

Adenovirus IL-13–Induced Airway Disease in Mice

A Corticosteroid-Resistant Model of Severe Asthma

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Interleukin 13 (IL-13) is considered to be a key driver of the development of airway allergic inflammation and remodeling leading to airway hyperresponsiveness (AHR). How precisely IL-13 leads to the development of airway inflammation, AHR, and mucus production is not fully understood. In order to identify key mediators downstream of IL-13, we administered adenovirus IL-13 to specifically induce IL-13–dependent inflammation in the lungs of mice. This approach was shown to induce cardinal features of lung disease, specifically airway inflammation, elevated cytokines, AHR, and mucus secretion. Notably, the model is resistant to corticosteroid treatment and is characterized by marked neutrophilia, two hallmarks of more severe forms of asthma. To identify IL-13–dependent mediators, we performed a limited-scale two-dimensional SDS-PAGE proteomic analysis and identified proteins significantly modulated in this model. Intriguingly, several identified proteins were unique to this model, whereas others correlated with those modulated in a mouse ovalbumin-induced pulmonary inflammation model. We corroborated this approach by illustrating that proteomic analysis can identify known pathways/mediators downstream of IL-13. Thus, we have characterized a murine adenovirus IL-13 lung model that recapitulates specific disease traits observed in human asthma, and have exploited this model to identify effectors downstream of IL-13. Collectively, these findings will enable a broader appreciation of IL-13 and its impact on disease pathways in the lung.

Keywords: IL-13; proteomic; asthma; neutrophils; corticosteroids

Asthma is a heterogeneous chronic inflammatory disease of the airways that is characterized by recurrent episodes of coughing, breathlessness, and wheezing. The classical features of asthma are airway inflammation, bronchoconstriction, increased mucus production, elevated serum immunoglobulin E (IgE), and airway hyperresponsiveness (AHR) (1). Despite our understanding of these pathophysiological endpoints in asthma, our comprehension of disease pathogenesis and processes remains limited. Clearly, asthma represents a multifactorial disease that is complex in origin, with genetic and environmental components that have only recently begun to be intensively interrogated.

Multiple studies have supported the significant increase in Th2 CD4⁺ T lymphocytes and eosinophils responsible for the production and release of multiple key cytokines, such as

CLINICAL RELEVANCE

This research details a novel, IL-13–dependent, steroid-resistant model of lung inflammation in mice that may facilitate the identification of druggable effectors, pathways, and/or biomarkers relevant for the treatment of steroid-resistant human asthma.

interleukin (IL)-4, IL-5, and in particular, IL-13 (reviewed in Ref. 2). Data derived from naïve mice administered recombinant murine IL-13, or from transgenic animals overexpressing IL-13, have shown that this cytokine induces various hallmarks of the human asthmatic phenotype, including AHR, goblet cell hyperplasia, mucus overproduction, and eosinophilia (3, 4). In agreement with these findings, genetic or pharmacologic blockade of IL-13 protects mice from many of these phenotypes (5–7). These studies complement data obtained in humans supporting a role for IL-13 in asthma. IL-13 levels are elevated in the BAL and the bronchial mucosa of individuals with atopic and non-atopic asthma, as well as patients with asthma and rhinitis compared with normal control subjects (8, 9). Moreover, single nucleotide polymorphisms within the IL-13 gene are associated with high serum IgE levels and with the development of asthma in ethnically diverse populations (10–12, and references therein). Clearly, IL-13 represents a critical driver of the allergic pulmonary inflammatory response.

Given the significance of these findings, the mechanisms and downstream mediators accounting for IL-13–dependent pulmonary inflammation in allergic asthma has been an area of intensive research. We generated a rapid and robust model of respiratory disease using adenovirus-expressing murine IL-13 as an approach to identify mediators and pathways triggered by IL-13. This platform is particularly attractive due to its ability to permit the expression of a protein of interest in a localized fashion for several days after a single *in vivo* administration in animals. Notably, this approach is of interest as a potential therapeutic gene delivery vehicle (13). The results described herein illustrate a rapid and robust model of IL-13–induced lung disease that recapitulates disease traits including airway inflammation, AHR, and mucus production, and may be relevant to steroid-resistant severe asthma. We characterized several identified candidate proteins but we focused on the complement cascade, based on its previously documented role in respiratory disease in mouse models. These findings substantiate the utility of proteomic approaches to yield information about pathways modulated in lung disease.

MATERIALS AND METHODS

IL-13 Adenovirus Challenge Model

Adenovirus particles (serotype 5) either containing the gene for murine IL-13 (Ad-IL-13) or not (Ad-null), were generated using the MRKAd5-hCMV-bGHpA shuttle vector and MRK[E3+] genomic

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vector, as previously described (14). Male BALB/c mice (6–8 wk of age; Charles River, St-Constant, PQ, Canada) were housed in a temperature- and humidity-controlled environment under specific pathogen-free conditions on a 12-hour light:dark cycle in groups of four to five, with standard rodent chow and water available *ad libitum*. A period of 3 days of acclimatization was allowed before experimentation. Experimental procedures were approved by the Animal Care Committee at the Merck Frosst Centre for Therapeutic Research, in accordance with the guidelines of the Canadian Council on Animal Care. Mice were divided into two groups and anaesthetized with a combination of ketamine/xylazine (80/10 mg/kg, respectively; Ayerst, Guelph, ON, Canada and Bayer, Toronto, ON, Canada) administered in a single intraperitoneal injection. Lightly anesthetized mice were then intranasally administered 50 μ l PBS containing 2.0×10^7 plaque forming units (pfu) of Ad-null or Ad-IL-13 virus under Biosafety level 2 containment conditions (Day 0). Mice were placed under warming lamps during the recovery period (~ 30 min) before re-housing for a period of up to 21 days in an adenovirus-dedicated environment. Lung function and terminal outcome measurements assessed in groups of Ad-null versus Ad-IL-13 mice were conducted on Days 4, 7, 10, 14, and 21 as indicated. For pharmacologic studies, dexamethasone sodium phosphate (Vetoquinol USA, Buena, NJ) was administered intraperitoneally at 3 mg/kg starting 1 day before adenovirus instillation, and then daily for 10 consecutive days.

Ovalbumin Challenge Model

Mice were sensitized and challenged to ovalbumin (OVA; Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) based on procedures described previously (15). Mice were sensitized intraperitoneally on Day 0 to 20 μ g OVA emulsified with 2 mg of aluminum hydroxide in 0.4 ml sterile PBS. Seven days later, mice were resensitized intraperitoneally to 10 μ g OVA with 1 mg of aluminum hydroxide. From Days 14 to 19 inclusive, mice were challenged via whole body aerosol exposure (Buxco, Wilmington, NC) to either a sterile solution of 0.5% PBS (Mediatech Inc, Herndon, VA) or 5% OVA (prepared in 0.5% PBS) for a period of 20 minutes per day for 6 consecutive days. Aerosols were generated employing a DeVilbiss compact compressor/nebulizer (Sunrise Medical Canada, Inc., Concord, ON, Canada). All endpoint measurements were conducted 24 hours after the last antigen aerosol challenge. For pharmacologic studies, dexamethasone sodium phosphate (Vetoquinol USA, Buena, NJ) was administered intraperitoneally at the indicated doses starting 1 day before OVA aerosol inhalation, and then 30 minutes before each of six aerosol challenges.

Assessment of Airway Responsiveness, Bronchoalveolar Lavage Fluid, and Tissue Harvests, Proteomics Analysis of Bronchoalveolar Lavage Fluid

The methods used are described in the online supplement.

Cell Culture

Normal human bronchial epithelial (NHBE) cells (Cambrex, East Rutherford, NJ) were grown submerged in bronchial epithelial cell growth medium (BEGM) containing additives (Cambrex) until confluent. Cells were then dissociated with trypsin/EDTA and seeded onto Transwell clear tissue culture inserts (Costar Co., Cambridge, MA) and maintained in submerged differentiation medium containing 50% Dulbecco's modified Eagle's medium in BEGM (with additives) and 50 μ M all-trans retinoic acid until confluent. Media on the apical side was removed, cells were grown at the air-liquid interface for 1 week, and recombinant murine IL-13 (various concentrations) was added to the medium-containing basolateral side. Cells were cultured for 7 days, after which the media were collected from the basolateral side for analysis of chemokine or C3 concentrations as described below. A549 cells (200,000 per well in 96-well plates) were grown overnight in RPMI media (Sigma-Aldrich) containing 0.5% charcoal/dextran-treated FBS (HyClone, Logan, UT). Recombinant murine IL-13 (Cell Sciences; Canton, MA) was added at various concentrations and the serum concentration was increased to 2.5%. Cells were then allowed to incubate for 30 hours at 37 °C (5% CO₂), after which supernatants were harvested and tested by sandwich enzyme-linked immunosorbent assay (ELISA) for the presence of C3 as described below.

ELISA and Quantitative Multiplex Chemokine Detection Assays

Levels of IL-13, eotaxin, eotaxin-2, and eotaxin 3 were assessed using Quantikine ELISAs (R&D Systems; Minneapolis, MN). Levels of C3a in mouse bronchoalveolar lavage (BAL) fluid were tested using a mouse/rat C3adesArg ELISA kit (Cedarlane, Burlington, ON, Canada). Levels of C3 in supernatants of cultured NHBE or A549 cells were assessed by sandwich ELISA using goat anti-human C3 antiserum (1:2,500 dilution; US Biologicals; Swampscott, MA) as the capturing antibody, rabbit anti-human C3 antiserum (1:5,000 dilution; Serotec, Raleigh, NC) as the detecting antibody and recombinant human C3 (US Biologicals) as the standard. Levels of chemokines in supernatants of NHBE cells were determined using multiplex technology from MesoScale, Inc. (Gaithersburg, MD) according to manufacturer's instructions.

Quantification of Mucus-Producing Cells

The right lung was processed and embedded in paraffin. Mid-trachea longitudinal sections (5 μ m thick) were affixed to microscope slides and stained with Alcian blue (AB) and periodic acid-Schiff (PAS) for identification of mucus-producing goblet cells. The total amount of AB/PAS staining was quantified (pixel intensity to define mucus area / membrane length; magnification = $\times 10$ to maximize the viewing field) under light microscope and normalized to total basement length using the AxioVision imaging software (Empix Imaging Ltd, Mississauga, ON, Canada).

Quantitative Reverse Transcriptase–Polymerase Chain Reaction

RNA from left lungs of Ad-IL-13 or Ad-null challenged mice was isolated on Day 10 using the RNEasy midi kit (Qiagen, Mississauga, ON, Canada) and was used as template for the synthesis of cDNA using the High Capacity cDNA archive kit (Applied Biosystems; Foster City, CA) according to the manufacturer's instructions. C3 and C3aR-specific cDNA levels were assessed with the TaqMan system (Applied Biosystems) and using pre-validated gene-specific expression assays available from the manufacturer.

Statistical Analysis

Endpoint comparisons between Ad-IL-13 and Ad-null were performed using one-way ANOVA followed by Bonferroni's Multiple Comparison post test. In the case of mucus quantitation, if no visible mucus was observed in lung histologic sections stained with AB/PAS in mice challenged with Ad-null, sections were assigned an arbitrary value of 0 for the purpose of data presentation. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Although IL-13 represents a pivotal mechanism for induction of allergic inflammation, its downstream mediators are less well understood. As an approach to identify potential downstream mediators and pathways modulated by IL-13, we generated a murine IL-13–expressing adenovirus serotype 5 (Ad-IL-13) specifically for *in vivo* administration. Anaesthetized male BALB/c mice were intranasally instilled a single dose of Ad-IL-13 or Ad-null and harvested at various time points. A time-dependent increase in IL-13 was detected in the BAL fluid that peaked 10 days (84 nM; ~ 8000 pg/ml) after instillation, and returned to baseline levels after 21 days (Figure 1). Elevations in IL-13 were specific to Ad-IL-13, since intranasal administration of Ad-null did not elicit IL-13 expression or induction in the lungs of mice. Biological activity of the IL-13 found in BAL fluid was confirmed using a STAT luciferase A549 lung epithelial cell reporter assay (data not shown). Thus, the Ad-IL-13 construct is active *in vivo* and specifically induces robust production of IL-13.

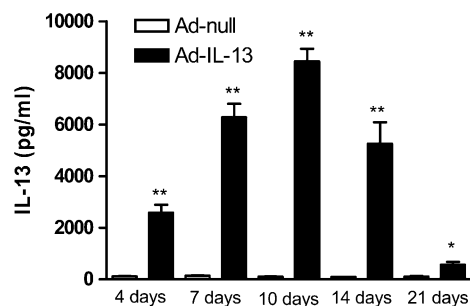


Figure 1. Expression of IL-13 in bronchoalveolar lavage (BAL) fluid of mice challenged with Ad-IL-13 or Ad-null. Time-course of IL-13 protein levels detected in BAL fluid of mice challenged with Ad-IL-13 ($n = 12$) or Ad-null ($n = 4$). Each value represents the mean $\pm$ SEM of determinations at various time points after instillation from two independent studies. * $P < 0.05$ and ** $P < 0.001$ compared with Ad-null-challenged mice.

Ad-IL-13 Treatment Induces a Robust Inflammatory Response in the Lung

To assess Ad-IL-13-dependent inflammation, groups of Ad-IL-13- and Ad-null-treated mice were harvested at various time points after instillation. Consistent with changes observed in IL-13 protein levels, challenge with Ad-IL-13, but not Ad-null, led to a time-dependent increase in the total number of cells recruited into the lungs of mice (Figure 2). Airway cellular inflammation peaked 10 days after instillation, at the time point demonstrating maximal IL-13 levels (Figure 2A). Notably, airway inflammation remained significantly elevated for up to 21 days after Ad-IL-13 instillation, even though BAL IL-13 levels returned to control levels at this time point. We characterized the cell types infiltrating the lung in response to IL-13 by differential cell analysis of BAL fluid at each time point. Ad-IL-13-treated mice displayed a dramatic increase in airway neutrophils, with small but significant increases in lymphocytes and eosinophils, while the macrophage numbers are unaltered and are similar to those observed in Ad-null-treated mice. Interestingly, while neutrophil infiltration peaks at Day 10 after instillation and drop down to near-baseline levels by Day 21, lymphocyte and eosinophil numbers peak later and remain elevated even at Day 21 after instillation. Conversely, BAL fluid from Ad-null-exposed mice was predominantly populated with (> 90%) macrophages at all time points, with small contributions from neutrophils and lymphocytes, but no detectable eosinophils (Figure 2B). Importantly, this differential profile is identical to that observed in lavage fluid from mice intranasally administered PBS and harvested on Day 10 (data not shown), demonstrating that the adenovirus itself does not alter differential cell profile *in vivo*.

Consistent with a role in the development of pulmonary inflammation, IL-13 has been shown to modulate the expression of several pro-inflammatory cytokines. We evaluated a select panel of Th1 and Th2 cytokines in the BAL after Ad-IL-13 instillation as an approach to identify alterations in representative cytokines over the course of the disease phenotype. Interestingly, IL-4 and IL-5, cytokines associated with the classical Th2 paradigm, were not modulated in response to Ad-IL-13. Moreover, the cytokines IL-2, IL-1 β , TNF- α , and IFN- γ were not modulated at any of the time points for either Ad-IL-13 or Ad-null (data not shown). In contrast, IL-10, IL-12, and keratinocyte chemoattractant (KC) were shown to be significantly increased by Ad-IL-13 treatment (Figures 3A–3C). Interestingly, KC is significantly elevated as early as Day 4 after

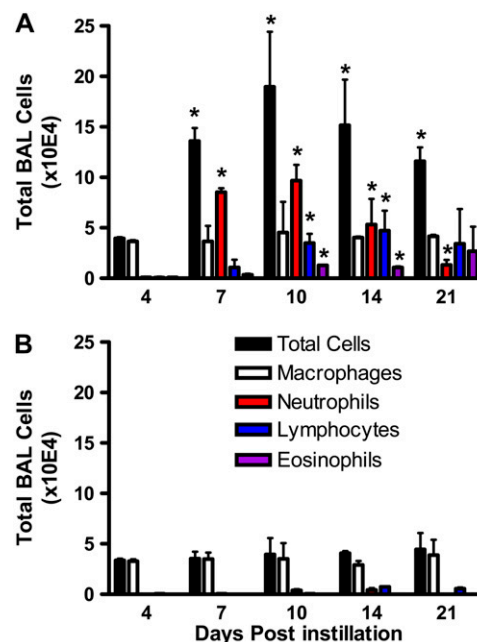


Figure 2. Cellular inflammation in BAL fluid of mice challenged with Ad-IL-13 or Ad-null. Total and differential cell counts in BAL fluid of mice challenged with (A) Ad-IL-13 ($n = 14$) or (B) Ad-null ($n = 6$) at various time points after instillation. Each value represents the mean $\pm$ SEM of determinations from three independent studies. * $P < 0.05$ compared with Ad-null-challenged mice for the specific cell type.

instillation and is maintained over the days evaluated. Adenovirus itself is well documented to induce innate immunity leading to cytokine (notably KC and IL-12) production (reviewed in Ref. 16). While slight alterations in the levels of these two factors were observed in Ad-Null-treated mice, in particular on Days 14 and 21 after instillation, these effects do not appear sufficient to induce neutrophil lung recruitment. Therefore, administration of Ad-IL-13 is associated with significant induction of selected cytokines and chemokines *in vivo*.

The significant neutrophilia observed in Ad-IL-13-treated mice is consistent with the presence of KC in BAL fluid of these mice. To assess whether IL-13 can drive the production of the KC-like chemokine IL-8 in human cells, we treated primary NHBE cells with IL-13 over a period of 7 days and assessed the release of IL-8 in the media. As shown in Figure 3D, IL-13 causes a significant increase in IL-8 release in these cells. IL-13 also causes the release of the eosinophil chemotactic factors eotaxin and eotaxin-3.

Ad-IL-13 Treatment Induces AHR and Mucus Release

It is well accepted that IL-13 is critical for the induction of AHR. To assess if IL-13 was associated with the development of AHR in this model of lung inflammation, groups of Ad-IL-13- and Ad-null-treated mice were evaluated for changes in lung function by assessing for changes in methacholine-induced lung resistance. As shown in Figure 4A, Ad-IL-13-exposed mice exhibit a dose-dependent increase in methacholine-induced AHR compared with Ad-null-exposed mice. To assess temporal kinetics, dose-response curves after intravenous methacholine infusion were constructed for Ad-IL-13- or Ad-null-treated mice at each time point. As shown in Figure 4B (plotted as the average lung resistance observed at a dose of 320 μ g/kg), methacholine-induced AHR in Ad-IL-13-treated animals does not differentiate from Ad-null-exposed animals until Day 10;

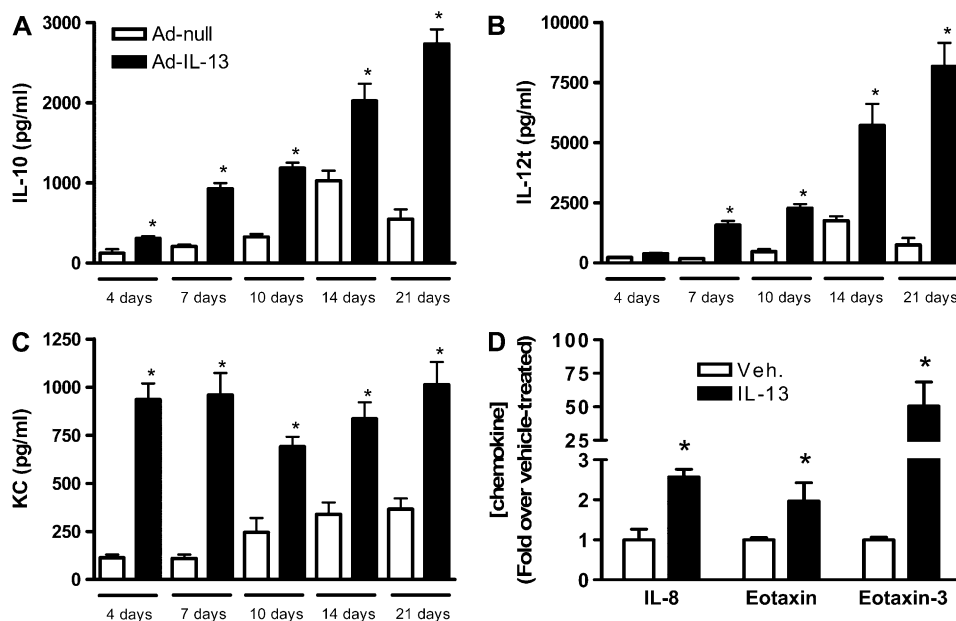


Figure 3. Cytokines secreted in BAL fluid of mice challenged with Ad-IL-13 or Ad-null. Time course of (A) IL-10, (B) IL-12, and (C) keratinocyte chemoattractant (KC) protein levels detected in BAL fluid of mice challenged with Ad-IL-13 ($n = 12$) or Ad-null ($n = 4$). Each value represents the mean $\pm$ SEM at various time points after instillation from two independent studies. * $P < 0.05$ compared with Ad-null-challenged mice. (D) Release of IL-8, eotaxin, and eotaxin-3 in NHBE cells after treatment with or without IL-13 for 7 days. Results are expressed as ratio over vehicle-treated group. Each value represents the mean $\pm$ SD of triplicate determinations. * $P < 0.05$ compared with vehicle-treated cells.

from this time until Day 21, AHR is observed in Ad-IL-13-exposed animals under conditions in which IL-13 levels and airway inflammation remain significantly elevated compared with Ad-null-treated animals (Figures 1 and 2A, Days 10, 14, and 21). These findings are consistent with previous reports (17, 18) and demonstrate that IL-13 production is sufficient for the development of AHR.

Airway obstruction is a key pathology that contributes to the development of AHR. To determine if Ad-IL-13 could induce changes in mucus production, we assessed AB/PAS-stained histologic lung sections from Ad-IL-13- versus Ad-null-exposed animals (Figure 5A). In contrast to a time-dependent elaboration of lung IL-13 levels coinciding with changes in airway inflammation and AHR, we observed at all time points a significant elevation in the degree of AB/PAS staining that progressively increases with time in Ad-IL-13-treated mice (Figure 5B).

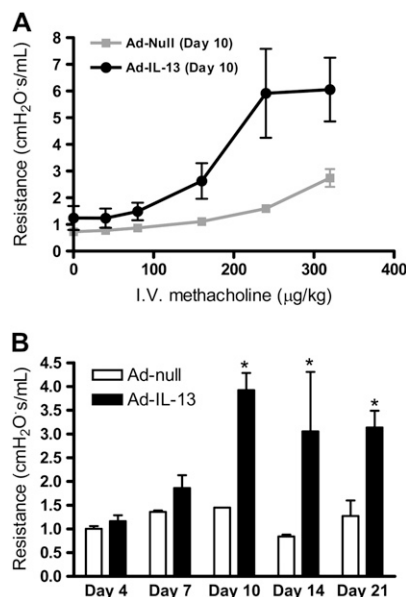


Figure 4. Airway hyperresponsiveness in mice challenged with Ad-IL-13 but not Ad-null. Representative dose response (A) and time course (B) depicting methacholine-induced airway hyperreactivity in mice challenged with Ad-IL-13 ($n = 6$) or Ad-null ($n = 2$). Each value represents the mean (plotted as the average lung resistance observed at a dose of 320μ g/kg) $\pm$ SEM at various time points after instillation. * $P < 0.05$ compared to Ad-null-challenged mice.

Ad-IL-13-Induced Lung Disease Is Resistant to Corticosteroid Treatment

One of the defining characteristics of allergic pulmonary inflammation in the mouse and in mild-to-moderate cases of asthma in humans is sensitivity to corticosteroids. Indeed, the anti-inflammatory properties of corticosteroids such as dexamethasone in the OVA airway eosinophil lung model are well established (19). We therefore assessed whether dexamethasone could also affect the various lung pathologies observed in the Ad-IL-13 model. As shown in Figure 6A, dexamethasone treatment (3 mg/kg) modulated Ad-IL-13-induced airway inflammation in a cell-specific manner. Indeed, differential cell analysis revealed that dexamethasone had no effect on the BAL neutrophil and macrophage cellular populations challenged with Ad-IL-13, while the eosinophil and lymphocyte inflammatory component was significantly reduced by dexamethasone treatment. The corticosteroid sensitivity of eosinophils and lymphocytes observed here (despite collectively accounting for only 15–25% of the overall Ad-IL-13 BAL inflammatory complement) is consistent with that observed in other eosinophil-dominated models of airway disease such as the Th2 model of ovalbumin-induced airway disease (see Figure E1 in the online supplement).

Interestingly, the effects of dexamethasone on eosinophils and neutrophils did not correlate with BAL fluid levels of the corresponding chemokines, KC, and eotaxin/eotaxin-2. Indeed, while levels of all three chemokines were elevated in BAL fluids of mice treated with Ad-IL-13 (Figure 6B), dexamethasone treatment was associated with a significant ($P < 0.05$) approximately 40% reduction in KC levels, no effect on eotaxin levels, and a near-significant ($P = 0.051$) 30% reduction in eotaxin-2 levels. Furthermore, dexamethasone had no effect on Ad-IL-13-induced IL-10 or IL-12 levels (data not shown). These findings highlight the complexity of the inflammatory response in this model and the intricate nature of the interplay between the various cytokines and chemokines that control cellular influx into the lung.

Consistent with a negligible effect on airway inflammation, we also observed that dexamethasone treatment did not reduce methacholine-induced AHR or mucus production in Ad-IL-13-challenged animals (Figures 6C and 6D, respectively). Collec-

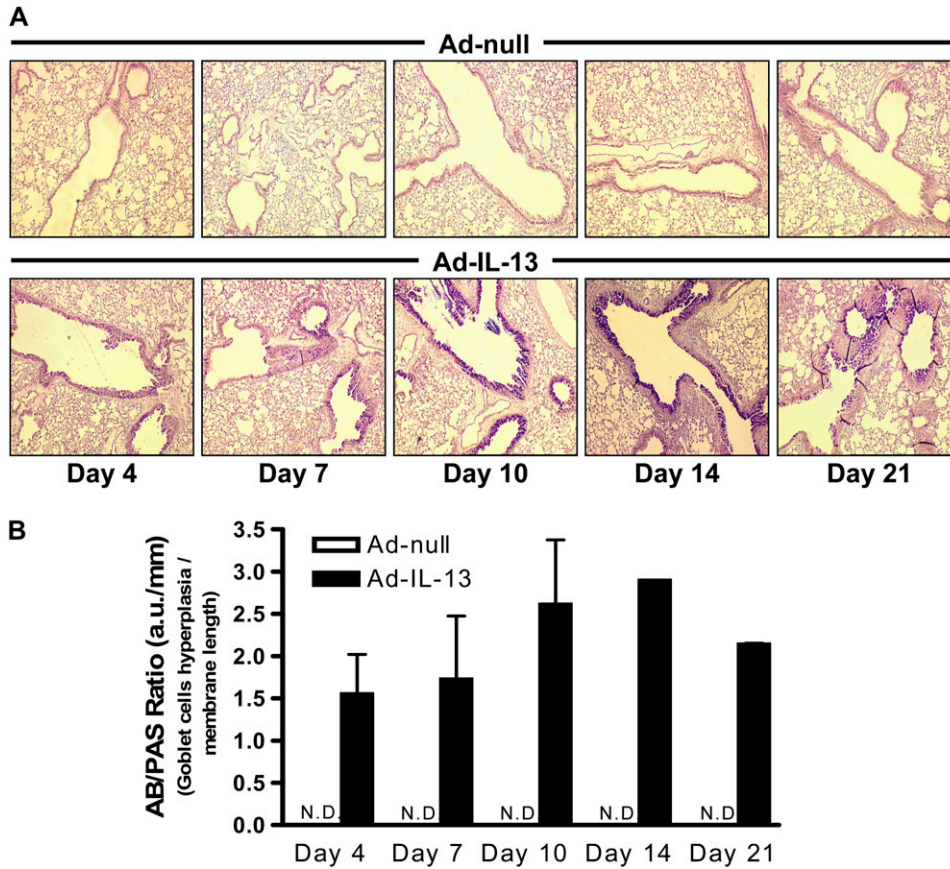


Figure 5. Mucus production in mice challenged with Ad-IL-13 or Ad-null. Representative time course (A) and quantitation (B) of mucus production in Ad-IL-13 ($n = 2$; > 15 airways/mouse) or Ad-null ($n = 2$; > 15 airways per mouse)-challenged mice was assessed histologically by Alcian Blue/Periodic Acid-Schiff (AB/PAS) staining in lung tissue sections at various time points after instillation. No AB/PAS staining was observed at any time point in Ad-null-exposed mice for the time course studies and thus was not determined (N.D.). a.u., arbitrary units.

tively, these findings demonstrate that Ad-IL-13 induces the hallmarks of airway disease, namely airway inflammation, AHR, and mucus production. However, in contrast to eosinophil-dominated models of airway disease, which are highly

sensitive to corticosteroids, the neutrophil-dominated Ad-IL-13 model is resistant to corticosteroid treatment at doses associated with attenuation of lung pathophysiology in the susceptible models.

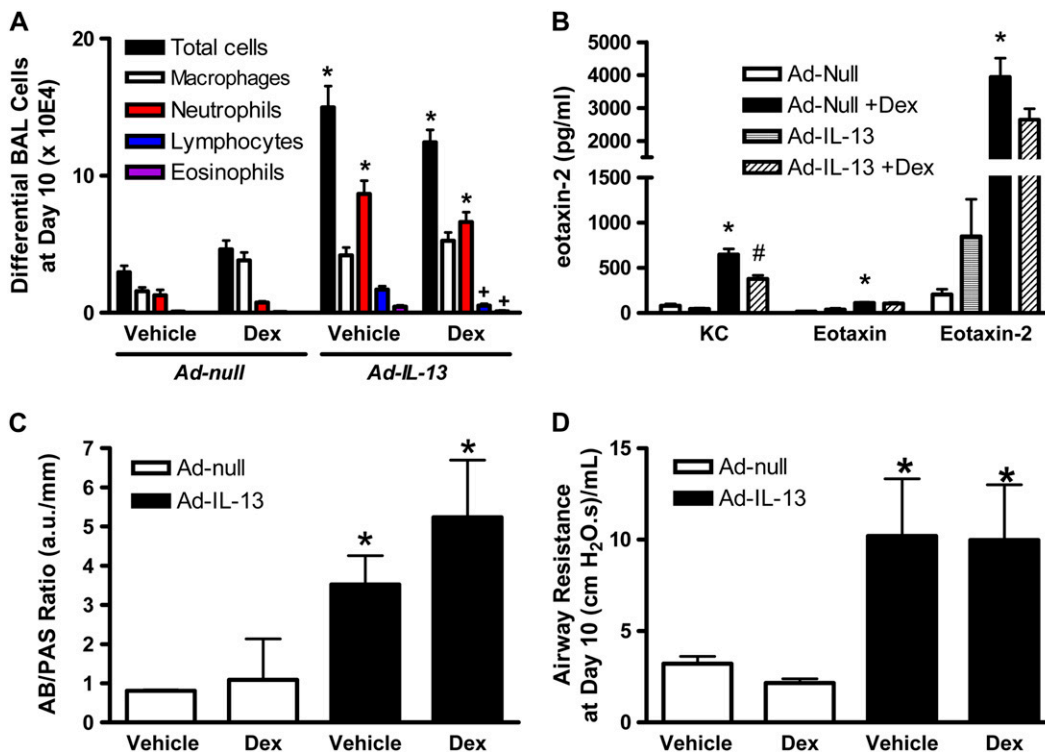


Figure 6. Effect of corticosteroid treatment on airway disease in mice challenged with Ad-IL-13 or Ad-null. Animals challenged with Ad-IL-13 ($n = 6$) or Ad-null ($n = 4$) were treated with dexamethasone (3 mg/kg) or vehicle and assessed on Day 10 after instillation for changes in Ad-IL-13-dependent airway inflammation (A); BAL KC, eotaxin, and eotaxin-2 levels (B); mucus production (C), and airway hyperresponsiveness (D). Each value represents the mean $\pm$ SEM from three independent experiments. Mucus production in Ad-IL-13 ($n = 2$; > 15 airways/mouse)– or Ad-null ($n = 2$; > 15 airways per mouse)-challenged mice was assessed histologically by AB/PAS staining in lung tissue sections. a.u., arbitrary units. $*P < 0.05$ compared with Ad-null-challenged mice for each specific cell type; $+P < 0.05$ compared with the vehicle-treated mice within each group.

Proteomic Analysis of BAL Fluid of Ad-IL-13–Treated Mice

While the analysis of gross pathologic endpoints is crucial for evaluating mechanisms for therapeutic efficacy, it provides a macroscopic view of the disease phenotype. To gain a deeper understanding of this model and identify mediators of IL-13, we wished to assess changes in BAL protein expression. Our rationale for employing BAL was predicated on the fact that this readily obtainable source could also provide a rapid means of identifying potential biomarkers for efficacy of anti-IL-13 therapeutics. To quantitate and compare proteomic profiles between Ad-IL-13– and Ad-null–challenged animals, we have used the resolving power of two-dimensional differential in-gel electrophoresis (2D-DIGE). A global analysis of all detected spots/proteins demonstrated that approximately 25% of detectable BAL proteins showed a statistically significant difference ($P < 0.05$) in abundance between Ad-IL-13 and Ad-null groups (Figure E2A). Due to the large number of changes observed (>900 spots) and the exhaustive verification efforts to assign identity to all the spots with statistically significant differences, we focused our attention on the most significantly modulated spots. Therefore, the criteria imposed for focused spot identification were arbitrarily chosen as (1) a fold change greater than 2 and (2) a P value less than 0.01. All proteins that met these criteria were followed up for identification using excised gel plugs after protein trypsin digestion and mass spectrometry MALDI-TOF-TOF analysis of the generated peptides. A select number of well-defined spots that had P values greater than 0.01 but less than 0.05 were also identified. Interestingly, when we compared the changes in the Ad-IL-13 BAL proteome to that of ovalbumin-sensitized and challenged animals (Figure E2), there was a significant degree of overlap (~60%) between these two models (Table 1). It is notable that a number of identified proteins have been previously documented to be modulated in allergic models (e.g., YM1, Clca3, SpD, C3b; see below). We validated this methodology by confirming specific protein changes by immunoblotting (data not shown).

As an example of the utility of this methodology for mechanistic evaluation, we capitalized on the identification of components of the complement pathway as being significantly modulated. In the analysis of the proteome changes, Complement C3b was shown to be significantly increased in both *in vivo* provocation models (Table 1). Since it has been demonstrated that C3b results from the cleavage of C3 into the products C3a and C3b in a C3 convertase–dependent manner (20), we reasoned that its significant elevation in BAL fluid would lend support to the presence of C3a as well, which has most recently been shown to be critical in mouse models for the development of pulmonary inflammation and airway hyperreactivity (21, 22). We therefore analyzed BAL fluid obtained from Ad-IL-13– and Ad-null–treated mice for C3a levels over time using an ELISA approach. Ad-IL-13, but not Ad-null, was associated with a time-dependent increase in C3a levels (Figure 7A). To further support the observation that complement pathway activation can occur downstream of IL-13, we assessed for mRNA changes in C3 and C3aR. We observed an approximately 2-fold increase in both C3 and C3aR expression (Figure 7B). Finally, to confirm that IL-13 could directly induce C3a release, we treated human epithelial cells (NHBE and A549) with increasing doses of recombinant human IL-13 and assessed human C3 levels in the culture supernatant. IL-13 treatment robustly induces C3 production ($EC_{50} = 75$ pM) in A549 cells (Figure 7C), although not in NHBE cells (not shown). Collectively, these findings demonstrate that expression of IL-13 results in the generation of C3a in the lungs of mice.

TABLE 1. IDENTIFICATION AND RELATIVE EXPRESSION OF BRONCHOALVEOLAR LAVAGE PROTEINS IN MOUSE MODELS OF LUNG DISEASE

	Ad-IL-13		OVA	
	Fold	P Value	Fold	P Value
Up-regulated proteins in Ad-IL-13				
Chitinase-3 like 4 or YM2	104.10	0.000083	67.6	0.000047
Chitinase-3 like 3 or YM1	48.92	0.0000987	36.7	0.000096
Resistin-like α	15.42	0.001	8.23	0.0007
Orosomucoid 1	11.93	0.0051	2.29	0.02
Polymeric Ig receptor	7.12	0.0022	7.36	0.001
Anterior gradient 2	5.78	0.0033		
Chloride channel calcium activated 3	4.95	0.00074		
Surfactant, pulmonary associated protein D	3.97	0.0048	5.78	0.000052
Chitinase-3 like 1	3.17	0.000071	2.01	0.11
β -actin	2.84	0.0033	4.43	0.0075
Kallikrein 26	2.29	0.009		
Complement C3b	1.63	0.032	2.58	0.02
Down-regulated proteins in Ad-IL-13				
Surfactant, pulmonary associated protein A	−4.68	0.00086	−4.02	0.0092
Lung carbonyl reductase	−3.66	0.003	−7.63	0.045
Uteroglobin	−2.72	0.027	−7.18	0.0058
Selenium binding protein 2	−2.25	0.0037	1.28	0.6
Serine protease inhibitor 2	−2.10	0.0016		
Serine protease inhibitor 1-1	−2.05	0.0086	−1.10	0.53
Kininogen	−2.05	0.0086	1.04	0.3
Haptoglobin β	−1.81	0.01	−2.78	0.0022
Major urinary protein 2	−1.44	0.022	−2.37	0.0009
Unchanged proteins in Ad-IL-13				
IgG light	−1.74	0.18	5.28	0.0022
Hemoglobin α	−1.65	0.6	−15.6	0.0016
IgG heavy	−1.41	0.24	6.65	0.0020
Peroxisome oxidin 5	−1.34	0.15	4.37	0.0034
Apolipoprotein H	−1.12	0.52	2.58	0.00053

Definition of abbreviations: Ad-IL-13, adenovirus particles containing the gene for murine IL-13; IgG, immunoglobulin G; OVA, ovalbumin.

DISCUSSION

We describe a novel murine model of pulmonary allergic inflammation that displays the cardinal features of allergic asthma, that is, airway inflammation, AHR, and mucus production. We show specifically that a single intranasal administration of Ad-IL-13 results in a time-dependent increase in IL-13 that peaks 10 days after instillation and disappears by Day 21. IL-13 expression is associated with significant changes in airway cellular inflammation, increases in airway hyperreactivity to methacholine, and a robust mucus phenotype. In addition to these physiological measurements, we have demonstrated alterations in the proteomic content of BAL fluid in this model that are consistent with an allergic response and comparable, though not identical, to the more classical ovalbumin allergen challenge model. We corroborated the utility of such a proteomic approach through the identification of pathways known to be involved in the development of specific disease traits associated with lung pathology, including confirmation of the role of the complement factor C3a.

The Ad-IL-13 model described herein displays notable, though not in all cases unique, features when compared with other murine asthma models: (1) it is dominated by a neutrophilic, rather than eosinophilic, airway inflammation; (2) it is not characterized by alterations in so-called “classical” Th2 cytokines; and (3) it appears to present inflammation-independent mucus production. At first glance, it is tempting to speculate that

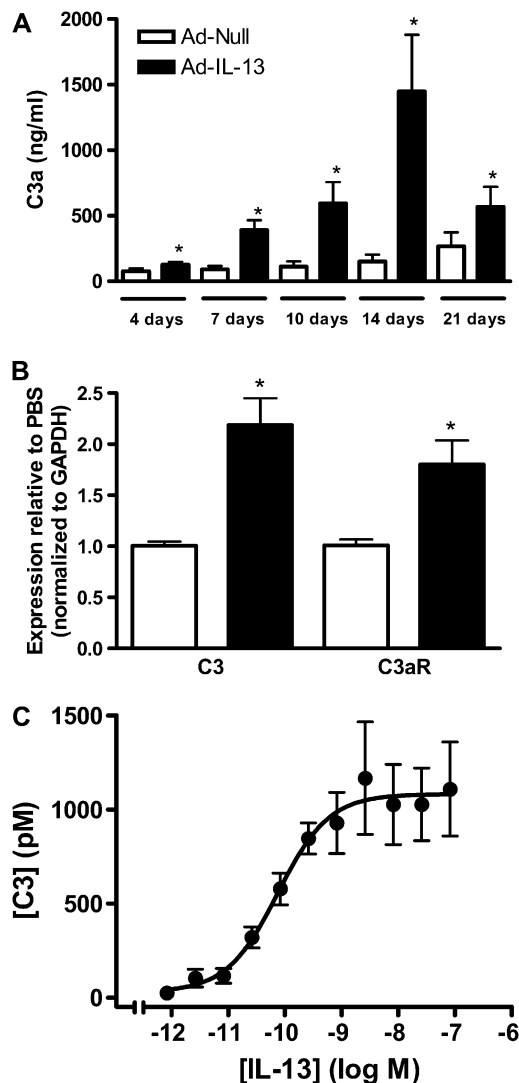


Figure 7. IL-13-dependent expression of C3, C3a, and C3aR. (A) Time course of C3a levels detected in BAL fluid of mice challenged with Ad-IL-13 ($n = 12$) or Ad-null ($n = 4$). Each value represents the mean $\pm$ SEM at various time points after instillation from two independent experiments. $*P < 0.05$ compared with Ad-null-challenged mice. (B) C3 and C3aR mRNA levels assessed by quantitative RT-PCR using left lung RNA from mice challenged with Ad-IL-13 ($n = 6$) or Ad-null ($n = 6$) on Day 10. Each value represents the mean $\pm$ SEM of determinations from two independent experiments. $*P < 0.05$ compared to challenge with Ad-null. (C) Representative experiment (out of four independent experiments) depicting IL-13-dependent C3 release from A549 cells. Each value represents the mean $\pm$ SD of three separate determinations.

high levels of IL-13 could account for some of the differences between the Ad-IL-13 levels and other murine models. Indeed, BAL fluid IL-13 levels were 2 to 8.5 ng/mL (Figure 1) on Days 4 through 14 after Ad-IL-13 challenge, compared with up to 0.7 ng/mL for the OVA model (not shown). On the other hand, lung-specific IL-13 transgenic mice exhibit BAL fluid levels of IL-13 that are comparable with those of mice treated with Ad-IL-13 despite not exhibiting the above characteristics (4), arguing against a significant effect of high IL-13 levels in the latter model.

Most murine asthma models, whether allergen-driven or induced through direct biochemical challenge with Th2 cytokines, are characterized by a robust lung eosinophilia accompanied by influx of T lymphocytes. Intranasal instillation of

Ad-IL-13, on the other hand, induces a robust increase in neutrophil influx, peaking on Days 7 and 10 and decreasing down to near-basal levels by Day 21 after instillation. Lymphocytes and eosinophils are also recruited to the lung, but levels of these cell types start to rise on Day 10 and peak by Days 14 and 21. This implies that neutrophils are the first cells to respond to IL-13 in this model and that eosinophils and lymphocytes are recruited later, perhaps in part by mediators released by the neutrophils. The distinct recruitment kinetics for different cell types offers one explanation for the divergence in predominant inflammatory cell type observed in other published models, in particular in cases in which a single time point is assessed. A more intriguing possibility is that the seemingly distinct inflammatory pattern observed in the Ad-IL-13 model is related to the concomitant viral infection that is a hallmark of this model. While our results with the Ad-null construct demonstrate that adenoviral infection on its own does not result in an appreciable inflammatory response, the data do not rule out the possibility that viral infection may alter the IL-13-dependent response, perhaps by promoting neutrophil recruitment. In this context, it is noteworthy that viral infections appear to play an important role in asthma pathogenesis and exacerbations, and are frequently associated with significant lung neutrophilia (23–25). Nevertheless, our finding that IL-13 treatment causes significant IL-8 release in cultured human primary epithelial cells highlights the potential relevance of IL-13-mediated neutrophilia in human asthma even in the absence of viral infection. Interestingly, it has been postulated that neutrophils, rather than eosinophils, are the principal effector cell type in individuals with severe asthma (26). That the Ad-IL-13 model may mimic more severe cases of asthma is corroborated by our observation that Ad-IL-13-mediated inflammation is largely resistant to dexamethasone treatment (Figure 6) while the eosinophil-based OVA model is sensitive to such treatment (19; Figure E1). Indeed, individuals with severe asthma who display airway neutrophilia also tend to show resistance to corticosteroid treatment (27). Whereas the (relatively minor) population of airway eosinophils and lymphocytes observed in the Ad-IL-13 model were sensitive to dexamethasone treatment, airway neutrophilia, mucus production, and AHR were not significantly affected. Since infiltrating neutrophils have been documented to contribute to airway mucus hypersecretion (28) and to correlate with AHR (29, 30), our findings may suggest their involvement in the development of these airway pathologies in this model and perhaps in resistant forms of human asthma. However, our data cannot exclude the possibility that the dexamethasone-resistant effects of IL-13 on AHR and mucus are independent of neutrophils, as suggested by data obtained by Kibe and coworkers (31).

The cytokine/chemokine BAL fluid signature exhibited in the Ad-IL-13 model is at first glance surprising. The lack of changes in IL-4 and IL-5 levels demonstrates that these cytokines are either upstream of IL-13 (likely for IL-4) or not affected by IL-13. On the other hand, IL-13 appears to mediate a rapid and sustained increase in expression of the chemokine KC, a neutrophil chemotactic factor thought to correspond to human IL-8, in the Ad-IL-13 model. While not classically thought of as being involved in Th2 immune reactions, IL-8 and KC can be up-regulated by IL-13 (see Figure 3D and Ref. 32), and BAL fluid levels of KC are elevated in several antigen-challenge paradigms (33–35). Furthermore, up-regulation of KC is consistent with the robust neutrophilia observed in this model, as discussed above. IL-12 is a Th1 cytokine and as such would not be expected to participate in this Th2-driven model of asthma. However, it has recently been shown that neutralization of IL-12 during the challenge, but not the sensitization,

phase of OVA-induced asthma abrogates inflammation and AHR, supporting a role of this cytokine in Th2 disease (36). Conversely, the relatively late onset of IL-12 production in Ad-IL-13–induced disease (levels are only significantly elevated at Day 7 after instillation, and appear to still be on the rise on Day 21) may implicate this cytokine as having a role in the resolution phase of disease, through skewing of a predominantly Th2 environment toward Th1. A similar argument could be invoked to explain the IL-13–dependent increase in IL-10, a well known anti-inflammatory cytokine whose expression kinetics mirror those of IL-12 in this model.

An intriguing aspect of the model described herein is the significant mucus production and goblet cell hyperplasia observed at a time point, 4 days post-Ad-IL-13 instillation, when no significant increases in inflammatory cells can be measured in the BAL fluid. These findings are consistent with previous observations demonstrating direct effects of IL-13 on epithelial cells to induce mucus production (17), suggesting that IL-13 alone is sufficient to drive this pathology. In addition, there are a number of alternative explanations that cannot be excluded, including the possibility that key mucus-inducing inflammatory cells infiltrate the airways before the 4-day time point and subsequently disappear or are present at levels that are too low to engender a significant observable increase in BAL fluid cells.

Proteomic analysis of BAL obtained from OVA-dependent mouse lung models (including the assessment of BAL protein changes influenced by corticosteroids) have been previously described (37–39) and clearly support these techniques as valid approaches to identify novel mediators and possibly therapeutic biomarkers. As BAL consists of a multitude of cellular and soluble components that reflect the lung microenvironment, proteomic analysis can reveal unique BAL profiles characteristic of normal and diseased lungs. Importantly, the identification of key soluble mediators directly implicated in the development of airway disease, as detailed in an elegant review by Magi and colleagues (40), lends support to the utility of this approach to dissect and broaden our understanding of these mechanisms. Furthermore, comprehensive differential proteomic analysis of BAL fluid from subjects with asthma and from healthy subjects illustrate that the majority of protein expression changes are closely associated with multiple aspects of pathophysiology of asthma, and thus strongly support this approach toward identifying novel, prognostic biomarkers and signaling pathways (41). However, it is important to note that the proteomic approach employed here investigates one time point of established airway disease, and may not necessarily be predicted to identify mechanisms associated with disease initiation and/or progression. Furthermore, there are technical limitations to analyzing samples by 2D gel, namely that only a fraction of all proteins in a given sample will be represented due to individual physiochemical properties (i.e., molecular weight, hydrophobicity, isoelectric point, abundance, etc.). Nonetheless, we performed a proteomic analysis to elucidate factors associated with airway disease. Notably, the analysis of BAL fluids of Ad-IL-13– and OVA-challenged mice provides evidence that the former model is in some ways a surrogate for the latter, more classical, asthma model, while also highlighting the significant differences that exist between the two models. The BAL fluid levels of a number of protein mediators shown to have a role in murine asthma were similarly altered in both OVA-challenged and Ad-IL-13–instilled mice. These include inflammatory modulators such as chitinases and uteroglobin (42, 43), chloride channel calcium activated 3 (CLCA3), a mediator of mucus production (44), lung carbonyl reductase, a protein involved in the regulation of oxidative stress (45)

and structural proteins including β -actin, and the lung surfactant proteins A and D (46).

Another striking example is the induction of complement in the two models, in particular the production of C3b, an important component of the enzyme C5 convertase that cleaves C5 into the anaphylatoxin C5a. Importantly, C3b is also a by-product of the formation of C3a from their common precursor C3. C3b is significantly increased in both models (Ad-IL-13: fold change = 1.63, $P = 0.032$; OVA: fold change = 2.58, $P = 0.02$). We chose to further investigate the role of complement as a proof-of-principle for the utility of the BAL proteomic approach, since the relevance of innate immune pathways in regulating adaptive immune responses is becoming increasingly appreciated (47). Furthermore, it has been convincingly demonstrated that complement mediators, in particular C3a, modulate adaptive immune responses to antigens, as well as amplify the Th2 immune response once initiated (21, 22, 47, 48). Lastly, we reasoned that analysis of a candidate protein less dramatically altered relative to other mediators (Table 1) could provide added confidence in the model for investigating pathways associated with airway disease. We were able to confirm that C3a levels are elevated in BAL fluid of both OVA-challenged (data not shown) and Ad-IL-13–instilled mice. Levels of the C3a precursor (C3) and receptor (C3aR) are increased at the RNA level in the lung of Ad-IL-13–treated mice. In addition, IL-13 can drive production of C3 in cultured epithelial cells, as previously demonstrated in TNF- α –stimulated fibroblasts (49), implying that one of the mechanisms through which C3a is produced *in vivo* is through direct stimulation of airways epithelial cells by IL-13. Combined with previous data showing that C3a can act upstream of IL-13 (21), there may be a positive feedback loop linking these two mediators in the pathogenesis and maintenance of the asthmatic phenotype.

While complement activation appears to be common to both allergen- and Ad-IL-13 challenge models, other BAL fluid proteins are differentially altered when comparing the two models. The down-regulation of the protease inhibitors serine protease inhibitors 1-1 (α_1 -antitrypsin) and 2 (contrapsin) and kininogen in the Ad-IL-13 model, but not in the OVA model, suggests the potential for remodeling in the former model, consistent with more severe forms of asthma, as described above. On the other hand, the up-regulation of IgG light and heavy chains in the OVA model but not in the Ad-IL-13 model is as expected for a model that depends on a humoral response to antigen. OVA model–specific modulation in the levels of hemoglobin α , peroxiredoxin, and apolipoprotein H similarly provide clues as to mechanism-based differences between the two models.

In conclusion, we describe a novel IL-13–driven inflammation model in the mouse that shares some of the hallmarks of more classical models such as the OVA challenge and transgenic IL-13 models. The neutrophilic nature of the inflammatory response as well as the partial resistance to corticosteroids indicate that this model may mimic disease traits associated with more severe cases of asthma, making it unique among previously described IL-13–driven models. Its further characterization (in conjunction with proteomic and genomic approaches) may provide opportunities to identify disease pathways and understand their potential network interrelationships so as to identify novel therapeutic targets and/or predictive efficacy biomarkers.

Conflict of Interest Statement: A.G.T. is an employee of Merck and Co. and owns stock options in Merck and Co. V.B. was an employee of Merck and Co. during the course of this study and declares no financial interests. S.W. is an employee of Merck Frosst Canada and Co. (a part of Merck and Co.) and has stock options in Merck and Co. P.T. an employee of Merck and Co. and owns stock options in Merck and Co. J.-P.F. an employee of Merck and Co. and owns stock options in Merck and Co. M.-C.M. is an employee of Merck and Co. and owns stock options in Merck and Co. J.H. was an employee of Merck and Co. during the course of

this study and declares no financial interests. V.P. was an employee of Merck and Co. during the course of this study and declares no financial interests. A.R. was an employee at Merck and Co during the course of these studies. R.S. is an employee of Merck and Co. and owns stock options in Merck and Co. L.D. was an employee at Merck and Co during the course of these studies. H.H. is an employee of Merck and Co. and owns stock and stock options in Merck and Co. J.L. is an employee of Merck and Co. C.R. was an employee of Merck and Co. during the course of these studies and owns stock and stock options of Merck and Co. G.P.O. is an employee of Merck and Co. and owns stock and stock options in Merck and Co. D.S. is an employee of Merck and Co. and owns stock and stock options in Merck and Co. C.M.T. is an employee of Merck and Co. and owns stock and stock options in Merck and Co.

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