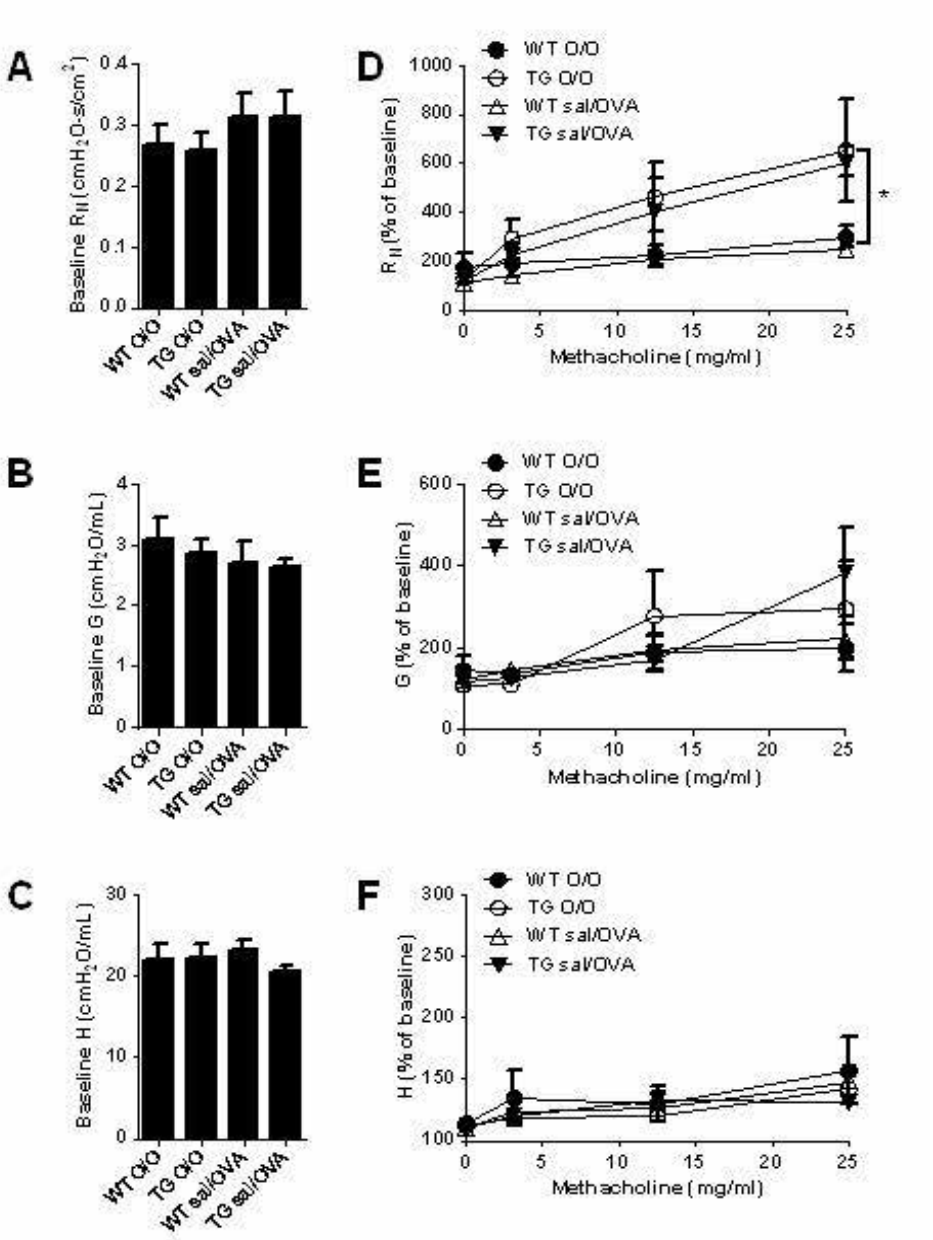


Figure 3. *Inducible airway epithelial NF-κB activation promotes airway hyperresponsiveness to inhaled methacholine, regardless of tolerized status.*
Forced oscillations using the flexiVent were used to assess baseline measures of R_N, G, and H (A-C) as well as the responsiveness to inhaled methacholine (D-F). n = 3-6 mice per/group. (*p=<0.05 for WT vs. TG (CAIKKβ) exposed to the same treatment regimen).

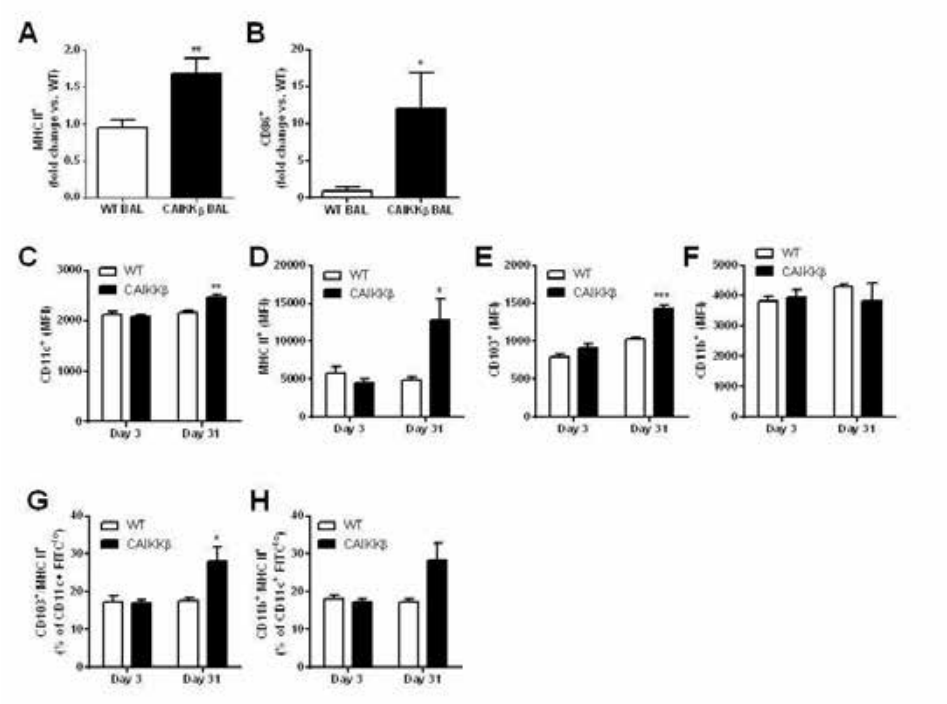


Figure 4. *Airway epithelial NF-κB activation drives dendritic cell maturation and promotes an inflammatory lung DC phenotype when overcoming inhalational tolerance.*

Naive dendritic cells were isolated from wild type mouse spleens and exposed to cell-free bronchoalveolar lavage fluid from wild type (WT) or CAIKKβ mice after 60 hours of Dox administration and analyzed by flow cytometry for MHC II (A) and CD86 (B). Mice were analyzed on day 3 (immediately following tolerization) or day 31 (immediately following sensitization) of the inhalational tolerance protocol and lung single cell suspensions were analyzed by flow cytometry for dendritic cell surface markers (C-G). Cells were gated for dendritic cell phenotype ($CD11c^{+}$, FITC autofluorescence^{LO}) and analyzed for co-positivity of CD103/MHC II (H) and CD11b/MHC II (I) positive populations. (n= 4-8, * = $p<0.05$, **= $p<0.01$ *** = $p<0.001$).

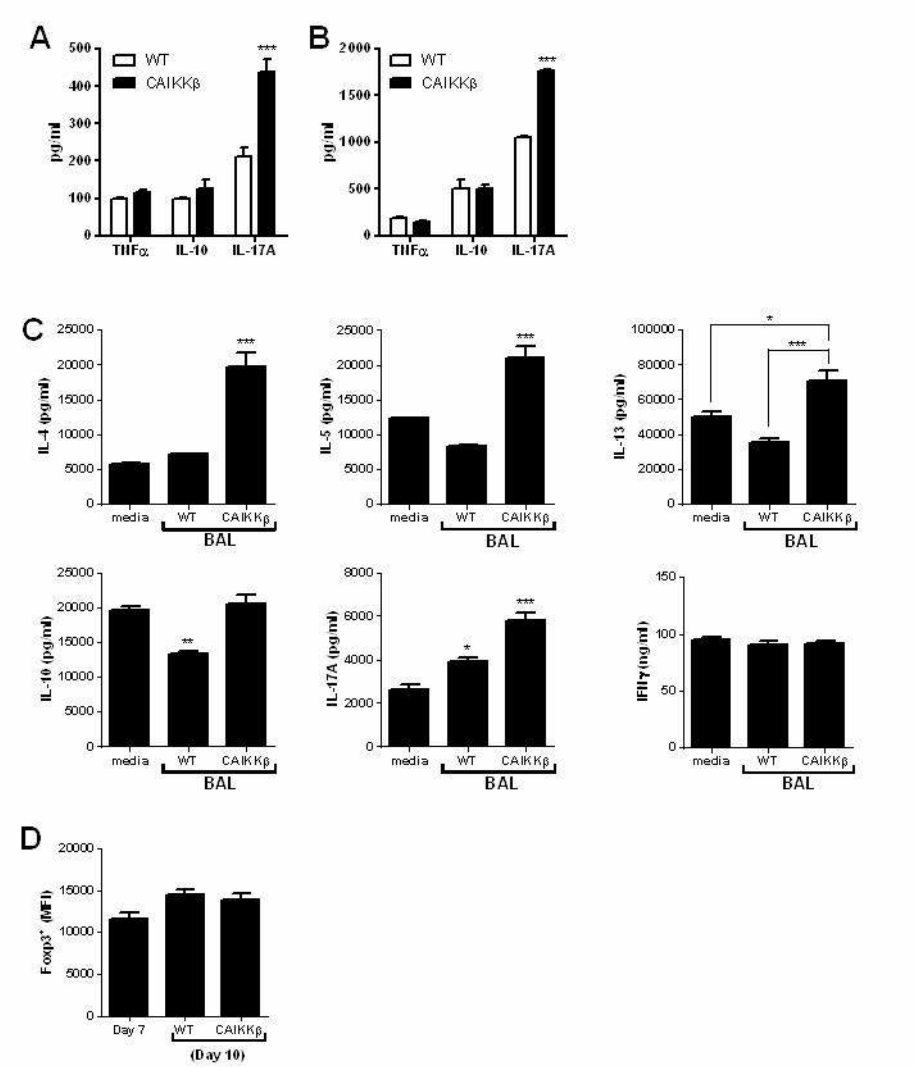


Figure 5. Airway epithelial NF-κB activation induces T cell activation, and induces polarized T_{REG} cells to secrete T_H2 and T_H17 cytokines. $CD11c^+$ FITC $autoflourescence^{LO}$ cells were isolated from the lungs of WT and CAIKKβ mice 60 hours after Dox administration and were co-cultured with $CD4^+CD25^-$ T cells from OT-II mice (A) or a 1:1 ratio of $CD4^+CD25^-$ and $CD4^+CD25^+$ cells (B). Cultures were stimulated with OVA and supernatants were analyzed 96 hours later by ELISA. n= 3-6. (C) $CD4^+$ T cells isolated from naive C57BL/6 mouse spleen and lymph nodes underwent in vitro T_{REG} polarization for 7 days. On day 7, cells were treated with fresh media (control) or cell-free BAL from wild type and CAIKKβ mice administered Dox for 60 hours. After 72 hours of restimulation, supernatants were analyzed by ELISA. n=6. (D) Polarized T_{REG} cells were stained for intracellular FoxP3 on Day 7, or on Day 10 after 72h treatment with WT or CAIKKβ BAL. n=3. (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$).

Airway epithelial NF-κB activation induces naïve T cell polarization and T_{REG} conversion. Having demonstrated the profound effect of soluble mediators present in the BAL from airway epithelial CAIKKβ mice on dendritic cell maturation both *in vivo* and *in vitro*, we next examined the impact of airway epithelial NF-κB activity on the T cells themselves. $CD4^+$ T cells isolated from OT-II mice, which express a transgenic T cell receptor that is specific for OVA, were co-cultured with pulmonary dendritic cells from wildtype or CAIKKβ mice enriched after 60 hours of Dox administration. The dendritic cells were pre-sorted by FACS to isolate the $CD11c^+FITC^{LO}$ DC population and to exclude the $CD11c^+$ $FITC^{HI}$ alveolar macrophages. The isolated pulmonary dendritic cells were cultured with either $CD4^+CD25^-$ naïve OT-II T cells (Fig. 5A) or a 1:1 ratio of $CD4^+CD25^-$: $CD4^+CD25^+$ OT-II T cells (Fig. 5B) and stimulated with whole OVA protein for 96 hours. Previous work by del Rio *et al.* (28) has demonstrated the ability of $CD4^+CD25^+$ cells to suppress $CD4^+CD25^-$ T cell proliferation. Analysis of the supernatants showed that dendritic cells from CAIKKβ mice induced OT-II T cells to produce an increased amount of IL-17A, regardless of whether the T cell population included $CD25^+$ cells (Fig. 5A-B).

The implication that pulmonary dendritic cells from CAIKKβ mice, following airway epithelial NF-κB activation, are capable of eliciting the activation of, and cytokine secretion from, $CD4^+$ T cells, regardless of the presence of $CD25^+$ cells that may include a T_{REG} population, was intriguing and required more rigorous study. Therefore, we utilized an *in vitro* model of T_{REG} polarization. $CD4^+$ T cells were isolated from the spleens and lymph nodes of naïve C57BL/6 mice and polarized to T_{REGS} using TGF-β and IL-2, as previously reported (10). On day 7, cells were washed and replated in the presence of anti-CD3, then treated with either fresh media or cell-free BALF from wildtype or CAIKKβ mice that was collected following 60 hours of Dox administration. After 72 hours of *in vitro* culture, the polarized T_{REG} cells secreted significantly more T_H2 cytokines (IL-4, IL-5, and IL-13) as well as more IL-17A in response to the CAIKKβ BALF compared to BALF from wildtype littermates (Fig. 5C). To examine changes in Foxp3 expression from the polarized T_{REG} cells after 72h treatment with BAL fluid, intracellular staining was performed. Neither treatment with BAL from wildtype or CAIKKβ mice changed the level of FoxP3 expression from that expressed on Day 7 of polarization (Fig. 5D).

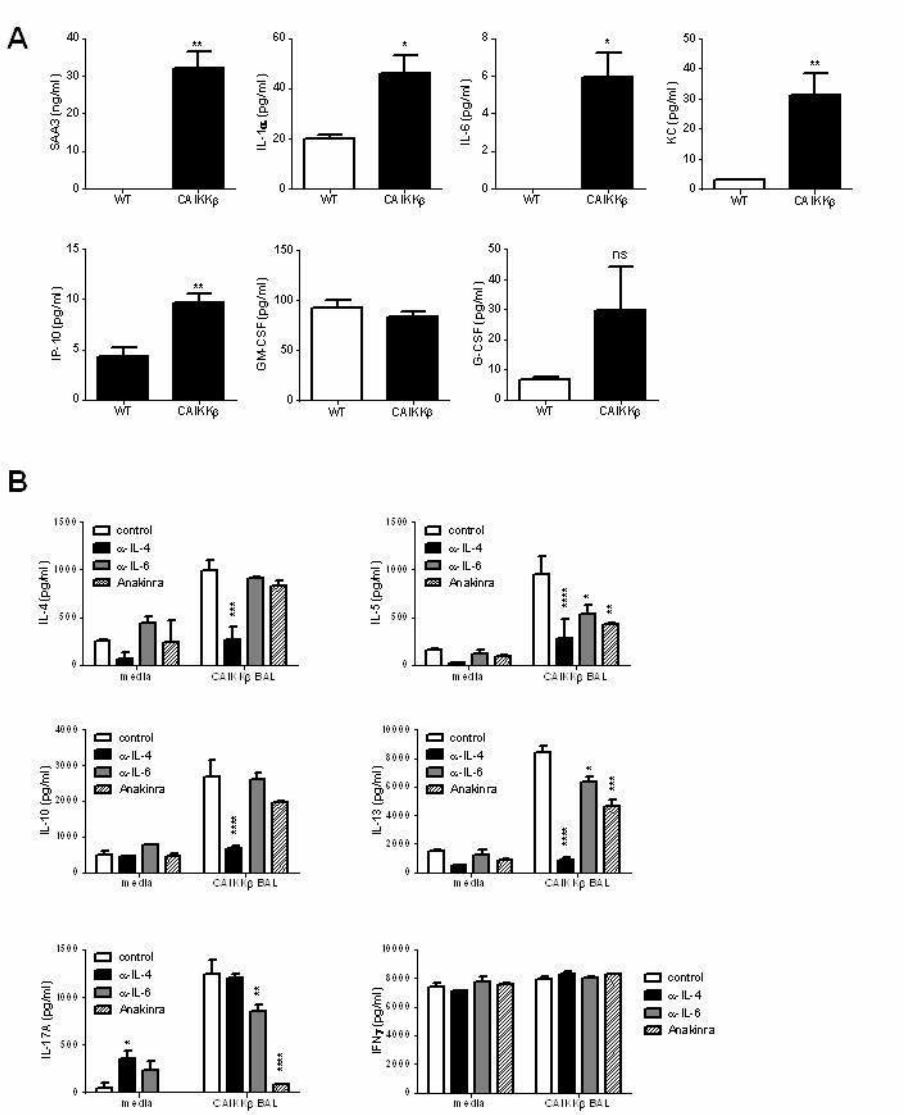


Figure 6. *Airway epithelial NF-κB activation induces release of acute inflammatory mediators into the lavageable airspace.* A) CAIKKβ and WT littermate mice were administered Dox for 60 hours. Cell-free BAL was collected and concentrated using centrifugal filtration. Cytokines from concentrate were analyzed by Milliplex panel. Cytokines that were measured but undetectable include: IL-1β, MCP-1, MIP-2, TNF-α, and IL-12p70. n = 3-4. (* = p<0.05, **= p<0.01). B) Polarized TREG cells from C57BL/6 mice were treated with media or cell-free BAL from CAIKKβ mice after 60 hours Dox administration, in the presence or absence of anti-IL-6 (10 μg/ml), anti-IL-4 (10 μg/ml), or Anakinra (200 ng/ml). Cells were incubated for 72 hours and supernatants were analyzed by ELISA.

Airway epithelial NF-κB activation induces release of acute inflammatory mediators capable of driving T_H2/T_H17 development. To search for the presence of soluble mediators released into the BAL of CAIKKβ mice that could be responsible for driving the inflammatory T cell phenotype and overcoming tolerization, we concentrated BAL from WT and CAIKKβ mice that had been administered Dox for 60 hours. Analysis of the concentrate revealed the presence of several candidate mediators (Fig. 6A), including the acute phase protein SAA3, the T_H17-polarizing cytokine IL-1α, the T_H17- and Th2-polarizing cytokine IL-6, and the leukocyte chemoattractors KC and IP-10, whereas the T_H2-polarizing cytokine IL-4 was undetectable (data not shown). To determine the role of IL-1α, IL-6, and IL-4 in the T cell-activating effects presented in Figure 5C, polarized T_{REG} cells were treated for 72 hours with cell-free BAL from CAIKKβ mice that had been on Dox for 60 hours, in the presence or absence of either Anakinra (which blocks IL-1 signaling), anti-IL-6, or anti-IL-4. Addition of Anakinra completely inhibited the production of IL-17A in this system (Fig. 6B), both from the BAL-treated cells and also the residual IL-17A from media-treated cells. Anti-IL-6 had a slighter, yet still significant effect on IL-17A production. Both Anakinra and anti-IL-6 affected the induction of IL-5 and IL-13. Neutralization of the T_H2-polarizing cytokine, IL-4, effectively reduced T_H2 cytokine production (IL-4, IL-5, IL-13, and IL-10), while having no effect on the production of IL-17A. Neither the neutralizing antibodies or Anakinra had any effect on the production of the T_H1 cytokine IFNγ.

Collectively, these results demonstrate that airway epithelial NF-κB activation yields several soluble mediators that are secreted into the lavagable airspace and that have polarizing effects on both lung dendritic cells and T cells, including T_{REG} cells, and as such, contribute to the breaking of inhalational tolerance.

Discussion

The generation of pulmonary allergic responses in the lung requires the close coordination of signals from airway epithelium, antigen-presenting dendritic cells, and naïve T cells that become polarized to inflammatory T_H2 and T_H17 subsets. While substantial study has been devoted to describing the early events that predicate allergic sensitization, the mechanisms by which the immune system overcomes an existing inhalational tolerance to elicit allergic responses is less clear. Recent work has implicated the role of inflammation, caused by infection or environmental cigarette smoke, and has even focused on dendritic cell activity as a key component of overcoming T_{REG} suppression (6, 20, 21).

The airway epithelial barrier acts as the first line of defense against inhaled agonists, and cytokines secreted from these cells as a result of antigen contact are critical mediators of the downstream inflammatory response. Airway epithelial

cells release chemokines, such as KC and CCL20, as well as cytokines that can polarize T cells toward T_H2 and T_H17 phenotypes (29). Our previous work (25) has demonstrated that after 60 hours of Dox administration, transgenic CAIKKβ mice upregulate lung gene expression of SAA3, CCL20, and GM-CSF. SAA3 participates in T_H2 and T_H17 polarization (11, 30, 31), CCL20 (also known as MIP-3α) is the ligand for CCR6 expressed by dendritic cells and is, in part, responsible for their recruitment to the lung (32), and GM-CSF is required for maturation of dendritic cells into potent antigen presenters and for inducing expression of co-stimulatory molecules to enhance DC activation of naive T cells (32, 33). Consequently, we find that the pulmonary dendritic cell phenotype in CAIKKβ mice following 60 hours of Dox administration is more mature and inflammatory in comparison to wild type counterparts, and that these DCs can induce naïve T cells to produce inflammatory cytokines and drive allergic sensitization.

A novel aspect of our data is that airway epithelial activation is sufficient to overcome inhalational tolerance to OVA. Sensitized mice, which had not been previously tolerized, demonstrated a robust allergic airway response, including airway eosinophilia and neutrophilia, TGF-β secretion and inflammatory gene expression, as well as increases in airway resistance in response to inhaled methacholine. Despite having gone through the inhalational tolerance regimen, the CAIKKβ mice developed these parameters of allergic airway disease subsequent to transgene expression and antigen exposure, followed several weeks later by inhaled antigen challenge. Furthermore, splenocytes from these mice showed undiminished production of IL-5 and IL-17A upon antigen stimulation.

Mediators secreted by the epithelium, or present in the lavageable airspaces and derived from other cells in the lung following NF-κB activation, were able to induce dendritic cell maturation and activation, as evidenced by increases in cell surface MHC II and CD86 in response to treatment with BALF obtained from CAIKKβ, but not wildtype, mice. Lung dendritic cell populations turn over approximately every seven days (13). Therefore, at the day 31 timepoint in the experimental tolerance model, the airway epithelium would be educating a distinct population from that which had originally been exposed to the OVA antigen. However, activation and maturation of pulmonary dendritic cells may only be one component of overcoming inhalational tolerance; changes must also be made to the existing T_{REG} population. We sought to determine whether airway epithelial NF-κB activation achieved this effect indirectly, via the activated DCs, or by exerting a direct effect on the T cell populations themselves. Previous studies have made distinctions between T_{REG} conversion, (generally via downregulation of the transcription factor FoxP3) and a separate effect whereby naïve T cell populations become refractory to T_{REG}-mediated suppression (6). To explore these effects in our model system, we first cultured FACS-sorted dendritic cells from wildtype and CAIKKβ mice with OT-II T cells, in the presence or absence of $CD25^+$ T cells. The $CD4^+CD25^-$ cells exhibit-

ed increased levels of IL-17A secretion, and this cytokine was produced even in the presence of $CD25^+$ T cells, which have been demonstrated to be repressive to $CD25^-$ T cell proliferation (28). In addition, we treated *in vitro*-polarized T_{REG} cells with BALF from wildtype and CAIKKβ mice that had been administered Dox for 60 hours. Just as the CAIKKβ BALF was able to induce maturation of dendritic cells, it also induced polarized T_{REG} cells to strongly upregulate their production of the T_H2 cytokines IL-4, IL-5, and IL-13, and the T_H17 cytokine IL-17A. These cytokines have well-studied participatory effects in the development of the allergic airway disease phenotype, as we have recently reviewed (34). It is interesting to note that treatment with CAIKKβ did not affect $Foxp3^+$ expression in these polarized cells, indicating perhaps that the soluble mediators inducing inflammatory cytokine production are not converting the polarized T_{REG} cells, but instead stimulating the $Foxp3^-$ cells present in culture. It is also possible that another set of cells altogether may be responsible for contributing to tolerance breaking. An emerging body of literature has revealed the importance of innate lymphocyte cells (ILCs), distinct populations of leukocytes that do not express rearranged antigen receptors or other classical T cell lineage markers, but which, in the lung, perform functions similar to $CD4^+$ T cells in animal models of allergic airway disease (35-37). Further study will be required to determine the exact cytokine(s) and its/their cellular source in our *in vivo* model that may be driving an allergic response in previously tolerized animals.

The results presented herein provide additional evidence for the multi-faceted effects of airway epithelial signaling on the immune response to inhaled antigen. Once considered merely a structural "barrier", the airway epithelium has begun to take center stage as a coordinator of the allergic lung response, and our results indicate that they do so not only in the generation of allergic responses in a naïve system, but also in overcoming previous tolerogenic immune education to promote asthma pathology.

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CHAPTER 7

Serum Amyloid A inhibits dendritic cell apoptosis to induce glucocorticoid resistance in CD4$^+$ T cells

Jennifer L. Ather, Karen A. Fortner, Ralph C. Budd, Vikas Anathy, and Matthew E. Poynter

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ABSTRACT

Pulmonary dendritic cells (DC) comprise the cellular interface between innate and adaptive immune responses. Inflammatory mediators produced by the airway epithelium control the activation, recruitment, and survival of pulmonary DC that present antigen to $CD4^+$ T cells during the genesis and exacerbation of allergic asthma. The epithelial-derived acute phase protein, serum amyloid A (SAA) induces DC maturation and T_H17 polarization. T_H17 responses are associated with severe forms of allergic asthma that are poorly-controlled by corticosteroids. In the studies presented herein, we sought to determine whether apo-SAA would enhance the survival of DCs during serum starvation and could then contribute to the development of a glucocorticoid-resistant phenotype in $CD4^+$ T cells. Bone marrow derived dendritic cells (BMDC) that were serum-starved in the presence of apo-SAA were protected from activation of caspase-3 and released less lactate dehydrogenase. In comparison with untreated serum-starved BMDCs, treatment with apo-SAA downregulated mRNA expression of the pro-apoptotic molecule *Bim*, increased production of the pro-survival heat shock protein 70 (HSP70), and induced secretion of the proinflammatory cytokines IL-6, TNF-α, and IL-1β. SAA-treated BMDC that were serum-starved for 48h remained capable of presenting antigen and induced OTII $CD4^+$ T cells to secrete IL-17A, IL-17F, IL-21, IL-22 and IFNγ in the presence of ovalbumin. IL-17A, IL-17F, IL-21 and IFNγ production occurred even when the $CD4^+$ T cells were treated with dexamethasone (Dex), whereas glucocorticoid treatment abolished cytokine secretion by T cells co-cultured with untreated serum-starved BMDC. Our results indicate that apo-SAA affects DC to both prolong their viability and increase their inflammatory potential under apoptosis-inducing conditions. These findings reveal mechanisms through which SAA enhances the $CD4^+$ T cell stimulating capacity of antigen-presenting cells that may actively participate in the pathogenicity of glucocorticoid-resistant lung disease.

Introduction

Dendritic cells (DC) occupy a unique niche in the immune system, functioning both as innate responders that take up antigen and secrete acute inflammatory mediators, and secondarily as modulators of the adaptive response, directly affecting the phenotype of effector and helper T cells (1-3). Maintaining healthy DC homeostasis ensures appropriate T cell responses, whereas dysfunction in DC maintenance can result in severe autoimmune disorders (2). Dysregulation in DC apoptosis, whether through over-expression of pro-survival Bcl-2 proteins or loss of the pro-apoptotic protein, **B**cl-2 **i**nteracting **m**ediator of cell death (Bim), can trigger autoimmune disease in mice (4). Bim-/- mice exhibit increases in autoreactive T and B cells, increased tumorigenesis, and fail to resolve immune responses due to a decrease in the apoptosis of activated T and B cells (5, 6). The loss of Bim in DCs results in reduced apoptosis and sustained T cell responses, both *in vivo* and *in vitro* (2). Bim-/- mice also exhibit a defect in T regulatory (Treg) cell generation; in these knockouts, a subset of $CD4^+$ T cells that are $Foxp3^+$ $CD25^{lo}$ is preferentially increased, and these cells show a diminished capacity to suppress IL-17 secretion from effector T cells compared to normal $Foxp3^+$ $CD25^{hi}$ $CD4^+$ T cells (7).

Under normal conditions, a naïve DC that encounters a harmless antigen will not mature, and will instead undergo apoptosis; likewise, mature DC treated with Toll-Like Receptor (TLR) agonists possess a "molecular timer" that limits their lifespan and, subsequently, their ability to present antigen to T cells (8). Using a model in which DC were transfected with Fas ligand (FasL) and allergen (Der p2), the DC that presented both antigen and a potent apoptotic trigger to T cells were able to induce T cell hyporesponsiveness and ameliorate the development of allergic airways disease in mice (9). This study suggests that interference with the normal apoptotic pathway during DC-T cell interactions could lead to inappropriate and prolonged antigen presentation, leading to exacerbated states of disease.

A variety of stimuli, from microbial products that stimulate TLRs to endogenous cytokines, can stimulate DC to mature and present antigen to T cells in an inflammatory context. The acute phase protein serum amyloid A (SAA), which is produced by a variety of cells in response to inflammatory insult, has been linked to a number of diseases, including Alzheimer's disease, Rheumatoid arthritis, atherosclerosis, and allergic airways disease (10-12). We have previously demonstrated that recombinant human apo-SAA is sufficient to cause BMDCs to upregulate inflammatory genes, induce cytokine secretion, and augment the surface expression of MHC II and the co-stimulatory molecules CD80 and CD86. Media from these cultured BMDC can, in turn, be used to stimulate naïve OTII $CD4^+$ T cells to secrete IL-17A and IFNγ in the presence of ovalbumin (OVA) (10). Furthermore, when administered to the lungs of mice along with OVA, apo-SAA is sufficient to

sensitize mice to OVA and promote a T_H17 allergic asthma response upon subsequent OVA challenge (10).

In the present study, we investigated the effect of apo-SAA on BMDC under conditions of serum starvation, which would normally induce apoptosis mediated by mitochondrial outer membrane permeabilization and caspase-3 activation (4). Our results demonstrate that apo-SAA treatment interferes with the induction of Bim, inhibits caspase-3 activation, and induces expression of the chaperone protein and cytokine, heat shock protein 70 (HSP70). In addition, the T_H17 $CD4^+$ T cell response generated from apo-SAA treated BMDC is resistant to steroid treatment, and this effect depends in part upon HSP70 expression. Therefore, SAA represents an endogenous mediator of DC lifespan and function that both quantitatively and qualitatively dictates the $CD4^+$ T cell response.

Materials and Methods

Mice.

Bim-/- mice on the C57BL/6J background were obtained from Dr. Karen Fortner and were generated as previously described (6). C57BL/6J mice and OT II TCR transgenic mice (C57BL/6-Tg(TcraTcrb)425Cbn), which produce $CD4^+$ T cells responsive to the peptide $ova_{323-339}$, an immunodominant MHC II antigenic epitope from the protein ovalbumin, were purchased from Jackson Laboratories (Bar Harbor, ME) and bred at the University of Vermont. Mice were housed in an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-approved facility, maintained on a 12h light/dark cycle, and provided food and water *ad libitum*. All animal studies were approved by the University of Vermont Institutional Animal Care and Use Committee.

Bone marrow-derived dendritic cells.

Bone marrow was flushed from the femurs and tibiae of C57BL/6 mice and cultured on 6-well plates at 1 x 10^6 cells/well (3 ml/well) in RPMI-1640 containing 10% serum and 5% conditioned media from X63-GMCSF myeloma cells transfected with murine GM-CSF cDNA (kindly provided by Dr. Brent Berwin, Dartmouth College). Media was replaced on days 2 and 4 and the adherent and lightly-adherent BMDCs, predominantly $CD11b^+CD11c^+$ by FACS, were collected on day 6. For serum starvation, BMDCs were plated at 1 x 10^6 cells/ml, washed with DPBS, and maintained in RPMI-1640 without serum, in the presence or absence of 1 μg/ml apo-SAA (Peprotech, Rocky Hill, New Jersey). As indicated, BMDCs were visualized on tissue culture plates by light microscopy using a 20X objective on a Nikon Eclipse TS100 inverted microscope and images were acquired using a Nikon/Leica 38mm Iso Port camera.

Flow cytometric analysis of apoptosis. Cells were labeled for DNA breaks and assessed by flow cytometry using the In Situ Cell Death Detection Fluorescein kit (Roche Diagnostics, Indianapolis, IN). Cells were analyzed on an LSR II FACS flow cytometer (BD Biosciences, San Jose, CA) equipped to distinguish as many as seven fluorophores 1–3 d following staining, and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Enzymatic activity assessment.
Cell-free supernatants from BMDCs were analyzed for the presence of lactate dehydrogenase using the Cytotox 96 Non-Radioactive Cytotoxicity Kit (Promega, Madison, WI). Cell lysates were collected in NP-40 buffer, and 50 μg of total protein was used to analyze the presence of cleaved caspase-3/7, utilizing the Caspase-Glo 3/7 Assay (Promega).
RT-qPCR.

Following washing in DPBS, RNA from BMDC was isolated using the PrepEase RNA Spin Kit (Affymetrix, Santa Clara, CA) and reversed transcribed to cDNA using the iScript kit (Bio-Rad, Hercules, CA). Primers were designed for mouse *Bim* (forward: CTACAGACAGAACCGCAAGGT; reverse: CCTGAGACTGTCGTATGGAAG), and *HSP70* (forward: ATCACCATCACCAACGACAAGG; reverse: TGCCCAAGCAGCTATCAAGTGC) (13), and quantitative PCR was performed on cDNA using iQ SYBR Green Supermix (Bio-Rad). To normalize cycle threshold (C_T) values, *Gapdh* was analyzed using an Assay-On-Demand primers and probe cocktail (Applied Biosystems, Foster City, CA) and iQ Supermix (Bio-Rad), and calculations were made using the $\Delta\Delta C_T$ method, as previously described (14).

Western blotting.
Cell lysates were collected in NP-40 buffer, total protein was quantitated using the Bradford method (Bio-Rad), and 30 μg of total protein was loaded onto 4-20% gradient Tris-Glycine precast gel (Bio-Rad). Gels were transferred to nitrocellulose membranes using the iBlot system (Life Technologies, Carlsbad, CA). Blots were probed with anti-HSP70 (Enzo Life Sciences, Farmingdale, NY), anti-Bim (Thermo Scientific, Waltham, MA) and anti-β-actin (Sigma-Aldrich, St. Louis, MO) primary antibodies and HRP-conjugated secondary antibodies (Thermo Scientific, Rockford, IL). Bands were visualized using enhanced chemiluminescence (Thermo Scientific) and exposure of blots to X-ray film.

Cytokine analysis.
Cytokines from cell supernatants were analyzed by ELISA for IL-1β and TNF-α (BD Biosciences), IL-6 (R& D Systems, Minneapolis, MN), and SAA3 (Millipore, Billerica, MA). A customized Milliplex assay was used to measure IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-21, IL-22, and IFNγ (Millipore).

OT II CD4⁺ T cell co-culture studies.
CD4⁺ T cells from OT II transgenic mice were isolated from spleen and peripheral lymph nodes by magnetic negative selection (Stem Cell Technologies, Vancouver, BC, Canada) and were co-cultured at 1 x 10^6 cells/ml in a 96-well plate with adherent BMDC that had been plated and serum-starved for 48 hours in the presence or absence of 1 μg/ml apo-SAA. At the time of CD4⁺ T cell addition, cells were also treated with whole OVA (100 μg/ml, Sigma-Aldrich), and those receiving corticosteroid treatment were supplemented with 0.1 μM dexamethasone (Sigma-Aldrich). Cells were cultured together for 72 hours, at which time supernatants were collected, centrifuged, and snap-frozen for later analysis. In separate experiments, BMDC received either 20 μM Z-Val-Ala-Asp(OMe)-CH_2F (zVAD) (Millipore) or the HSP70 inhibitor (KNK437, an inhibitor of the transcription factor, Heat Shock Factor-1, which regulates expression of the *Hsp70* gene) (15) (Millipore) or 10 μg/ml anti-TNF-α neutralizing antibody (BD Biosciences) at the beginning of serum starvation.

Statistical analysis.
Data were analyzed by two-tailed unpaired student's t-test, a one-way ANOVA, or a two-way ANOVA using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Statistically significant results by ANOVA were further analyzed by Bonferroni post-hoc analysis (where indicated). A p value smaller than 0.05 was considered statistically significant.

Results

BMDC treated with apo-SAA are resistant to serum starvation-induced apoptosis. To more closely recapitulate the conditions encountered under homeostatic conditions, BMDC were cultured in serum-free media for up to 72 hours. Under this condition, untreated cells released lactate dehydrogenase (LDH) into the supernatant in increasing amounts over time (Fig 1A). In contrast, LDH secretion was reduced in serum-starved BMDC treated with apo-SAA (Fig 1A). Furthermore, visualization of the cells by light microscopy revealed a marked difference in cellular morphology over time, with the serum-starved apo-SAA-treated cells exhibiting more dendritic processes, whereas the untreated serum-starved cells were more rounded in appearance (Fig 1B). To assess early enzymatic markers of apoptosis, cleaved caspase 3 was analyzed from whole cell lysates at 6 hours. Caspase-3 activity was significantly reduced in apo-SAA treated cells compared to untreated controls (Fig 1C).

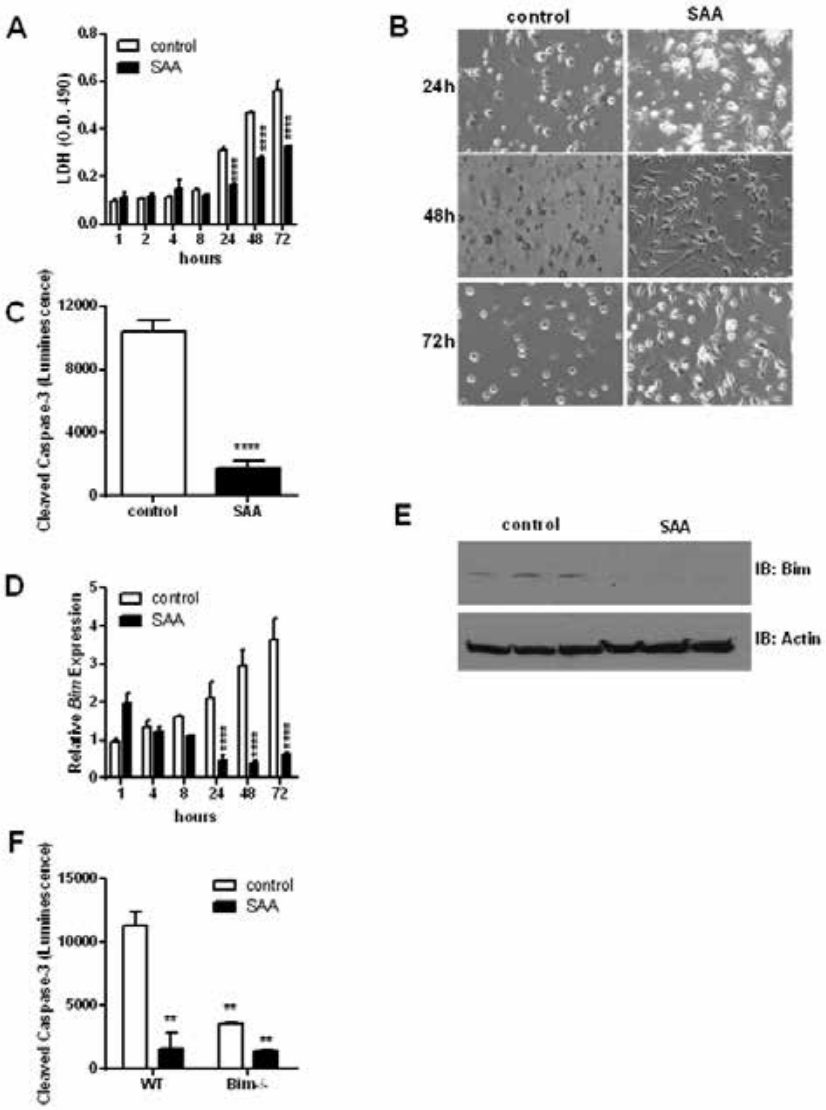

Figure 1. *apo-SAA inhibits Bim expression and protects BMDC from serum starvation-induced apoptosis*. A) LDH levels in supernatant from BMDC serum-starved in the presence (SAA) or absence (control) of 1 μg/ml apo-SAA for the indicated times. B) Light photomicrographs of BMDC in 12-well plates at 24, 48, and 72 hours post serum-starvation in the absence or presence of apo-SAA. C) Caspase-3 activity in BMDC serum starved for 6 hours in the presence or absence of apo-SAA. D) Time course of *Bim* expression in serum-starved BMDC compared to 1 hour control. E) Immunoblot (IB) for Bim and β-actin from 30 μg of whole cell lysate from BMDC that were serum starved for 24 hours in the presence or absence of apo-SAA. F) Caspase-3 activity in wild type (WT) and Bim-/- BMDC that were serum starved for 6 hours in the presence or absence of apo-SAA. n = 3-5 replicates per condition. ** = $p<0.005$, **** = $p<0.0001$ compared to control cells (or WT control, G) at the same timepoint.

apo-SAA treatment downregulates expression of the pro-apoptotic protein Bim. Apoptosis in BMDC that is caused by nutrient deprivation has been documented to rely upon the pro-apoptotic protein Bim (4). Therefore, BMDC were serum-starved for up to 72 hours and analyzed for mRNA abundance of a panel of pro- and anti-apoptotic genes. No differences were observed in the expression of the anti-apoptotic proteins *Bcl-2*, *Bcl-X_L*, and *TIAP* or the pro-apoptotic genes *Bad* and *Bax* as a consequence of apo-SAA stimulation in serum-starved BMDCs (data not shown). In contrast, whereas untreated serum-starved controls upregulated *Bim* expression over time, apo-SAA treated BMDC displayed marked *Bim* downregulation (Fig 1D). Western blot analysis at 24 hours confirmed the lack of Bim protein in apo-SAA treated BMDC (Fig 1E). To further demonstrate the role of Bim in the downstream activation of caspase-3, BMDC from Bim-/- mice were serum-starved for 6 hours and whole protein lysate was analyzed for caspase-3 activity. An absence of capase-3 activation was measured in the BMDC from the Bim-/- mice, both under conditions of serum starvation or when serum starved and treated with apo-SAA (Fig 1F). The absence of Bim leads to a lack of caspase-3 cleavage in BMDC that have been serum-starved, mimicking the effect observed when serum-starved BMDC were treated with apo-SAA.

HSP70 expression is critical for apo-SAA induced caspase-3 inactivation. Since the pro-survival protein HSP70 has been documented to cause dysfunction in apoptosis downstream of cytochrome *c* release from the mitochondria (16), we analyzed mRNA expression of *HSP70* in serum-starved BMDC. After treatment with apo-SAA, *HSP70* was strongly upregulated at 8 and 24 hours post treatment (Fig 2A). The increases in *HSP70* expression at these timepoints were reflected by increased protein production at the same times (Fig 2B). Addition of an HSP70 inhibitor (HSP70*i*), blocked mRNA expression of HSP70 both in control and in SAA-treated cells (2C) and also dose-dependently restored caspase-3 activation in serum-starved, apo-SAA treated BMDC (Fig 2D). Inhibition of HSP70 also induced an increase in TUNEL staining in apo-SAA-treated cells (Fig 2E). We next examined whether HSP70 modulates the capabilities of apo-SAA to induce pro-inflammatory cytokine production. BMDC that were serum-starved in the presence of apo-SAA showed a strong secretion of IL-6, TNF-α, and IL-1β into the cell-free supernatant after 24 hours (Fig 2F). The secretion of IL-6 and TNF-α was inhibited by the HSP70*i*; however, IL-1β was markedly increased in the presence of SAA and HSP70*i*.

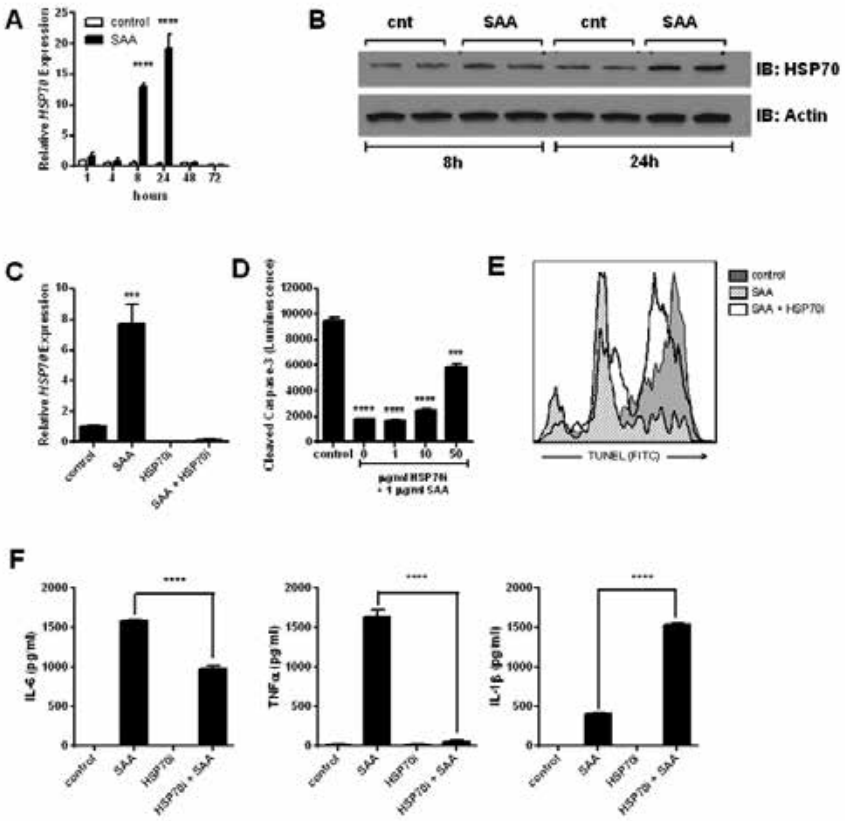

Figure 2. *apo-SAA-induced HSP70 modulates caspase-3 activity and is required for cytokine secretion.* A) Time course of *HSP70* expression in serum-starved BMDC exposed to 1 μg/ml apo-SAA (SAA) or not (control) compared to 1 hour control. B) Immunoblot (IB) for HSP70 and β-actin from 30 μg of whole cell lysate from BMDC serum starved for 8 or 24 hours in the presence (SAA) or absence (control) of apo-SAA. C) mRNA expression of HSP70 in cells serum starved for 8 hours after treatment with apo-SAA (SAA), 25 μg/ml HSP70 inhibitor (HSP70*i*), or both. D) Caspase-3 activity in BMDC that were serum starved for 6 hours in the presence or absence of apo-SAA, +/- 25 μg/ml HSP70 inhibitor (HSP70*i*). E) Assessment of DNA strand breaks by TUNEL assay in serum-starved BMDC in the presence or absence of apo-SAA, +/- HSP70*i* after 72 hours. F) IL-6, TNF-α, and IL-1β levels from supernatants of BMDC that were serum starved for 24 hours +/- apo-SAA, +/- HSP70*i*. n = 3-6 replicates per condition. *** = $p<0.005$, **** = $p<0.0001$ compared to control (or compared to SAA in 2F).

BMDC treated with apo-SAA drive a pro-inflammatory CD4⁺ T cell response that is resistant to dexamethasone. Dysfunction in dendritic cell apoptosis can result in an inappropriate inflammatory response from T cells (17). We have previously demonstrated that BMDC treated with apo-SAA upregulate MHC II and co-stimulatory cell surface molecules and can readily induce OT II CD4⁺ T cells to secrete IL-17 in the presence of OVA (10). Here, we investigated the OT II CD4⁺ T cell

responses to BMDC that had been serum-staved for 48 hours in the presence or absence of apo-SAA. apo-SAA-treated BMDC induced CD4+ T cells to secrete enhanced amounts of the T_H17 cytokines IL-17A, IL-17F, IL-21, and IL-22, whereas they did not enhance the production of the T_H2 cytokine IL-13, and only marginally increased the levels of the T_H1 cytokine IFNγ (Fig 3). Treatment of the serum-starved BMDC co-cultures with the corticosteroid dexamethasone (Dex) at the time of CD4+ cell stimulation decreased the production of nearly all cytokines measured (Fig 3). However, pre-treatment of the BMDC with apo-SAA blocked steroid responsiveness; apo-SAA was still able to induce secretion of IFNγ, IL-17A, IL-17F, and IL-21 (Fig 3). Only the production of IL-13 and IL-22 remained sensitive to the Dex treatment. It is interesting to note that Dex did not diminish control levels of IL-21, and in fact *enhanced* its secretion in the presence of apo-SAA. These results were also specific to the BMDC-CD4+ T cell co-culture system; apo-SAA treatment of polyclonally stimulated T cells alone did not enhance cytokine production beyond control levels, and neither did it induce the CD4+ cells to be refractory to Dex (data not shown). Furthermore, the addition of a TNF-α-neutralizing antibody to the co-culture system had no effect on OVA-induced T-cell cytokine production or to the Dex sensitivity of the CD4+ T cells (data not shown).

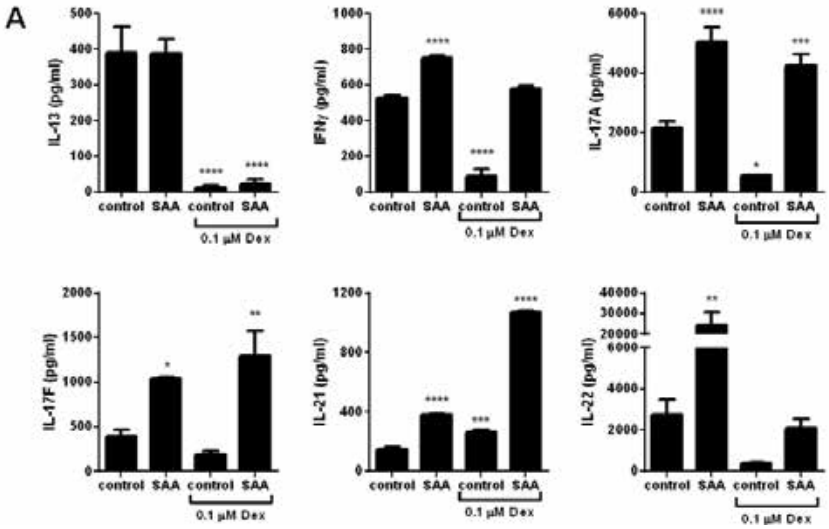

Figure 3. *BMDC serum starved in the presence of apo-SAA can induce T_H17 cytokine secretion from OT II CD4+ T cells that is resistant to dexamethasone.*

BMDC were serum starved for 48 hours in the presence (SAA) or absence (control) of 1 μg/ml apo-SAA prior to co-culture with OT II CD4+ T cells and OVA, +/- 0.1 μM Dex. Supernatants from co-cultures were collected 72 hours later and analyzed for IL-13, IFNγ, IL-17A, IL-17F, IL-21, and IL-22. (IL-4 and IL-5 were undetectable in supernatants.) n= 3-5 replicates per condition. * = p< 0.05, ** = p<0.01, *** = p<0.005, **** = p<0.0001 compared to control.

Caspase-3 inhibition is sufficient to induce IL-17A and IL-22 production in CD4⁺ T cells. In a recent review, Martin *et al*. discussed the emerging hypothesis that caspase-3, rather than controlling cell fate in apoptosis, is responsible for modifying endogenous cell proteins to limit the inflammatory capacity of damage-associated molecular patterns (DAMPs) upon release from the dying cell (18). Since apo-SAA caused marked diminution of caspase-3 activation, which could lead to an increase in the inflammatory potential of cell DAMPs, we sought to determine whether caspase-3 inhibition itself would be sufficient to enhance

CD4⁺ T cell activation and induce corticosteroid resistance. However, Bim deficiency in DC itself was not sufficient to induce corticosteroid resistance in CD4⁺ T cells (Fig 4A) and neither did serum-starved Bim-/- cells produce IL-1β or TNF-α without stimulation (data not shown). In addition, wild type BMDC were serum starved for 48 hours in the presence or absence of the pan-caspase inhibitor zVAD, prior to co-culture with OT II CD4⁺ T cells and OVA. As presented in Figure 4B, zVAD-treated cells upregulated IL-17A, IL-21, and IL-22. It should be noted that the levels of IL-17A induced by zVAD (1729.7 ± 348.5 pg/ml) are not as high as those induced by SAA treatment (5038.0 ± 501.0 pg/ml, Fig. 3) and the same is true for IL-22 (3404.3 ± 1059.9 pg/ml in response to zVAD, and 24,459.5 ± 5975.5 pg/ml in response to SAA). Additionally, zVAD treatment was not sufficient to induce corticosteroid insensitivity; unlike with the apo-SAA treatment, dexamethasone substantially inhibited the production of all cytokines measured, except for IL-21 (Fig 4B). These results indicate that blockade of caspase-3 activation alone in BMDC is insufficient to induce corticosteroid resistance from CD4⁺ T cells. Figure 4B also demonstrates an overall additive effect of SAA and zVAD treatment together (except with IL-22, where it does not differ in response to treatment with control or zVAD alone). It would appear that the SAA-induced effects leading to cytokine production are further augmented by caspase inhibition.

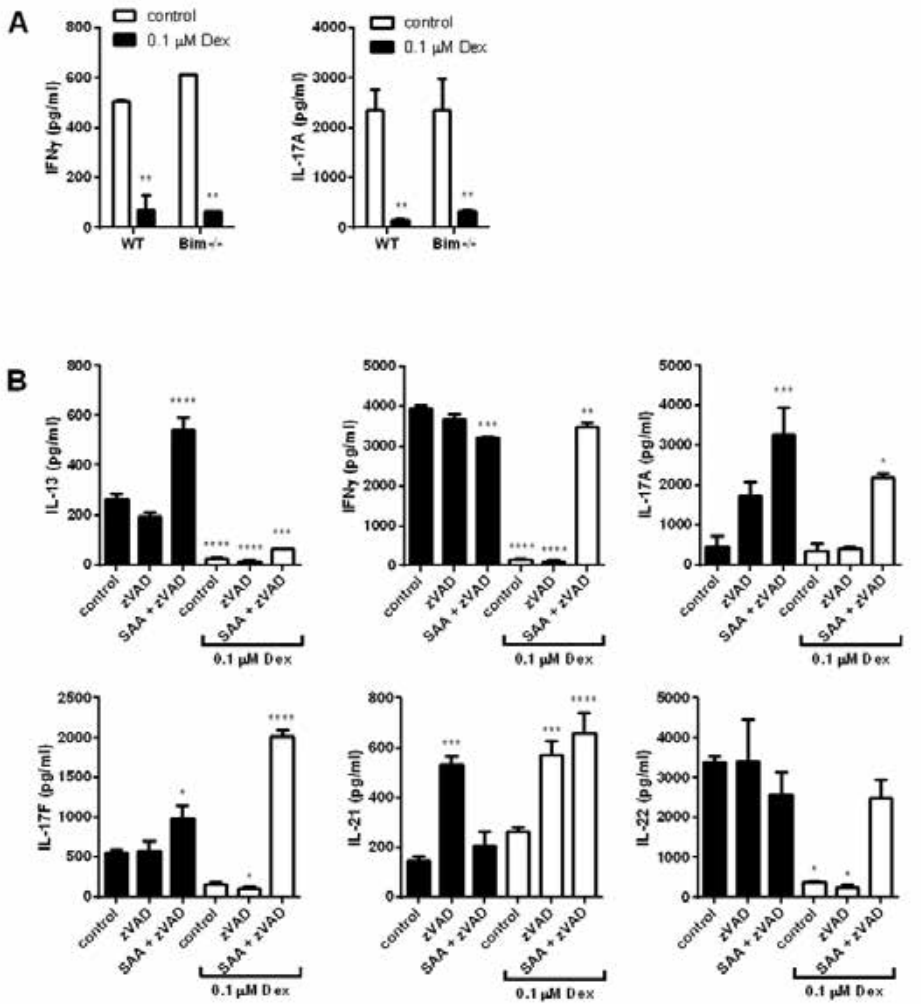

Figure 4. *Caspase-3 inhibition is not sufficient to induce dexamethasone resistance.* A) BMDC from WT or Bim-/- mice were serum starved for 48 hours prior to co-culture with OT II CD4+ T cells and OVA, +/- 0.1 μM Dex. Supernatants from co-cultures were collected 72 hours later and analyzed for IFNγ and IL-17A. B) BMDC from WT mice were serum starved for 48 hours in the presence or absence of 20 μM zVAD prior to co-culture with OT II CD4+ T cells and OVA, +/- 0.1 μM Dex. Supernatants from co-cultures were collected 72 hours later and analyzed for IL-13, IFNγ, IL-17A, IL-17F, IL-21, and IL-22. (IL-4 and IL-5 were undetectable in supernatants.) n= 3-5 replicates per condition. * = p< 0.05, ** = p<0.01, *** = p<0.005, **** = p<0.0001 compared to control.

HSP70 expression is not necessary for SAA- inducted production of IL-17A and IL-17F from OT II CD4+ T cells, but is required for corticosteroid resistance. Heat shock proteins, including HSP70, can function as DAMPs to exert cytokine-like effects on DCs and encourage autoimmune disease (19). In addition, HSP70 comprises part of the

chaperone protein complex that governs the folding and cellular localization of the glucocorticoid receptor (GR) (20-22). Since apo-SAA potently induced the upregulation of HSP70, we explored the possibility that this protein played a role in cytokine release and steroid insensitivity in our BMDC/CD4+ T cell co-culture system. Therefore, BMDC were serum starved for 48 hours in the presence or absence of apo-SAA, alone or with an HSP70 inhibitor (HSP70*i*). Inhibition of HSP70 blocked production of IL-13, IFNγ, IL-21, and IL-22, both in controls and in apo-SAA-treated cells (Fig 5). IL-17A and IL-17F were still strongly induced by apo-SAA in the presence of HSP70*i*, suggesting a differential regulation of these cytokines. However, when the experiment was conducted in the presence of Dex, the corticosteroid insensitivity induced by apo-SAA treatment disappeared across the board (Fig 5A), suggesting that HSP70 was indeed required for CD4+ T cell steroid resistance in this model.

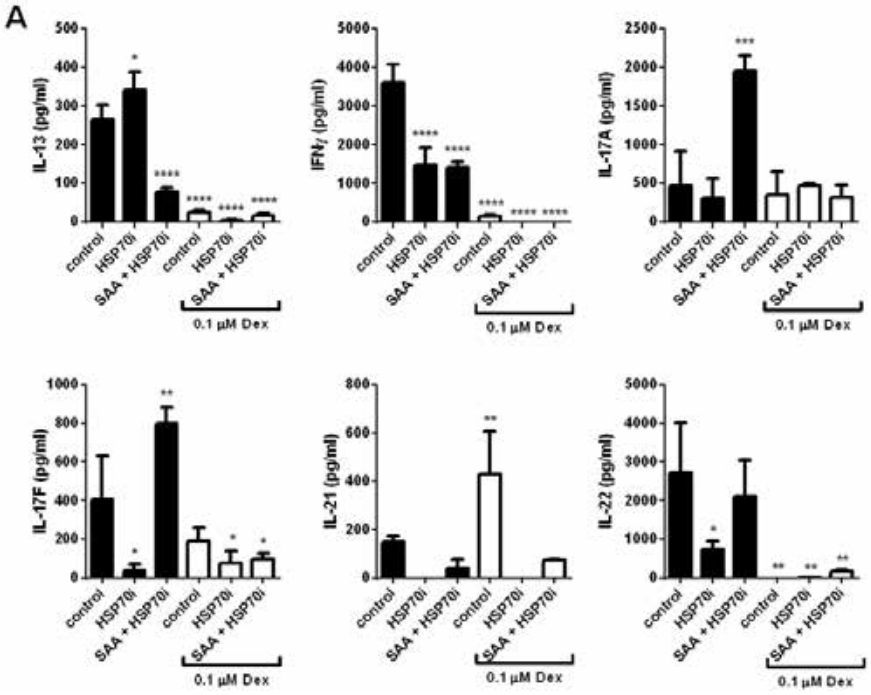

Figure 5. *HSP70 is required for dexamethasone-resistance of apo-SAA-induced T_H17 cytokine secretion.* BMDC were serum starved for 48 hours in the presence (SAA) or absence (control) of 1 μg/ml apo-SAA, +/- 25 μg/ml HSP70*i*, prior to co-culture with OTII CD4+ T cells and OVA, +/- 0.1 μM Dex. Supernatants from co-cultures were collected 72 hours later and analyzed for IL-13, IFNγ, IL-17A, IL-17F, IL-21, and IL-22. (IL-4 and IL-5 were undetectable in supernatants.) n= 3-5 replicates per condition. * = $p< 0.05$, ** = $p<0.01$, **** = $p<0.0001$ compared to control.

Discussion

Recent studies have highlighted the importance of apoptosis not only in the clearance of dying cells, but also in the removal of cellular proteins such as HSPs, HMGB1, and S-100 proteins (18) that can function as DAMPs outside of the cell (23). Apoptotic processes active under homeostatic conditions, therefore, protect the organism from endogenous inflammatory stimuli and also assist in the resolution of the inflammatory response.

Serum amyloid A has long been implicated as a dangerous pre-cursor protein that can aggregate in tissue and lead to amyloid plaques, mostly notably in systemic amyloidosis and Alzheimer's disease (11, 24). In a previous publication, we have explored the inflammatory potential of recombinant apo-SAA *in vitro* and in a mouse model of allergic airways disease, implicating SAA as a DAMP that induces NLRP3 inflammasome activation, IL-1β production, and asthma-like disease with a mixed T_H2/T_H17 response in mice (10). Furthermore, we have demonstrated the importance of the NLRP3/ASC/Caspase-1 inflammasome and IL-1 responsiveness in order to induce IL-17A production by apo-SAA (10). Here, we have more closely explored the effect of apo-SAA specifically on dendritic cells, and found that it can increase DC lifespan, downregulate *Bim* expression and caspase-3 activity while upregulating HSP70, and that this unique intracellular DC milieu induces antigen-specific $CD4^+$ T cells to secrete T_H17 cytokines that are resistant to corticosteroid treatment.

Much of the research on the mechanism of glucocorticoid activity has focused specifically on T cells, which undergo apoptosis in a Bim-dependent manner upon treatment with corticosteroids such as Dex (25). Glucocorticoids pass through the cell membrane in order to bind to the GR, which resides in the cytosol in the company of a chaperone protein complex that includes HSPs. These molecular chaperones are shed from the receptor once ligand binding occurs, and this reveals the nuclear localization sequence that allows the GR to migrate to the nucleus and bind to glucocorticoid response elements (GREs) on DNA, thereby modulating gene function directly (21, 25). Our *in vitro* co-culture system is intended to model interactions between DCs and CD4+ T cells as they occur *in vivo*, a situation in which both cell types are exposed to administered corticosteroids. As the scope of the studies presented herein were limited to analyzing effects of apo-SAA only in BMDC, it remains an important objective of future studies to explore the indirect effects of corticosteroids specifically on the T cells, and to determine how the apo-SAA-treated DC are influencing the ligand-binding and processing of the GR in $CD4^+$ T cells. In addition, since T cell viability may be affected by Dex, reduced numbers of live cells could account for the decreases in cytokine production. Nonetheless, the capacity for SAA to induce a DC phenotype that permits $CD4^+$ T cell cytokine production, even in the presence of inhibitory concentrations of Dex,

remains a significant finding. The alterations in metabolism, the cell surface molecules expressed, as well as the mediators, including gasses such as reactive oxygen and nitrogen species, lipids such as PGE_2, and cytokines, released by apo-SAA-activated BMDCs (10, 26), are all candidates for affecting corticosteroid responsiveness of $CD4^+$ T cells. In addition, it is of special interest how BMDC-induced HSP70 plays a role in this process, as it was clearly shown to be critical in inducing corticosteroid resistance in our model.

The differential expression and regulation of the $CD4^+$ T cell cytokines produced as a consequence of BMDC exposure to apo-SAA is also of interest. apo-SAA induced the robust secretion of IL-17A and IL-17F from $CD4^+$ T cells. In mouse models, IL-17A is capable of promoting neutrophilic asthma and exacerbating allergic airway disease (27). In addition, mice unable to respond to IL-17A or IL-17F do not develop allergic airway disease in several models (27, 28). Finally, adoptive transfer of *in vitro*-polarized $CD4^+$ T cells secreting IL-17A induce corticosteroid-insensitive allergic airway disease following antigen challenge (29). BMDC that were pre-treated with apo-SAA were able to induce staggering amounts of IL-22 from $CD4^+$ T cells, to an extent not seen in our other models. T cells from HIV-1 resistant patients produced both large amounts of IL-22 and an acute-SAA cleavage product that downregulated cell surface expression of CCR5 and rendered cells more resistant to HIV-1 viral infection (30). Other reports have revealed that IL-22 is a critical instigator of lung damage, reducing pulmonary function in *Aspergillus fumigatus* models of allergic airways disease (31), and also that IL-22, IL-17A and IL-17F, can each induce proliferation of human airway smooth muscle cells (32). Our findings revealed that IL-21 secretion appeared to be differentially regulated from the T_H17 cytokines measured. IL-21 production was enhanced by Dex treatment (Fig 3), induced by caspase-3 inhibition alone (Fig 4B) and blocked by inhibition of HSP70 (Fig 5). IL-21 is closely related to IL-2, though functionally distinct, as IL-21 promotes the differentiation of T_H17 $CD4^+$ T cells and appears to be involved in autoimmune pathologies (33-35). Previous studies have also implicated IL-21 as a Dex-resistant cytokine (36). The role of HSP70 in IL-21 induction has not previously been published, although it has been demonstrated that HSP70 can activate transcription factors such as NF-κB and stimulate the release of other cytokines such as IL-6, IL-1β, and TNF-α. Our current study agrees that HSP70 plays a role in the modulation of these cytokines in response to apo-SAA treatment of BMDC (Fig 2E). Previously, we have demonstrated that HSP70 is released into the lavageable airspaces of mice exposed to the pollutant nitrogen dioxide (NO_2) (14) and may contribute to the ability of NO_2 to induce DC maturation (37) and allergic sensitization (38). It is possible that HSP70 executes multiple functions in our system: as a pro-survival and pro-inflammatory cytokine as well as a GR chaperone.

The studies presented herein reveal that an endogenous protein, SAA, can induce antigen-presenting cells to create a pro-inflammatory environment that is resistant to apoptosis, and therefore, resistant to resolution of the inflammatory state. This in turn drives production of T_H17 cytokines from $CD4^+$ T cells in response to antigen, a response that is insensitive to corticosteroids, the first line of therapy for the treatment of myriad inflammatory diseases, including asthma. Although further studies are required to define the precise mechanism of glucocorticoid insensitivity in $CD4^+$ T cells, the chaperokine HSP70 appears to play a key role, and modulation of this protein may provide a method by which to circumvent corticosteroid resistance in allergic, autoimmune, and inflammatory diseases.

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CHAPTER 8

Discussion and Future Directions

NF-κB in the Airway Epithelium

The role of NF-κB as a widely-expressed transcription factor that initiates inflammatory responses has made it of great interest to those studying allergic airway disease. Recent reviews have highlighted the importance of NF-κB in lung pathogenesis (1-3), and the studies presented in this thesis have contributed in a meaningful way to the identification of airway epithelial NF-κB as an orchestrator of allergic sensitization. In Chapters 2 and 4, the data presented demonstrate that inducible NF-κB activation in the airway epithelium of our transgenic mouse model implicate that production of NF-κB-dependent cytokines and chemokines, particularly SAA, GM-CSF, IL-1β, CCL20 and IL-6, activate antigen-presenting dendritic cells and drive a mixed T_H2/T_H17 phenotype of allergic airway disease; and as seen in Chapter 6, this disease manifests regardless of prior tolerance to an innocuous inhaled antigen. The consequences of airway epithelial NF-κB activation induce the activation of neighboring DCs, which in turn qualitatively modulate the cytokine production from $CD4^+$ T cells as well as their sensitivity to glucocorticoid treatment.

Inhibition of NF-κB in various disease states has had mixed results (4). Modulation of NF-κB activation has broad consequences, perhaps too broad for its inhibition to serve as a solution for the treatment of allergic asthma. Though it acts as an instigator of inflammation, NF-κB regulated genes also have important roles in tissue repair and homeostatic function (3). The wide spectrum of activities of NF-κB regulated proteains indicates that more focused targets for therapeutic intervention will be required.

Innate Lymphoid Cells in the Lung

The classic understanding of cytokines such as IL-4, IL-5, IL-13, and IL-17, which are important in generating the hallmark symptoms of allergic asthma, is that they are the products of polarized $CD4^+$ T cells. However, the identification of innate lymphoid cells (ILCs) as sources of the aforementioned cytokines has altered this dogma. ILCs have lymphoid morphology, but lack rearranged antigen receptors (TCR) and other classical T cell markers such as CD3, CD4 and CD8. Several classes of ILCs have been elucidated that produce a repertoire of cytokines very similar to those produced by $CD4^+$ T cells, but in a much more rapid and innate context (5, 6). ILCs are similar to NK cells, and may arise from some of the same progenitors – progenitor cells containing the transcription factor Id2 appear to be critical to both NK cells and ILC2 cells, for example (6) - and are not only important in the innate immune response, but are also crucial to lymphoid tissue development, tissue repair, and homeostasis (5).

	ILC1	ILC2	ILC3 (IL22)	ILC3 (IL17)
Locations	Intestinal mucosa, tonsils	Lung, adipose tissue, LNs, peripheral blood.	Mucosal tissues	Mucosal tissues
Functions	Protective against colitis	Protective against helminth infection.	Protective against tissue damage in allergic airway disease. Anti-inflammatory in intestine.	Protective against fungal infection.
Cell surface markers	CD127, IL12Rβ1	CD90, CD25, Ly6A, CD117, ST2	CD90, CD25, CD117, NKp46	CD90, CD25, CD117, NKp46
Transcription Factors	Tbet	GATA3, Rorα	RORγt (Tbet)	RORγt
Require:	IL-12, IL-15	IL-33, IL-25, TSLP	IL-23, IL-1β	IL-23, IL-1β
Produce:	IFNγ	IL-5, IL-13	IL-22 (IFNγ)	IL-17A

Figure 1. An overview of the known ILC populations in mice.

The classes of ILCs that have thus far been examined are reminiscent of $CD4^+$ T cell T_H1, T_H2, and T_H17 lineages, and a summary of the currently known locations, functions, and cell surface markers of these cells in mice is presented in Figure 1. ILC1 cells are similar to T_H1 $CD4^+$ T cells, in that they produce large amounts of IFNγ (7, 8). ILC2 cells (for a brief time termed "nuocytes") mimic T_H2 function (9, 10); they respond strongly to IL-25 and IL-33, and in response to these cytokines produce IL-5 and IL-13 (11). The ILC3 family is divided into two subsets; one that expresses RORγt, produces IL-22 (especially in the gut) and resides in mucosal tissue (12), and another, that also expresses RORγt and is a major source of IL-17A; these cells appear to drive pathology in colitis models and in Crohn's Disease patients. Recent studies have begun to reveal the importance of ILCs in lung protection and pathology. ILC2 cells accumulate rapidly in the lung following H1N1 infection, where selective depletion of ILC2s using an anti-CD90 antibody demonstrated a critical role for ILC2s in protecting barrier integrity of the airway epithelium (13). ILC2 cell are also important in models of allergic airway disease (14, 15), as ILC2s accumulate in the lungs of mice in house dust mite – and OVA-induced asthma models and act as prime sources of IL-5 and IL-13 (14). ILC3 cells are likewise important in allergic airway disease models (16), and also have protective functions against tissue damage in allergic airway disease (16) and against fungal infections (17).

Though there seems to be a degree of plasticity between ILC subsets, one finding is clear; these cells contribute to the inflammatory responses in the lung in allergic asthma models and may serve as an important source for cytokines that have traditionally been implicated as the product of $CD4^+$ T cells. As our transgenic CAIKKβ mice rapidly upregulate polarizing cytokines, it will be of great importance to determine if these cytokines are having an effect on the recruitment and activa-

tion of ILCs, and to elucidate what role ILCs are playing in allergic sensitization induced by our CAIKKβ model. Of particular interest is the source of IL-4 that is so critical to $CD4^+$ T cell polarization to T_H2, as seen in our in vitro experiments from Chapter 6 (Figure 6B). It is still up for debate whether ILCs produce IL-4 (11), though it is well documented that IL-4 is critical for T_H2 polarization (18, 19).

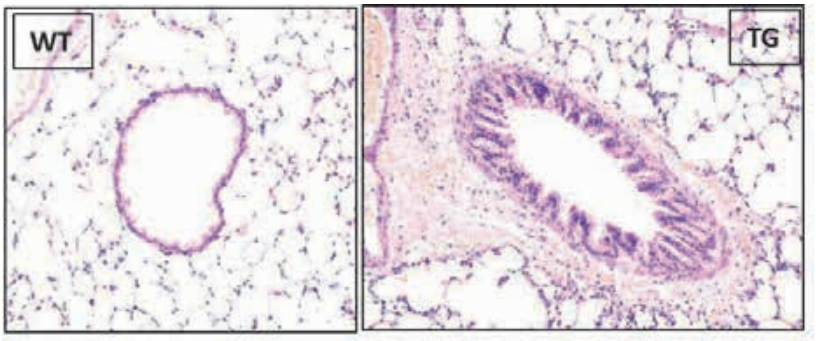

Figure 2. Morphological changes in the airways of CC10-SAA3 transgenic (TG) mice compared to wild type (WT) littermates following 10 weeks of transgene induction. 20X visualization of representative H&E stained lung sections.

Serum Amyloid A

Most recently, our work has ventured away from the use of the commercially available recombinant human apo-SAA, used in Chapters 5 and 7. We have generated a transgenic mouse expressing a CC10-promoter- regulated, Dox-inducible form of SAA3 (CC10-TetOp-SAA3), that secretes SAA3 from non-ciliated airway epithelial cells. Preliminary studies have shown that long term administration of Dox (~10 weeks) results in morphological changes in the airways and pulmonary endothelium of transgenic mice compared to wild type littermates (Figure 2), concurrent with increases in BAL protein and LDH release, and an increase in gene expression of matrix metalloproteinase-9 (MMP-9) (Figure 3). These SAA3-induced changes in airway epithelial barrier function may account for observed increases in parameter G (heterogeneity) in response to inhaled methacholine challenge (Figure 4). In addition, we have begun to utilize these mice in several models of allergic airway disease, including the Alum/OVA model, in which the sensitized CC10-TetOp-SAA3 mice recruit neutrophils (as well as eosinophils) into the airways following antigen challenge, and this neutrophilia appears to be resistant to glucocorticoid treatment (data not shown). Further studies will address the extent of steroid resistance in these mice, as well as examine barrier function alterations and airway remodeling.

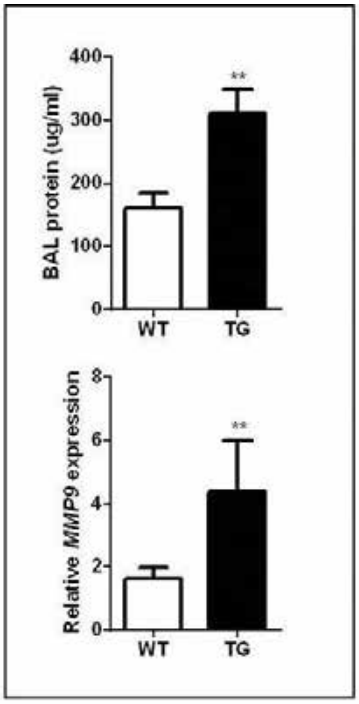

Figure 3. Total protein from BAL and MMP9 gene expression from the whole lung of wild type (WT) and CC10-SAA3 transgenic (TG) mice following 10 weeks of transgene induction.

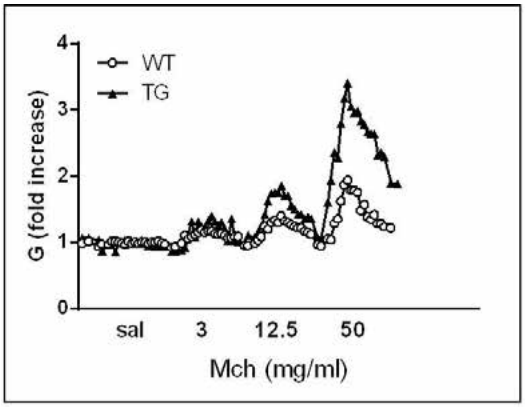

Figure 4. Parameter G (heterogeneity) in response to methacholine challenge in CC10-SAA3 transgenic (TG) vs. wild type (WT mice following 10 weeks of transgene induction.

In addition to over-expressing SAA3 in the airway, we have also generated a global SAA3-/- mouse, and are currently breeding to extend our population in order to utilize these mice in preliminary studies. With these two genetically modified mouse strains, we can begin to address steroid refraction in an *in vivo* context. Additionally, we can address whether endogenously produced SAA3, specifically, is required for NLRP3 inflammasome activation and function.

The NLRP3 Inflammasome

Since it was first described roughly a decade ago, studies of the NLRP3 inflammasome have emerged rapidly, and not without significant debate over the findings. What we do know from these respective studies is that endogenous damage associated molecular patterns (DAMPs) have the same ability as those molecules produced by pathogens (PAMPs) to prime and activate the immune system and generate innate and adaptive responses. This is of particular importance for auto-

immune disorders as well as for asthma, and may help explain why certain individuals become allergically sensitized to harmless proteins, when most others do not. Intervention at the inflammasome level may provide a valuable treatment opportunity; and the reduction in IL-1β may prove crucial in controlling asthmatic T_H17, steroid-refractory phenotypes. The SAA-induced allergic sensitization model utilized in Chapter 5 proved to be dependent upon IL-1 signaling, providing another potential target, downstream of the initial NF-κB activation, at which therapeutic intervention may be advantageous. In addition, Chapter 7 demonstrated a role for HSP70 in the responses to apoSAA in dendritic cells. It is of note that other groups have linked another heat shock protein, HSP90, to inflammasome function (20). Specifically, immunoprecipitation studies have demonstrated that HSP90 associates with NLRP3, and also that not only is HSP90 essential to NLRP3 function, but HSP90 is also critical to the function of the Nod2 and IPAF inflammasomes as well (20). Though we have yet to investigate the role of HSP70 in NLRP3 inflammasome activity, a preliminary immunoprecipitation study indicates that HSP70 and NLRP3 do interact *in vitro* in dendritic cells exposed to SAA (Figure 5).

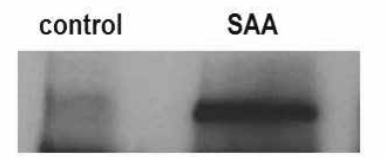

Figure 5. Mouse BMDCs treated for 24 hour ± SAA. Cell lysates were immunoprecipitated with anti-HSP70, and immunoblotted for NLRP3.

Glucocorticoid Resistance in Asthma.

There are multiple mechanisms modulating glucocorticoid responsiveness, including transcriptional expression of the glucocorticoid receptor (GR), phosphorylation status of the GR, and the ability of the GR to translocate to the nucleus upon ligand binding. GC insensitivity can result from changes at all three of these checkpoints. In allergic asthma, GC insensitivity has been associated with a more severe, T_H17 phenotype (21), and studies implicate that this can be due to a reduced affinity of the GR for glucocorticoids, or a reduced overall expression of the GR (22, 23). Recent *in vitro* studies have also highlighted the importance of the GR isoform, demonstrating that certain isoforms (i.e. GR-C) have a greater apoptotic potential, specifically with greater downstream expression of *Bim*, than others (24).

Historically, the focus of problems with the GC response has centered on the target cells themselves; specifically, the ability (or lack thereof) of GC treatment to cause apoptosis in neutrophils (25) and $CD4^+$ T cells (24), leading to a dampening of inflammation. Less is known about the effect of GCs on dendritic cells. The studies presented in Chapter 7 indicate a critical role for DCs in initiating steroid-refractory T cell cytokine production. Clearly, future work will need to determine exactly what changes in the DCs can account for downstream GC-resistant T cell responses. Studies have reported that *in vitro* polarized T_H17 $CD4^+$ T cells are non-responsive to dexamethasone, and adoptive transfer of these cells into mice results in Dex-resistant inflammation and AHR (22). Interestingly, these studies indicated that T_H17 cells express the GR normally, and that nuclear translocation and DNA binding were all intact (22). This lack of a GR defect in the polarized T cells may indicate another mechanism of action, perhaps produced by the polarizing DCs, that is responsible for glucocorticoid resistance in T_H17 cells.

Concluding Remarks

The multi-faceted manifestations of asthma, from the varying symptoms, the broad range of onset age, and the variety of adaptive $CD4^+$ T cell responses, ensure that there will be no "quick fix" treatment that will work for all patients. Genetic predispositions and pathogenic infections that predicate allergic sensitization are compounded by the effects of cigarette smoking, environmental pollutants, and the rising epidemic of obesity. Only as we further understand the individual, innate responding mediators that have both exacerbating and polarizing effects, can we design new treatments for individual subsets of patients. The work presented in this thesis examines the effects of NF-κB activation specifically in the airway epithelium and explores the NF-κB-induced acute mediator SAA in allergic sensitization, inflammasome activation, and T_H17 polarization. Through several mechanisms studied in this thesis, modulation of SAA expression presents a potential treatment for diminishing the inflammation associated with allergic airway disease. Likewise, targeted inhibition of SAA-induced HSP70 may increase the efficacy of glucocorticoid treatment in severe asthmatics.

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CHAPTER 9
Summary

The generation of allergic airway disease requires specific interactions between the initial responder cells (the epithelium), the underlying antigen presenting cells (dendritic cells) and naïve T cells. Chapter 1 provides an in depth literature review exploring the complex relationships between these cells in the lung, and how an inflammatory milieu instigated by one cell type can lead to aberrant adaptive immune responses that manifest in allergic airway disease.

Airway epithelial NF-κB activation modulates airway inflammation and acute lung injury in mice.

Chapter 2 documents the characterization of a novel transgenic mouse expressing a CC10-specific, Doxycycline-inducible, constitutively active form of IKKβ (the CAIKKβ mouse). Following Dox administration, these mice activate NF-κB in the airway epithelium, resulting in the recruitment of neutrophils into the lung, upregulation of inflammatory cytokines and chemokines, induction of airway hyperresponsiveness in response to methacholine challenge, and concurrent airway smooth muscle thickening. Chapter 3 examines the role of airway epithelial NF-κB in a model of NO_2-induced acute lung injury. We determine that the phenotype induced in the CAIKKβ mouse after 1 week of Dox administration is similar to the acute lung injury seen in mice exposed to 25ppm of nitrogen dioxide (NO_2) for 6 hours a day for 3 days. NO_2-induced acute lung injury also requires NF-κB activation, as indicated by the results demonstrating that CC10-IκBα$_{SR}$ mice, in which airway epithelial NF-κB expression is repressed, have an ameliorated inflammatory response to NO_2 (except at very high NO_2 doses).

Airway epithelial NF-κB activation is sufficient to induce allergic sensitization to an innocuous inhaled antigen in mice.

Chapter 4 demonstrates that the inflammatory milieu generated by airway epithelial NF-κB activation allows for allergic sensitization to an innocuous inhaled antigen. In this chapter, we begin to elucidate the effect of airway epithelial NF-κB activation on nearby cells: specifically, dendritic cells (DCs). Soluble mediators released by the airway epithelium allow for DC maturation, activation, and effective antigen presentation to $CD4^+$ T cells, resulting in a mixed T_H2/T_H17 polarization complete with airway eosinophilia and neutrophilia, as well as airway hyperresponsiveness (AHR). We move away from the use of intraperitoneally-injected aluminum hydroxide (Alum) as an adjuvant, and begin to explore sensitization specifically as it takes place in the lung.

The acute mediator Serum Amyloid A induces NLRP3-dependent allergic airway disease in mice.

Chapters 5 takes a closer look at the NF-κB-regulated, airway epithelial-produced soluble mediators that play a role in our animal models of allergic asthma. Chapter 5 reveals that Serum Amyloid A (SAA) is a potent inducer of inflammation, NLRP3 inflammasome activation, and is an instigator of allergic airway sensitization to OVA. SAA treatment can induce dendritic cells to undergo maturation and activation, and to robustly produce IL-1β, leading to an IL-1 receptor signaling-dependent T_H17 phenotype.

Airway epithelial NF-κB activation is sufficient to break inhalational tolerance in mice.

Having demonstrated in Chapter 4 that airway epithelial NF-κB activation allowed for allergic sensitization to an innocuous inhaled antigen in *naïve* mice, we were left with the question of whether induced NF-κB activation in airway epithelium could promote allergic airway disease in mice that had previously been tolerized to the antigen OVA. Chapter 6 reveals that airway epithelial NF-κB activation can drive allergic airway disease irrespective of previous antigen tolerance. The effects of soluble mediators produced by the airway epithelium on DCs appears to be critical in overcoming tolerance, and there is a key role for IL-4 and IL-1 receptor signaling in generating downstream T_H2 and T_H17 responses, respectively.

The acute mediator Serum Amyloid A protects dendritic cells from apoptosis, driving $CD4^+$ T cell activation and resistance to glucocorticoid treatment.

Chapter 7 returns to the polarizing effects of SAA and indicates that, *in vitro*, SAA affords protection against apoptosis. Serum-starved dendritic cells undergo caspase 3-dependent apoptosis that is inhibited in SAA-treated cells. In addition, SAA treatment induces inflammatory cytokine production from serum-starved DCs, including IL-1β, IL-6, and HSP70. Finally, SAA-treated, serum-starved DCs effectively present antigen and drive steroid-refractory IL-17 production from $CD4^+$ T cells.

Closing Remarks

The data presented in this thesis contribute to the understanding of how NF-κB-dependent inflammatory mediators, generated by airway epithelial cells in response to injury or antigenic insult, can modulate surrounding leukocytes and polarize the adaptive $CD4^+$ T cell phenotype in allergic airway disease. From these

studies, we conclude that the inflammatory milieu generated as a consequence of airway epithelial NF-κB activation triggers the maturation and prolongs the survival of dendritic cells, and this in turn results in $CD4^+$ T cells that produce both T_H2 and T_H17 cytokines. Importantly, we have determined that the generation of this NF-κB regulated response can not only induce sensitization in naïve mice, but also overcome established antigen tolerance. It is the latter situation that likely accounts for the development of allergic airway disease in adults. Therefore, inhibition of select soluble mediators secreted by the epithelium may improve inflammation in asthmatic patients, and may also modulate the patient response to glucocorticoid treatment.

Curriculum vitae

EDUCATION

University of Vermont, College of Medicine, Burlington VT
M.S. in Pathology Graduated Sept. 2007.

University of Vermont, College of Agriculture and Life Sciences, Burlington, VT
B.S. in Animal Science. Graduated May 2004.

WORK EXPERIENCE

Lab Manager, University of Vermont, Department of Medicine, Burlington VT. (October 2007 – present). Duties include: Supervising of students, post-doctoral fellows, and other employees in an immunology lab setting. Coordinating projects with other laboratories and overseeing the use and maintenance of shared equipment. Pursuit of individual project design, execution, and publication. Attendance of pertinent workshops, seminars, and conferences. Maintenance of experimental mouse colony, including breeding and genotyping. Oversight of lab expenditures and finances.

Lab Technician II, University of Vermont, Animal Science Department, Burlington VT. September 2004 –June 2005.
Duties include: Performing experiments in mastitis research, such as PCR, Northern, Southern, and Western Blots, cell culture, and ELISA. In charge of ordering supplies and reallocating laboratory purchases. Assistance of post-doctorate, graduate student, and undergraduate student projects as well as independent research.

Internship: "Healthy Farms: Healthy Agriculture", (May 2003-September 2003) University of Vermont, Animal Science Department, Burlington VT. Duties included: researching information on dairy farm biosecurity and assembling into an easy access binder format.

Undergraduate Research Projects:

"Cytokine Upregulation During Mastitis: Analysis by Mini-Array" (Sept. 2003 – May 2004). Duties included: analyzing RNA isolated from cell culture via PCR, mini-array, and Northern Blot techniques.

"Tools for Promoting Biosecurity in Vermont's Equine Community" (Sept. 2003 – May 2004). Duties included: researching information on horse farm biosecurity and assembling into an easy-access binder format and CD-Rom format. Conducting a preliminary impact survey, and presenting project at Everything Equine and Horses 2004 and the 2004 National Association of County Agriculture Agents in Orlando, Florida.

"Vermont Horses Count" 2002 Equine Survey (Sept. 2002 – Sept 2003). Duties included: Creating a computer database for input of survey information, additional research for, and co-authoring of, the survey publication, and presenting results at the 2003 Vermont Equine Summit.

"Cloning the Bovine Lactoferrin Promoter" (May 2002 – May 2003). Duties included: Analytical laboratory techniques, such as ELISA, PCR, Southern Blotting, cloning, and radiation certification for handling of Phosphorous-32.

Staff Assistant, University of Vermont, Animal Science Department, Burlington VT. (Sept. 2000 – May 2002). Duties included: copying, filing, and running errands. Designing, creating, printing, and folding department brochures. Miscellaneous computer work (i.e. formatting documents, typing letters) and assisting professors with presentation design.

Receptionist, Brown Animal Hospital, Burlington VT May 2001-August 2001

TEACHING EXPERIENCE

Writing Tutor, UVM Writing Center. Duties included minimum 3 hours a week one-on-one tutoring with students. Specialized in assisting students with lab reports and papers for science classes.

SCHOLARSHIPS AND AWARDS

UVM Department of Pathology Graduate Research Fellowship 2006-2007
Animal Science Faculty Award 2004
Undergraduate Research Endeavors Competitive Award (URECA) 2003-2004

Publications

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