Published in final edited form as:

Clin Exp Allergy. 2015 July ; 45(7): 1245–1258. doi:10.1111/cea.12491.

Airway epithelial NF- κ B 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*

*Vermont Lung Center, Division of Pulmonary Disease and Critical Care, Department of Medicine

[†]Department of Surgery, University of Vermont, Burlington, VT 05405

Abstract

Background—Inhalational antigen tolerance typically protects against the development of allergic airway disease but may be overcome to induce allergic sensitization preceding the development of asthma.

Objectives—We examined *in vivo* whether pre-existing inhalational antigen tolerance could be overcome by activation of the transcription factor NF- κ B in conducting airway epithelial cells, and used a combination of *in vivo* and *in vitro* approaches to examine the mechanisms involved.

Methods—Wildtype and transgenic mice capable of expressing constitutively active I κ B kinase β (CAIKK β) in airway epithelium were tolerized to inhaled ovalbumin. Twenty-eight days later, the transgene was transiently expressed and mice were exposed to inhaled OVA on day 30 in an attempt to overcome inhalational tolerance.

Results—Following ovalbumin challenge on days 40–42, CAIKK β mice in which the transgene had been activated exhibited characteristic features of allergic airway disease, including airway eosinophilia and methacholine hyperresponsiveness. Increases in the CD103⁺ and CD11b^{HI} lung dendritic cell populations were present in CAIKK β mice on day 31. Bronchoalveolar lavage from mice expressing CAIKK β mice induced CD4⁺ T cells to secrete T_H2 and T_H17 cytokines, an effect that required IL-4 and IL-1 signaling, respectively. CAIKK β mice on Dox demonstrated increased numbers of innate lymphoid type 2 cells (ILC2) in the lung, which also exhibited elevated mRNA expression of the T_H2-polarizing cytokine IL-4. Finally, airway epithelial NF- κ B activation induced allergic sensitization in CAIKK β mice on Dox that required IL-4 and IL-1-signaling *in vivo*.

Conclusions—Our studies demonstrate that soluble mediators generated in response to airway epithelial NF- κ B activation orchestrate the breaking of inhalational tolerance and allergic antigen sensitization through the effects of soluble mediators, including IL-1 and IL-4, on pulmonary dendritic cells as well as innate lymphoid and CD4⁺ T cells.

Address correspondence and reprint requests to: Matthew E. Poynter, PhD Division of Pulmonary Disease and Critical Care Department of Medicine University of Vermont Given E410A 89 Beaumont Avenue Burlington, VT 05405. Matthew.Poynter@uvm.edu.

Conflict of Interest The authors declare no conflict of interest.

Keywords

allergic airway disease; NF- κ B; inhalational antigen tolerance; dendritic cells; T regulatory cells

Introduction

Despite recent insights into the molecular mechanisms of allergic asthma, incidence of the disease continues to rise (1). 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 circumstance in which an individual previously-tolerized to an inhaled antigen develops allergic airway disease in response to subsequent inhalational exposure to the same antigen. Importantly, we hypothesize that activated airway epithelial cells are capable of orchestrating an immune response that is sufficient to allow allergic sensitization in a previously-tolerized individual through the secretion of soluble mediators. We have adapted a mouse model system to experimentally assess this situation.

Under 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 (2, 3). This tolerogenic response requires the activity of CD4⁺CD25⁺ T regulatory cells (T_{REG}s), which can suppress naïve T cell proliferation and cytokine production in response to an innocuous antigen (4). Indeed, adoptive transfer of T_{REG} cells prior to sensitization confers resistance to the development of allergic asthma (5). A critical role has been implicated for dendritic cells (DCs) as producers of soluble factors that influence naïve T cells (6). Three DC populations have been described in the lung, each with distinct immune functions (7-9): plasmacytoid DCs (pDCs), which are poorly phagocytic, and are required for the generation of inhalational tolerance (10, 11); and CD103⁺ and CD11b^{HI} myeloid DCs that promote inflammatory and allergic responses (7, 8). In the absence of activating stimuli, DCs present harmless antigens to T cells, without providing the co-stimulatory signals necessary for the generation of effector CD4⁺ T cell responses; instead, a T_{REG} population undergoes expansion (12, 13). 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 (8, 14).

Activation of pulmonary DCs is directly influenced by the pulmonary epithelium. In response to microbial products, physical insult, or injury, airway epithelial cells secrete cytokines such as CCL20, TNF α , IL-6, and GM-CSF, driving DC recruitment, maturation, and activation (15, 16). In particular, airway epithelial activation of the transcription factor nuclear factor kappa-B (NF- κ B) serves as a critical regulator of allergic airway disease and is induced by many agonists that promote inflammatory cytokine production (16-18). Previously, we have reported that airway epithelial NF- κ B activation in an inducible transgenic mouse model is sufficient to sensitize mice to OVA (19). We therefore sought to determine whether airway epithelial NF- κ B activation would allow for allergic sensitization in mice that had previously been tolerized to 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 cell polarization and cytokine production. Our results implicate airway epithelial NF- κ B activity as a profound modulator in overcoming inhalational tolerance and developing allergic airway disease, through the influence of soluble mediators on DCs, innate lymphoid cells, and CD4⁺ T cells.

Methods

Mice

CC10-rtTA $\times$ TetOP-CAIKK β bitransgenic mice (16), which express a constitutively active I κ B kinase B (CAIKK β) 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 were purchased from Jackson Laboratories (Bar Harbor, ME). 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

CAIKK β mice and wildtype littermates received oropharyngeal aspiration on Day 0, 1, and 2 of sterile saline (50 μ l) as a negative control or 100 μ g 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 μ g OVA in 100 μ l 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.

Bronchoalveolar lavage (BAL) collection and lung processing

Lungs were lavaged with 1 ml DPBS (Life Technologies, Carlsbad, CA) from which cells were counted by hemocytometer. Differential analysis was performed by cyto-spin and H&E stain.

Quantitative RT-PCR

Total RNA was extracted from whole lungs or flow-sorted ILC2 cells using the PrepEase RNA Spin kit (Affymetrix, Santa Clara, CA) and reverse transcribed to cDNA using the iScript kit (Bio-Rad Laboratories, Hercules, CA). Primers were designed for mouse *Il4*, *Il5*, *Il13*, and *Cxcl1*. *Muc5ac* and *Gapdh* were analyzed using Taqman Assay on Demand (Life Technologies). PCR was performed using SYBR Green Supermix (Bio-Rad) or iQ Supermix (Bio-Rad) and normalized to *Gapdh* using the C_T method, as previously described (19).

Splenocyte restimulation

Splenocytes were isolated using Lymphocyte Separation Media (MP Biomedicals, Solon, OH) following mechanical disruption as previously described (20). 4×10^6 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 treated with 100 μ g/ml OVA. Supernatants were collected after 96 hours of incubation at 37°C in 5% CO₂.

ELISA analysis

Analysis of cytokine content from unconcentrated BAL and cell supernatants was performed using ELISA kits for TGF- β , IL-5, IL-10, IL-13, IL-17A, IFN γ (R&D Systems, Minneapolis, MN), and IL-4 (BD Biosciences, San Diego, CA). BAL was concentrated using Amicon Ultra centrifugal filters (Millipore, Billerica, MA), and subsequently analyzed for cytokine content using custom Milliplex panels (Millipore) for SAA3, KC, IL-6, MIP-2, G-CSF, GM-CSF, TNF- α , IL-1 α , IL-1 β , IP-10, TNF- α , IL-12p70, and MCP-1. Serum immunoglobulin ELISAs were performed as previously described (21).

Methacholine responsiveness

Mice were anesthetized and mechanically ventilated using the forced oscillation technique (17, 19). 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 the GentleMacs dissociator and Lung Digestion Kit (Miltenyi, Auburn, CA) per manufacturer instructions. Total cells 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), CD103-APC (eBioscience, San Diego, CA), CD90.2-eFluor450 (eBioscience), and (Ly6A)Sca-1-PE Cy7 (Biolegend). Dead cells were excluded by UV-Blue Live/Dead stain (Life Technologies). Briefly, 1×10^6 cells were blocked with 2.5 μ g/ml Fc block (anti-CD16/CD32, BD Pharmingen) before staining for 30 minutes in 100 μ l of antibody solution. Following staining, cells were fixed in DPBS with 1% paraformaldehyde. Cells were analyzed using a Becton Dickinson LSR II FACS equipped to distinguish as many as 7 fluorophores. DCs were identified as the CD11c⁺, FITC-autofluorescence^{LO} population as described in (8). Macrophages were excluded as the CD11c⁺, FITC-autofluorescence^{HI} population. Flow data were analyzed using FlowJo (Treestar, Ashland, OR). In separate experiments, 1×10^6 lung cells were stained for lineage negative, live (UV-blue negative), and ILC2 surface markers using the Lineage negative cocktail (Ly6G (Ly6C), CD45R (B220), Ter119, CD3, and CD11b, Biolegend), NK1.1-Alexa700 (Biolegend), CD11c-Alexa700 (Biolegend), CD90.2-eFluor450 (eBioscience), and

Ly6A-PE Cy7 (Biolegend). The same antibodies were used for flow sorting of ILC2 cells, which were first stained at a starting concentration of 20×10^6 cells. ILC2 cells were sorted using the Becton Dickinson FACS Aria high speed cell sorter and were processed for total RNA isolation and RT-qPCR analysis.

Cell culture

CD11c⁺ cells were isolated from the spleens of naïve C57BL/6 mice by positive magnetic selection (Stem Cell Technologies, Vancouver, BC, Canada) and treated with cell-free BAL fluid from wild type or CAIKK β mice for 24 hours prior to flow analysis. Lavage was performed for cell experiments with culture media (RPMI-1640 supplemented with 10% FBS (Cell Generation), penicillin/streptomycin, L-glutamine, folic acid, and 2-ME). CD4⁺ T cells were isolated from the spleens of naïve C57BL/6 mice by negative magnetic selection (Stem Cell Technologies), and plated with 5 μ g/ml anti-CD3 (BD) and 4 μ g/ml anti-CD28 (BD) and treated with wild type or CAIKK β BAL, in the presence or absence of 10 μ g/ml anti-IL-4 (Bio X Cell, West Lebanon, NH) or 200 ng/ml Anakinra (Biovitrum, Stockholm, Sweden). After 72 hours, cell-free supernatants were collected for analysis.

Antibody inhibition of allergic sensitization model

All mice received Dox for 60 hours (Day 0 thru 3) prior to 30 minutes of 1% OVA nebulization on Day 3. CAIKK β mice received either 1 mg/mouse anti-rat IgG isotype control (Bio X Cell), 1 mg/mouse anti-IL-4 (Bio X Cell), or 110 μ g/mouse IL-1 TRAP (Regeneron Pharmaceuticals, Tarrytown, NY) on Day 0 and 3 by i.p. injection. Mice were challenged with on Day 14, 15, and 16 with 30 minutes of nebulized 1% OVA in sterile saline and mice were analyzed on Day 18.

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- κ B activation sensitizes mice to allergic airway disease despite previous tolerization

To examine the responses of mice tolerized to OVA, we utilized two models. The first is a well-documented regimen for antigen-specific tolerance induction (Fig. 1a), which has previously demonstrated that intraperitoneal (i.p.) injection of Alum/OVA cannot sensitize mice to allergic airway disease that have already been exposed to inhaled OVA in the absence of an adjuvant (20). In our hands, tolerized mice that underwent a subsequent sensitization regimen via i.p. Alum/OVA did not develop airway eosinophilia after challenge, nor did they upregulate OVA-specific serum IgE or inflammatory genes in the lung (supplemental figure 1). Additionally, splenocytes isolated from these mice following antigen challenge produce less inflammatory cytokines, with a predominance for IL-10 secretion (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- κ B activation.

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- κ B activation (16) (Fig. 1b). Transgenic CAIKK β mice were administered Dox-containing chow for a single period of 60 hours, ensuring transient transgene activation, but without the recruitment of inflammatory cells that is observed after long periods of Dox administration (16). Despite having been tolerized, transgenic CAIKK β mice administered 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 before transgene activation (Fig. 2a, TG sal/O+Dox), consistent with our earlier findings (19). In contrast, wild type 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 and in which the transgene would be activated. In addition to airway leukocyte accumulation, CAIKK β mice secreted pro-fibrotic TGF- β into the BAL (Fig. 2b), whereas wild type mice did not. CAIKK β mice also expressed the inflammatory gene *Muc5ac*, the T_H2 cytokine *Il13*, and *Cxcl1* (Figs. 2c-e) in the lung following challenge. Furthermore, upon antigen restimulation, splenocytes from previously tolerized transgenic mice strongly induced production of IL-17A that was similar to those produced by transgenic mice that had not undergone inhalational tolerance (Fig. 2f).

Airway epithelial NF- κ B activation promotes antigen-induced methacholine hyperresponsiveness irrespective of prior tolerization

Previously, we reported that transient airway epithelial NF- κ B 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, mice underwent methacholine 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), CAIKK β 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), demonstrating that airway methacholine hyperresponsiveness develops in CAIKK β mice in which the transgene has been activated despite previous antigen-induced immunological tolerance.

Airway epithelial NF- κ B activation drives myeloid dendritic cell maturation

Overcoming inhalational antigen tolerance requires the expansion of an antigen-specific CD4⁺ T cell population that is polarized to specific effector phenotypes as a consequence of encounters with activated professional antigen-presenting cells, such as DCs, and exposure to soluble polarizing mediators. To examine specific pulmonary DC populations affected by airway epithelial NF- κ B activation in our tolerance model, we analyzed surface markers of lung cells immediately following tolerization (day 3) or immediately following sensitization of Dox-treated mice that had been previously tolerized (day 31). As anticipated, no

differences were measured between wild type and transgenic mice on day 3. On day 31, there was an equal increase in the CD11b⁺/MHC II⁺ and CD103⁺/MHC II⁺ (Fig. 4a) DC populations present in the lungs of CAIKK β mice, both of which are causal in development of allergic asthma (7). To examine mechanisms by which NF- κ B activation in the airway epithelium promotes adaptive immune responses and overcomes 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 CAIKK β mice following 60 hours of Dox administration. DCs were cultured in these BAL fluids for 24 hours and then stained for surface markers of maturation. Flow cytometric analysis revealed that DCs treated with BAL from CAIKK β mice displayed elevated levels of surface MHC II and the costimulatory molecule CD86 compared to BAL from wild type littermates (Fig. 4b).

Airway epithelial NF- κ B activation induces release of acute inflammatory mediators capable of driving T_H2/T_H17 development

Analysis of concentrated BAL from WT and CAIKK β mice that had been administered Dox for 60 hours revealed the presence of several candidate mediators that could be responsible for driving the inflammatory T_H17 and T_H2 CD4⁺ T cell phenotype and overcoming tolerization (Fig. 4c). These included the acute phase protein SAA3, the T_H17-polarizing cytokine IL-1 α , the T_H17- and T_H2-polarizing cytokine IL-6, and the leukocyte chemoattractors KC and IP-10. However, the most potent T_H2-polarizing cytokine, IL-4 (21), was undetectable in BAL fluid from CAIKK β mice (data not shown). Despite their low level or their inability to be quantitated, we sought to determine whether IL-1 and IL-4 in the BAL fluid were participating in T-cell polarization. Therefore, naïve CD4⁺ T cells were polyclonally stimulated for 72 hours in cell-free BAL fluid (obtained by lavaging the lungs of mice with tissue culture media) from wild type or CAIKK β mice that had been on Dox for 60 hours, in the presence or absence of either Anakinra (which blocks IL-1 signaling), or anti-IL-4. Addition of Anakinra completely inhibited the production of IL-17A from the CAIKK β BAL fluid-treated CD4⁺ T cells (Fig. 5). Anakinra also augmented the production of the T_H2 cytokines IL-4, IL-13, and IL-10, in response to wild type BAL treatment, and further augmented IL-10 in response to CAIKK β BAL treatment (Fig. 5). Conversely, IL-13 levels were decreased in response to CAIKK β BAL treatment in the presence of Anakinra. Neutralization of the T_H2-polarizing cytokine, IL-4, effectively reduced T_H2 cytokine production (IL-4, IL-13, and IL-10), while inducing a small increase in the production of IL-17A from CAIKK β BAL-treated CD4⁺ T cells (Fig. 5). It is important to note that the presence of anti-IL-4 in the media would effectively block the measurement of IL-4 in the ELISA; therefore, it is only appropriate to conclude that IL-13 and IL-10 are significantly blocked by anti-IL-4 administration. Neither the anti-IL-4 nor Anakinra had significant effects on the production of the T_H1 cytokine IFN γ .

Airway epithelial NF- κ B activation increases innate lymphoid type 2 (ILC2) cells in the lung

The T_H2-polarizing effects induced by CAIKK β BAL treatment were effectively inhibited by neutralizing IL-4 (and enhanced by inhibiting IL-1 signaling). Since IL-4 cannot be produced by cultured mouse tracheal epithelial cells from CAIKK β mice treated with Dox (16) and IL-4 is not considered to be a product of activated DCs (22), we sought an alternative cellular source of T_H2-polarizing IL-4 in lungs of the CAIKK β mice. Recent

studies have highlighted the role of innate lymphoid cells in the generation of allergic responses, particularly the role of ILC2s in generating T_H2 -polarizing responses in the lung (23, 24). Therefore, we generated lung single cell suspensions from wild type and CAIKK β mice after 60 hours of Dox administration. Flow cytometric analysis revealed a population of Lineage-negative, CD90.2⁺, (Ly6A) Sca-1⁺ cells consistent with ILC2s (25). As seen in Figure 6a, these cells were more abundant in the CAIKK β mice compared to wild type littermates. Replicate experiments indicated a consistent ~3 fold increase in lung ILC2s in the CAIKK β mice (Fig. 4b). Lung single cell suspensions from wild type and CAIKK β mice that received Dox were sorted for ILC2s using the same staining. ILC2s from CAIKK β mice showed increases in gene expression of both IL-4 and IL-5, with no difference in IL-13 (Fig. 4c).

Airway epithelial NF- κ B activation drives allergic sensitization that is dependent upon IL-4 and IL-1-signaling during the sensitization phase

Given the efficacy of anti-IL-4 and Anakinra to inhibit the T_H2/T_H17 responses of CD4⁺ T cells driven by soluble mediators in the BAL of CAIKK β mice that received Dox (Figure 5), we sought to determine whether IL-4 and IL-1 signaling were required in our in vivo model of airway epithelial NF- κ B allergic sensitization. Wild type and CAIKK β littermates received Dox for 60 hours prior to a 30 minute 1% OVA nebulization. Additionally, transgenic mice received either isotype control (Iso), anti-IL-4 (α IL-4), or IL-1 TRAP (TRAP) on Day 0 and 3 by i.p. injection (Fig. 7a). Forty-eight hours following challenge on Days 14-16, CAIKK β mice that received Dox and isotype control (TG Iso) exhibited robust increases in BAL cell recruitment (Fig. 7b), including eosinophils, lymphocytes, and neutrophils (Fig. 7c), compared to wild type controls. However, administration of anti-IL-4 or IL-1 TRAP at sensitization was sufficient to inhibit total cell recruitment, including eosinophils, lymphocytes and neutrophils (Fig. 7b-c). Restimulated splenocytes from transgenic CAIKK β mice on Dox that received isotype control antibody secreted significant amounts of the T_H2 cytokines IL-5 and IL-13 compared to wild type controls (Fig. 7d). Anti-IL-4 or IL-1 TRAP administered during sensitization did not significantly diminish the production of IL-5 and IL-13 from restimulated splenocytes. Interestingly, the secretion of IL-17A from restimulated splenocytes was strongly modulated by the administration of either α IL-4 or IL-1 TRAP during initial sensitization: α IL-4 drove a robust increase in the amount of IL-17A produced by splenocytes (compared to TG Iso mice), whereas IL-1 TRAP significantly diminished the production of IL-17A (Fig. 7d). Production of the T_H1 cytokine IFN γ was not significantly different between any of the transgenic groups (Fig. 7d). Finally, administration of either α IL-4 or IL-1 TRAP at sensitization was sufficient to inhibit gene expression of the mucin genes *Muc5ac* and *Clca1* in the lung, compared to transgenic mice on Dox that received isotype control (Fig. 7e).

Collectively, these results demonstrate that airway epithelial NF- κ B activation can initiate a cascade of signaling events to induce the secretion of soluble mediators that affect ILC2s, DCs, and T cells, including IL-1 and IL-4, which facilitate allergic antigen sensitization and 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 DC activity as a key component of overcoming T_{REG} suppression (4, 14, 15).

The airway epithelial barrier is the first line of defense against inhaled agonists, and airway epithelial cells release cytokines that can polarize T cells toward T_H2 and T_H17 phenotypes (26). Our previous work (19) has demonstrated that after 60 hours of Dox administration, transgenic CAIKK β mice upregulate inflammatory genes that participate in cell recruitment and maturation of DCs, and enhance DC activation of naïve T cells (27, 28). A novel aspect of the data presented herein is that airway epithelial activation is sufficient to overcome inhalational tolerance to OVA. Sensitized, Dox-treated transgenic 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 CAIKK β mice developed these parameters of allergic airway disease subsequent to transgene activation and antigen exposure, followed several weeks later by inhaled antigen challenge. Furthermore, splenocytes from these mice showed substantial production of IL-17A upon restimulation.

An antigen-specific allergic response was initiated in CAIKK β mice following transgene expression and antigen inhalation. Understanding how the airway epithelium orchestrates this antigen-specific CD4⁺ T cell response is critical to gaining insight into the pathogenesis of allergic asthma. Mediators secreted by the epithelium, or present in the lavageable airspaces and derived from other cells in the lung following NF- κ B activation, were able to induce DC maturation, as evidenced by increases in cell surface MHC II and CD86 in response to treatment with BAL obtained from Dox-treated CAIKK β mice. Lung DC populations turn over approximately every seven days (9). Therefore, at the day 31 time point in the experimental tolerance model, the airway epithelium would be educating a distinct DC population from that which had originally been exposed to the OVA antigen. We sought to determine whether airway epithelial NF- κ B activation elicited the skewing towards T_H17 and T_H2 cells indirectly, via the activated DCs, or by exerting a direct effect on T cells themselves. Cell-free lavage from Dox-treated CAIKK β mice demonstrated an increase in cytokines that not only facilitate DC recruitment, but may also contribute to T cell polarization. Preliminary studies revealed that treatment with CAIKK β BAL did not affect Foxp3⁺ expression in polarized T_{REG} cells (data not shown) indicating that perhaps the soluble mediators inducing inflammatory T cell cytokine production are not converting a polarized T_{REG} population, but instead acting upon Foxp3⁻, naïve CD4⁺ T cells. When naïve CD4⁺ T cells were treated with cell-free lavage from wild-type and CAIKK β mice after 60 hours of Dox administration, these cells produced increased levels of IL-4, IL-10,

IL-13, IL-17A, and IFN γ secretion. These cytokines have well-studied participatory effects in the development of the allergic airway disease phenotype, as we have recently reviewed (29). Blockade of IL-4 under these conditions abrogated production of the T_H2 cytokines IL-4, IL-10, and IL-13, and had an enhancing effect upon CAIKK β BAL-induced CD4⁺ T cell production of IL-17A. Conversely, when Anakinra was utilized to block signaling via the IL-1 receptor, IL-17A production was greatly reduced, whereas T_H2 cytokine production was increased. These results indicate that lung DCs are activated as a consequence of airway epithelial NF- κ B activation and that the lung environment in the Dox-treated CAIKK β mice is conducive to the promotion of T_H2 and T_H17 responses, even if they occur in the draining lymph node.

Cellular sources of T_H2- and T_H17-polarizing mediators in the lung remain controversial. IL-1 α and IL-1 β can be produced by activated DCs (30) and IL-1 α can be released by airway epithelium to facilitate allergic sensitization (31). Although IL-1 may function as an autocrine stimulator of epithelial-derived DC-activating cytokines (31), IL-1 also has a well-documented function in T cell activation (32) and T_H17 polarization (33). Therefore, the two principal cell types we have documented to be activated in the lungs of the CAIKK β mice, airway epithelium and DCs, are candidate sources for the T_H17-polarizing IL-1. IL-6 can be produced by pulmonary DCs (34) and by airway epithelium (35); indeed, we did measure elevated levels of IL-6 in the BAL fluid from CAIKK β mice (Fig. 4b). IL-6 can function as an inducer of T_H2 polarization (36) and facilitates the generation of T_H17 cells, in part through the upregulation of IL-1 receptor on CD4⁺ T cells (33). However, our attempts to block IL-6 in the *in vitro* system in which polyclonally-stimulated CD4⁺ T_{REG} cells were cultured in the presence of Dox-treated CAIKK β BAL fluid, resulted in only modest reductions in the amount of IL-4 and IL-17A production elicited by BAL fluid from the CAIKK β mice (data not shown). IL-4 has long been implicated in the generation of the T_H2 response, however IL-4 is unlikely to be produced by either epithelium or DCs and we were unable to detect this cytokine at critical sensitization time points in our model. Nevertheless, IL-4 must be important in our model since its neutralization abrogated the production of T_H2 cytokines induced by BAL fluid from CAIKK β mice, and also effectively inhibited the generation of allergic sensitization in Dox-treated CAIKK β mice *in vivo*. Indeed, a small amount of IL-4 is appreciated to have substantial impact on T_H2 polarization (37). An emerging body of literature has revealed the importance of innate lymphocyte cells (ILCs), distinct populations of lymphocytes 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 (23, 24, 38). The lungs of CAIKK β mice, following Dox exposure, demonstrate increases in ILC2 cells, and this population of cells possesses upregulated expression of IL-4 and IL-5 compared to wild type littermates. We can surmise that since there are no alterations in the ILC2-activating mediators, IL-33 and IL-25 (39), in the CAIKK β mice (19), the enhanced number and elevated expression of IL-4 by ILC2 cells may account for the T_H2-polarizing activity of BAL from CAIKK β mice. Additionally, recent data indicates a critical role for crosstalk between ILC2s and DCs in generating T_H2 responses; specifically, that cytokine production from ILC2s can license migration of activated DCs to the draining lymph nodes (40). Approaches to demonstrating a functional role for ILC2 cells in the induction of an allergic CD4⁺ T cells response are

limited by the fact that CD90-mediated ILC depletion (25) also effectively induces long-term elimination of T cells (41), which are pathogenic contributors to the adaptive immune response in allergic asthma. Therefore, further study and alternative approaches will be required to determine whether the presence of ILC2 cells in our *in vivo* model is critical to the induction of an allergic response in previously tolerized animals.

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. The capacity of activated airway epithelium to secrete soluble mediators and to coordinate innate and adaptive immune cells reinforces the notion that the epithelium is a potential “leverage point” (42) at which to target therapeutic interventions in the complex system that is the allergic lung.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank Roxana Del Rio Guerra of the University of Vermont Flow Cytometry Center for assistance with cell staining and FACS sorting experiments.

This work was funded by grants R01 HL089177 and R01 HL107291, as well as a Clinical Innovator award from the Flight Attendant Medical Research Institute (FAMRI).

Abbreviations

BAL	bronchoalveolar lavage
DC	dendritic cell
T_{REG}	T regulatory cell
OVA	ovalbumin
ILC2	innate lymphoid type 2 cell

References

1. Martinez FD, Vercelli D. Asthma. *Lancet*. 2013; 382(9901):1360–72. Epub 2013/09/18. [PubMed: 24041942]
2. Tournoy KG, Hove C, Grooten J, Moerloose K, Brusselle GG, Joos GF. Animal models of allergen-induced tolerance in asthma: are T-regulatory-1 cells (Tr-1) the solution for T-helper-2 cells (Th-2) in asthma? *Clin Exp Allergy*. 2006; 36(1):8–20. [PubMed: 16393260]
3. Van Hove CL, Maes T, Joos GF, Tournoy KG. Prolonged inhaled allergen exposure can induce persistent tolerance. *Am J Respir Cell Mol Biol*. 2007; 36(5):573–84. [PubMed: 17218615]
4. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science*. 2003; 299(5609):1033–6. [PubMed: 12532024]
5. Ostroukhova M, Seguin-Devaux C, Oriss TB, Dixon-McCarthy B, Yang L, Ameredes BT, et al. Tolerance induced by inhaled antigen involves CD4(+) T cells expressing membrane-bound TGF-beta and FOXP3. *J Clin Invest*. 2004; 114(1):28–38. [PubMed: 15232609]

6. Oriss TB, Ostroukhova M, Seguin-Devaux C, Dixon-McCarthy B, Stolz DB, Watkins SC, et al. Dynamics of dendritic cell phenotype and interactions with CD4+ T cells in airway inflammation and tolerance. *J Immunol.* 2005; 174(2):854–63. [PubMed: 15634907]
7. Hammad H, Lambrecht BN. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol.* 2008; 8(3):193–204. [PubMed: 18301423]
8. Jakubzick C, Randolph GJ. Methods to study pulmonary dendritic cell migration. *Methods Mol Biol.* 595:371–82. [PubMed: 19941125]
9. Condon TV, Sawyer RT, Fenton MJ, Riches DW. Lung dendritic cells at the innate-adaptive immune interface. *J Leukoc Biol.* 90(5):883–95. [PubMed: 21807741]
10. Reizis B, Colonna M, Trinchieri G, Barrat F, Gilliet M. Plasmacytoid dendritic cells: one-trick ponies or workhorses of the immune system? *Nat Rev Immunol.* 11(8):558–65. [PubMed: 21779033]
11. de Heer HJ, Hammad H, Soullie T, Hijdra D, Vos N, Willart MA, et al. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med.* 2004; 200(1):89–98. [PubMed: 15238608]
12. Oh SY, Park JU, Zheng T, Kim YK, Wu F, Cho SH, et al. Cbl-b regulates airway mucosal tolerance to aeroallergen. *Clin Exp Allergy.* 41(3):434–42. [PubMed: 20738317]
13. Tsitoura DC, DeKruyff RH, Lamb JR, Umetsu DT. Intranasal exposure to protein antigen induces immunological tolerance mediated by functionally disabled CD4+ T cells. *J Immunol.* 1999; 163(5):2592–600. Epub 1999/08/24. [PubMed: 10452998]
14. van Rijt LS, Vos N, Willart M, Muskens F, Tak PP, van der Horst C, et al. Persistent activation of dendritic cells after resolution of allergic airway inflammation breaks tolerance to inhaled allergens in mice. *Am J Respir Crit Care Med.* 184(3):303–11. [PubMed: 21562124]
15. Robays LJ, Maes T, Joos GF, Vermaelen KY. Between a cough and a wheeze: dendritic cells at the nexus of tobacco smoke-induced allergic airway sensitization. *Mucosal Immunol.* 2009; 2(3):206–19. [PubMed: 19262504]
16. Pantano C, Ather JL, Alcorn JF, Poynter ME, Brown AL, Guala AS, et al. Nuclear factor-kappaB activation in airway epithelium induces inflammation and hyperresponsiveness. *Am J Respir Crit Care Med.* 2008; 177(9):959–69. [PubMed: 18263801]
17. Poynter ME, Cloots R, van Woerkom T, Butnor KJ, Vacek P, Taatjes DJ, et al. NF-kappa B activation in airways modulates allergic inflammation but not hyperresponsiveness. *J Immunol.* 2004; 173(11):7003–9. [PubMed: 15557197]
18. Poynter ME, Irvin CG, Janssen-Heininger YM. Rapid activation of nuclear factor-kappaB in airway epithelium in a murine model of allergic airway inflammation. *Am J Pathol.* 2002; 160(4):1325–34. [PubMed: 11943717]
19. Ather JL, Hodgkins SR, Janssen-Heininger YM, Poynter ME. Airway epithelial NF-kappaB activation promotes allergic sensitization to an innocuous inhaled antigen. *Am J Respir Cell Mol Biol.* 44(5):631–8. [PubMed: 20581095]
20. Odemuyiwa SO, Ebeling C, Duta V, Abel M, Puttagunta L, Cravetchi O, et al. Tryptophan catabolites regulate mucosal sensitization to ovalbumin in respiratory airways. *Allergy.* 2009; 64(3):488–92. [PubMed: 18721245]
21. Le Gros G, Ben-Sasson SZ, Seder R, Finkelman FD, Paul WE. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *J Exp Med.* 1990; 172(3):921–9. Epub 1990/09/01. [PubMed: 2117636]
22. MacDonald AS, Maizels RM. Alarming dendritic cells for Th2 induction. *J Exp Med.* 2008; 205(1):13–7. Epub 2008/01/16. [PubMed: 18195077]
23. Klein Wolterink RG, Kleinjan A, van Nimwegen M, Bergen I, de Bruijn M, Levani Y, et al. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *Eur J Immunol.* 2012; 42(5):1106–16. Epub 2012/04/28. [PubMed: 22539286]
24. Peebles RS Jr. A New Horizon in Asthma: Inhibiting ILC Function. *Sci Transl Med.* 2013; 5(174):174fs7. Epub 2013/03/01.
25. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nature immunology.* 2011; 12(11):1045–54. Epub 2011/09/29. [PubMed: 21946417]

26. Wang Y, Bai C, Li K, Adler KB, Wang X. Role of airway epithelial cells in development of asthma and allergic rhinitis. *Respir Med.* 2008; 102(7):949–55. [PubMed: 18339528]
27. Kato A, Schleimer RP. Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. *Curr Opin Immunol.* 2007; 19(6):711–20. [PubMed: 17928212]
28. Min L, Mohammad Isa SA, Shuai W, Piang CB, Nih FW, Kotaka M, et al. Cutting edge: granulocyte-macrophage colony-stimulating factor is the major CD8+ T cell-derived licensing factor for dendritic cell activation. *J Immunol.* 184(9):4625–9. [PubMed: 20357255]
29. Ckless K, Hodgkins SR, Ather JL, Martin R, Poynter ME. Epithelial, dendritic, and CD4(+) T cell regulation of and by reactive oxygen and nitrogen species in allergic sensitization. *Biochim Biophys Acta.* 1810(11):1025–34. [PubMed: 21397661]
30. Hodgkins SR, Ather JL, Paveglio SA, Allard JL, LeClair LA, Suratt BT, et al. NO₂ inhalation induces maturation of pulmonary CD11c+ cells that promote antigenspecific CD4+ T cell polarization. *Respir Res.* 11:102. [PubMed: 20659336]
31. Willart MA, Deswarte K, Pouliot P, Braun H, Beyaert R, Lambrecht BN, et al. Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J Exp Med.* 2012; 209(8):1505–17. Epub 2012/07/18. [PubMed: 22802353]
32. Ben-Sasson SZ, Hu-Li J, Quiel J, Cauchetaux S, Ratner M, Shapira I, et al. IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proc Natl Acad Sci U S A.* 2009; 106(17):7119–24. Epub 2009/04/11. [PubMed: 19359475]
33. Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, et al. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity.* 2009; 30(4):576–87. Epub 2009/04/14. [PubMed: 19362022]
34. Lewkowich IP, Lajoie S, Clark JR, Herman NS, Sproles AA, Wills-Karp M. Allergen uptake, activation, and IL-23 production by pulmonary myeloid DCs drives airway hyperresponsiveness in asthma-susceptible mice. *PloS one.* 2008; 3(12):e3879. Epub 2008/12/09. [PubMed: 19060952]
35. Neveu WA, Bernardo E, Allard JL, Nagaleekar V, Wargo MJ, Davis RJ, et al. Fungal allergen beta-glucans trigger p38 mitogen-activated protein kinase-mediated IL-6 translation in lung epithelial cells. *Am J Respir Cell Mol Biol.* 2011; 45(6):1133–41. Epub 2011/06/07. [PubMed: 21642586]
36. Rincon M, Anguita J, Nakamura T, Fikrig E, Flavell RA. Interleukin (IL)-6 directs the differentiation of IL-4-producing CD4+ T cells. *J Exp Med.* 1997; 185(3):461–9. Epub 1997/02/03. [PubMed: 9053446]
37. Brown MA. IL-4 production by T cells: you need a little to get a lot. *J Immunol.* 2008; 181(5):2941–2. Epub 2008/08/21. [PubMed: 18713963]
38. Vivier E, Spits H, Cupedo T. Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair? *Nat Rev Immunol.* 2009; 9(4):229–34. Epub 2009/03/26. [PubMed: 19319141]
39. Licona-Limon P, Kim LK, Palm NW, Flavell RA. TH2, allergy and group 2 innate lymphoid cells. *Nature immunology.* 2013; 14(6):536–42. Epub 2013/05/21. [PubMed: 23685824]
40. Halim TY, Steer CA, Matha L, Gold MJ, Martinez-Gonzalez I, McNagny KM, et al. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity.* 2014; 40(3):425–35. Epub 2014/03/13. [PubMed: 24613091]
41. Seaman WE, Wofsy D, Greenspan JS, Ledbetter JA. Treatment of autoimmune MRL/lpr mice with monoclonal antibody to Thy-1.2: a single injection has sustained effects on lymphoproliferation and renal disease. *J Immunol.* 1983; 130(4):1713–8. Epub 1983/04/01. [PubMed: 6187819]
42. Meadows, D. Leverage Points: Places to Intervene In a System. The Sustainability Institute; 1999.

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 CAIKK β 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.

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 48 hours after challenge, and restimulated with OVA for 96 hours before IL-17A from cell-free supernatants was analyzed by ELISA (f). n = 6-7 mice per/group. *p<0.05, **p<0.01, ***p<0.001, compared to wild type tolerized mice on Dox, WT O/O.

Figure 3. Inducible airway epithelial NF- κ B 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 (CAIKK β) 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

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 (a). Cells were gated for dendritic cell phenotype (CD11c⁺, FITC autofluorescence^{LO}) and analyzed for co-positivity of CD103/MHC II and CD11b/MHC II positive populations. 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 and CD86 (b). 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 (c). Cytokines that were measured but undetectable include: IL-1β, MCP-1, MIP-2, TNF-α, and IL-12p70. n= 4-8 mice/group, * = p<0.05, ** = p<0.01, *** = p<0.001 compared to WT.

Figure 5. Airway epithelial NF- κ B activation induces release of acute inflammatory mediators into the lavageable airspace that polarize CD4⁺ T cell cytokine secretion

CD4⁺ T cells isolated from naive C57BL/6 mouse spleen and lymph nodes were plated in the presence of anti-CD3 and anti-CD28 and treated with cell-free BAL from CAIKK β mice after 60 hours Dox administration, in the presence or absence of anti-IL-4 (10 μ g/ml), or Anakinra (200 ng/ml). Cells were incubated for 72 hours and supernatants were analyzed by ELISA. n = 9 mice/group. * = p<0.05, ** = p<0.01, *** = p<0.001.

Figure 6. Airway epithelial NF- κ B activation induces increased lung ILC2 populations, and ILC2 increases in IL-4 and IL-5 mRNA expression

Lung single cell suspensions from wild type and CAIKK β mice that received 60 hours of Dox administration were analyzed for the presence of ILC2 cells (Lin-CD90.2⁺Sca-1⁺) (a, b). mRNA expression from these lung ILC2s was analyzed by RT-PCR for IL-4, IL-5, and IL-13 (c). n=3-4 mice/group. * = p<0.05, ** = p<0.01, ns = not significant, compared to WT.

Figure 7. Airway epithelial activation requires IL-4 and IL-1-signaling to drive allergic sensitization to OVA

Wild type and transgenic CAIKKβ littermates were sensitized to OVA as previously published (19), with the addition of neutralizing antibodies during the sensitization phase (a). On Day 0 and 3, transgenic mice received either 10 mg/mouse anti-rat IgG1 isotype control (Iso), 10 mg/mouse anti-IL-4 (αIL-4), or 110 μg/mouse IL-1 TRAP (TRAP). Total BAL cell counts (b) as well as differential counts from cytopins (c) were performed. Splenocytes were restimulated with OVA for 96 hours and supernatants were analyzed by ELISA (d). Quantitative-PCR from whole lung was examined for gene expression of *Muc5ac* and *Cxcl1* (e). n=4-6 mice per group. * = p < 0.05, ** = p < 0.01, *** = p < 0.005, **** = p < 0.001.