Complement C3a Regulates Muc5ac Expression by Airway Clara Cells Independently of Th2 Responses

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Rationale: The factors that control the secretion of epithelial mucins are essential to understanding obstructive airway diseases such as asthma. Although the complement anaphylatoxin C3a and its receptor have been shown to promote many features of allergic lung inflammation, the contribution to mucin expression has not been elucidated.

Objectives: To determine if the C3a receptor with its ligand regulates airway epithelial mucin production.

Methods: Mice deficient in the C3a receptor were examined in a model of allergic airway disease for the presence of goblet cells and the gel-forming secreted mucin Muc5ac.

Measurements and Main Results: Lungs from antigen-challenged C3a receptor-deficient mice revealed a dramatic decrease in goblet cells and Muc5ac compared with challenged wild-type control animals. These differences were dependent on C3a binding to its receptor since intranasal challenge with C3a induced the formation of goblet cells only in wild-type but not C3a receptor-deficient mice. Increased numbers of goblet cells were also found in C3a-stimulated RAG-1-deficient mice demonstrating a mechanism independent of T lymphocytes and Th2 cytokines, mediators which have been shown to regulate mucin expression. A direct physiological role for C3a in these models was further demonstrated in cultures of airway epithelial Clara cells, which not only express the C3a receptor but also produce Muc5ac in response to C3a.

Conclusions: These studies identify a novel C3a receptor-dependent mechanism in the development of airway epithelial goblet cells and regulation of Muc5ac production and implicate C3a as a mediator of airway obstruction in asthma.

Keywords: T cells; allergy; lung; inflammation

Mucus hypersecretion by airway epithelium and airway obstruction due to mucus plugs contribute significantly to the pathology of asthma and can result in a fatal outcome (1, 2). Airway mucus is composed mainly of mucin glycoproteins; specialized airway epithelial cells, called goblet cells, are the major source of mucins in the proximal airway (3). Goblet cell numbers are increased in the epithelium during asthma and result in a higher volume of stored mucin in the airway than is observed under normal conditions (4). Of the 20 known human mucin genes (reviewed in Reference 5), attention has focused predominantly on the gel-forming secreted mucins MUC5AC and MUC5B because their mRNA is well expressed in normal airway tissues and their gene products have been identified in mucus from the lungs of patients with asthma (4, 6, 7).

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The effect of the complement anaphylatoxin C3a and its receptor on airway epithelial responses, especially in regard to mucin regulation and obstructive airway disease, has not been examined.

What This Study Adds to the Field

This study identifies a novel C3a receptor-dependent mechanism for regulating goblet cells and Muc5ac production and implicates C3a as a major mediator in airway obstruction in asthma.

Our studies, as well as those of others, have revealed a major role for the complement C3a anaphylatoxin and its receptor, C3aR, in allergic lung disease. These investigations have demonstrated the presence of C3a in the airways of patients with asthma (8–10), increased expression of C3aR by human and mouse lung tissue during allergic airway inflammation (11, 12), and attenuation of inflammatory responses in rodents genetically deficient in the third component of complement (C3) or C3aR in experimental models of pulmonary allergy (8, 13–15). These studies did not investigate the regulation of goblet cells and mucin production by C3a and its receptor, however. Given that an earlier report indicated that C3a stimulates secretion of mucus glycoprotein *in vitro* (16), we decided to investigate C3a as a possible mediator of mucus production in asthma. Mice deficient in either C3 or the C3aR were examined for the presence of airway epithelial goblet cells and Muc5ac. Data from these knockout mice showed a reduction in the numbers of goblet cells and expression of Muc5ac after antigen challenge compared with their wild-type control mice. Furthermore, intranasal challenge of mice or stimulation of primary airway epithelial cultures with C3a induced the expression of epithelial mucins, suggesting a novel regulatory mechanism dependent on the C3aR but independent of Th2 responses. Collectively, these studies provide strong evidence that C3a, on binding to its receptor, contributes to mucus production during obstructive airway diseases such as asthma. Some of the results presented here have been previously published in the form of an abstract (17).

METHODS

Mice

C3aR- and C3-deficient (C3aR^{-/-}, C3^{-/-}) mice were backcrossed onto the C57BL/6 background for 12 generations, and wild-type littermates (C3aR^{+/+}, C3^{+/+}) were used as controls (18,19). C3aR^{-/-} mice were crossed onto the recombination activation gene-1 (RAG-1)-deficient (RAG-1^{-/-}; Jackson Laboratories, Bar Harbor, ME) (20) strain to generate RAG-1^{-/-}-C3aR^{-/-} mice and their C3aR-sufficient (RAG-1^{-/-}-C3aR^{+/+})

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controls. Experiments were conducted according to The University of Texas Health Science Center at Houston Animal Welfare Committee and National Institutes of Health guidelines.

Model of Allergic Airway Disease

Four-week-old mice were sensitized and challenged with an antigen preparation consisting of *Aspergillus fumigatus* cell culture filtrate, prepared free of living organisms, and ovalbumin (Sigma, St. Louis, MO) as described previously (14, 21).

Isolation of Airway Epithelial Clara Cells

Airway epithelial Clara cells were isolated from mouse lung as described (22). The Clara cell populations were more than 85% pure as determined by fluorescence-activated cell sorter (FACS) analysis using an antibody specific for Clara cell secretory protein (CCSP; Upstate Biologicals, Lake Placid, NY). Clara cell cultures were free of contaminating macrophages, leukocytes, and mast cells as determined by FACS analysis using antibodies specific for Mac-1 (Becton Dickinson, Franklin Lakes, NJ), CD45 (Becton Dickinson), and c-kit (Becton Dickinson), respectively.

Analysis of C3aR mRNA expression by reverse transcriptase-polymerase chain reaction (RT-PCR) and C3aR protein expression by FACS and immunofluorescence was conducted as reported (19). Analysis of C3aR protein expression by Western blot was conducted by resolving 10 μ g of lysates from Clara cells on an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel under reducing conditions and transferring to a nitrocellulose membrane. The membrane was incubated with a rabbit anti-mouse C3aR IgG (11), detected with a goat anti-rabbit horseradish peroxidase-labeled IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and visualized with ECL-Plus (Amersham, Piscataway, NJ). The membrane was then reprobed

using an anti- β -actin monoclonal antibody (clone AC-15) to assess variations in sample loading.

Isolation and Culture of Tracheal Epithelial Cells

Isolation, culture, and differentiation of tracheal epithelial cells from C3aR^{+/+} and C3aR^{-/-} mice were conducted as described (23).

Stimulation with C3a and IL-13

For *in vivo* experiments, 8-week-old mice were instilled intranasally with either 10 ng/ml mouse IL-13 (Becton Dickinson) or 2.5 μ g/kg body weight of human C3a or its desarginated form, C3a(desArg) (Advanced Research Technologies, San Diego, CA). For *in vitro* experiments using isolated Clara cells or polarized airway epithelium, cells were incubated with media alone, 10 nM C3a or C3a(desArg), or 10 nM C3a plus 0.5, 1, or 2 μ g/ml of the C3aR nonpeptide antagonist N²-([2,2-diphenylethoxy]acetyl)-L-arginine (SB 290157; Calbiochem, San Diego, CA) (24). The purity of the C3a peptides was greater than 99% as determined by HPLC and mass spectrometry.

Periodic Acid Fluorescent Schiff Staining

To identify goblet cells, tissues were stained using the periodic acid fluorescent Schiff (PAFS) procedure described previously (25). Slides were imaged with an Axioskop FS compound fluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Muc5ac Immunofluorescence

Deparaffinated and rehydrated lung sections, frozen sections of filters containing tracheal epithelial cells, and permeabilized Clara cells were blocked with 10% goat serum, incubated with MUC5AC monoclonal

Figure 1. Goblet cell formation and Muc5ac expression in lungs from antigen-challenged C3aR^{-/-} and C3aR^{+/+} mice. Four-week-old mice were sensitized and challenged with a mixed antigen preparation consisting of *Aspergillus fumigatus* cell culture filtrate, and lungs were isolated 24 hours after the last challenge. Five-micron sections were stained with periodic acid fluorescent Schiff (PAFS) (A, B) or Muc5ac (C) and visualized by fluorescent microscopy. Sections shown ($\times 200$ original magnification) are representative of three separate experiments. Mucus production (D) or Muc5ac expression (E) was quantified by measuring PAFS or Muc5ac staining as described in METHODS. Values are presented as mean index $\pm$ SEM (PBS, $n = 3$; wild-type, $n = 9$; antigen-C3aR^{-/-}, $n = 9$; antigen-C3aR^{+/+}, $n = 9$), and significant differences between wild-type and knockout mice are indicated as ** $p < 0.01$ as determined by Student's *t* test. PBS controls shown were wild-type mice that were sensitized with the mixed antigen preparation and then challenged with PBS. PBS = phosphate-buffered saline.

antibody (Clone 45M-1; Lab Vision Corp., Fremont, CA), and detected with goat anti-mouse IgG-Alexafluor-546 (Invitrogen, Carlsbad, CA). Samples were imaged as described for the PAFS staining.

Mucus and Muc5ac Index

For *in vivo* experiments, an index of mucus- and Muc5ac-positive cells was determined by analyzing multiple airways from five separate lung sections per mouse, with and without PAFS- or Muc5ac-positive cells, to provide an overall assessment of mucin production in the lungs (26). For *in vitro* experiments using polarized cultures of airway epithelium, an index of Muc5ac-positive cells was determined by analyzing multiple fields per sample, with and without Muc5ac-positive cells, to provide an overall assessment of mucin production by the cultured cells. To calculate the mucus or Muc5ac index, mucin containing airway epithelium was measured at $\times 200$ magnification (lung sections) or $\times 400$ magnification (polarized epithelial cultures), and the area of PAFS or Muc5ac staining was divided by the total area of the airway epithelium (25).

Transmission Electron Microscopy

Filters of tracheal epithelial cells were prepared as described (27, 28). Briefly, 1% osmium tetroxide was dissolved in perfluorocarbon (Fluorinert FC-72; 3M Corp., St. Paul, MN) to prevent disruption of the mucus layer by aqueous solvents, and samples were fixed for 1 hour, followed by three rinses in perfluorocarbon. Samples were dehydrated, transitioned to Eponate-12 (Ted Pella, Inc., Redding, CA), and cured overnight at 65°C. Ultra-thin sections were stained with uranyl acetate and lead citrate and imaged on a Leo 912 Omega energy-filter transmission electron microscope (Carl Zeiss).

Statistical Analysis

Statistical analysis was performed using Prism software (Graphpad, San Diego, CA), and statistical significance was assessed using the two-tailed, unpaired Student's *t* test.

RESULTS

Antigen-challenged C3^{-/-} and C3aR^{-/-} Mice Have Reduced Airway Epithelial Goblet Cell Numbers and Muc5ac Production

Our previous studies using a rodent model of allergic airway disease demonstrated that mice deficient in C3, the parent protein of the ligand C3a, or C3aR had reduced airway hyperresponsiveness and inflammation (14, 15). Given that airway goblet cell production of mucins is a feature of airway obstruction in asthma (4) and that this parameter was not fully evaluated in the previous studies, wild-type, C3^{-/-}, and C3aR^{-/-} mice were examined to delineate the contribution of C3a to the generation of airway epithelial goblet cells. Mice were sensitized and challenged with a mixed antigen preparation composed of *A. fumigatus* cell culture filtrate and ovalbumin (21) or with phosphate-buffered saline (PBS) as a negative control. Twenty-four hours after the last challenge, lungs were isolated, sectioned, and stained with PAFS to detect the presence of mucus glycoprotein produced by goblet cells (Figures 1A and 1B). Lungs from PBS-challenged control mice exhibited low PAFS positivity, consistent with the observation that nonsensitized, wild-type rodent airway epithelia do not contain mucus-producing goblet cells

Figure 2. Goblet cell formation in lungs from C3a-stimulated C3aR^{-/-} and RAG-1^{-/-}C3aR^{-/-} mice. Eight-week-old mice were instilled intranasally with 2.5 μ g/kg body weight of human C3a, and lungs from C3a-stimulated C3aR^{+/+} and C3aR^{-/-} mice (A) or RAG-1^{-/-}C3aR^{+/+} and RAG-1^{-/-}C3aR^{-/-} mice (B) were removed from the chest cavity after 24 hours. Five-micron sections were stained with periodic acid fluorescent Schiff (PAFS) and visualized by fluorescent microscopy. Sections shown ($\times 630$ original magnification) are representative of two separate experiments. Mucus production from C3a-stimulated mice was quantified by measuring PAFS staining as described in METHODS (C). Values are presented as mean index $\pm$ SEM (PBS-C3aR^{+/+}, n = 2; C3a-C3aR^{+/+}, n = 6; C3a-C3aR^{-/-}, n = 6; PBS-RAG-1^{-/-}C3aR^{+/+}, n = 2; C3a-RAG-1^{-/-}C3aR^{+/+}, n = 6; C3a-RAG-1^{-/-}C3aR^{-/-}), and significant differences between C3a (C3aR^{+/+}) and C3a (C3aR^{-/-}) mice or C3a (RAG-1^{-/-}C3aR^{+/+}) and C3a (RAG-1^{-/-}C3aR^{-/-}) mice are indicated as *p < 0.05 as determined by Student's *t* test. PBS controls shown were C3aR^{+/+} and RAG-1^{-/-}C3aR^{+/+} mice that were stimulated with PBS. PBS = phosphate-buffered saline.

(5, 29). Challenge of wild-type mice with the antigen resulted in increased numbers of mucin-glycoprotein-producing goblet cells as demonstrated by the presence of PAFS in the airway epithelium. In contrast, challenged $C3aR^{-/-}$ and $C3aR^{-/-}$ mice had reduced numbers of goblet cells, and quantitation of the PAFS staining from the lungs of these mice further verified a significant reduction in the amount of mucus relative to the challenged wild-type control animals (Figure 1D).

Studies have indicated that the levels of the human mucin gene product MUC5AC are up-regulated in goblet cells in asthma (4). Similarly, levels of the mouse ortholog to MUC5AC, Muc5ac, are also induced after antigen challenge in rodent models of allergic airway disease (29). Muc5ac expression was examined in the lungs of antigen-challenged $C3aR^{+/+}$ and $C3aR^{-/-}$ mice. Consistent with the PAFS stain, an increase in Muc5ac signal was only observed in the airway epithelium of antigen-challenged $C3aR^{+/+}$ mice, whereas very few Muc5ac-positive cells were detected in the PBS controls and the challenged $C3aR^{-/-}$ animals (Figures 1C and 1E).

C3a Induces Goblet Cell Formation Independently of T Lymphocytes and IL-13

To examine if C3a interactions with its receptor were sufficient to regulate goblet cell formation *in vivo*, $C3aR^{+/+}$ and $C3aR^{-/-}$ mice were instilled intranasally with C3a. Twenty-four hours after stimulation, lungs were isolated and stained with PAFS (Figure 2A). $C3aR^{+/+}$ mice stimulated with C3a exhibited increased PAFS positivity of airway epithelial cells relative to the PBS controls. In contrast, $C3aR^{-/-}$ mice stimulated with C3a

revealed minimal signal for PAFS. Quantitation of the PAFS staining from the lungs of these mice further demonstrated that the regulation of airway epithelial mucus production was dependent on C3aR because increased mucus levels were only observed in the airways of wild-type, but not $C3aR^{-/-}$, mice exposed to C3a (Figure 2C). As a control for specificity, mice were also stimulated with C3a(desArg), which does not bind the C3aR with high affinity and is greatly reduced in ability to elicit inflammatory responses compared with intact C3a (30). Stimulation with C3a(desArg) did not demonstrate an increase in the numbers of goblet cells and the airway epithelium in these mice resembled that observed in the PBS control mice (Figure 2A).

The production of IL-13 by Th2 cells can regulate airway epithelial goblet cell formation (29, 31, 32), and our previous study has documented that Th2 responses and IL-13 levels are greatly attenuated in antigen-challenged $C3aR$ -deficient mice (14). Therefore, the ability of C3a to regulate goblet cell formation was examined in the absence of T lymphocytes and Th2 expression of IL-13. $C3aR^{-/-}$ mice were bred onto the T- and B-lymphocyte-deficient RAG-1 $^{-/-}$ mouse strain (20). Both RAG-1 $^{-/-}$ - $C3aR^{+/+}$ and RAG-1 $^{-/-}$ - $C3aR^{-/-}$ mice were then stimulated intranasally with C3a. As revealed by the PAFS stain and levels of PAFS-positive cells (Figures 2B and 2C), lungs from these mice demonstrated that formation of goblet cells after stimulation with C3a was dependent on the presence of the C3aR but was not dependent on T lymphocytes. Moreover, analysis of bronchoalveolar lavage fluid from these mice by ELISA failed to detect IL-13 after stimulation with C3a (data not shown).

Figure 3. Goblet cell formation in lungs from IL-13-stimulated $C3aR^{-/-}$ and RAG-1 $^{-/-}$ - $C3aR^{-/-}$ mice. Eight-week-old mice were instilled intranasally with 10 ng/ml of mouse IL-13, and lungs from IL-13-stimulated $C3aR^{+/+}$ and $C3aR^{-/-}$ mice (A) or RAG-1 $^{-/-}$ - $C3aR^{+/+}$ and RAG-1 $^{-/-}$ - $C3aR^{-/-}$ mice (B) were removed from the chest cavity after 24 hours. Five-micron sections were stained with periodic acid fluorescent Schiff (PAFS) and visualized by fluorescent microscopy. Sections shown ($\times 630$ original magnification) are representative of two separate experiments. Mucus production from IL-13-stimulated mice was quantified by measuring PAFS staining as described in METHODS (C). Values are presented as mean index $\pm$ SEM (PBS- $C3aR^{+/+}$, $n = 2$; IL-13- $C3aR^{+/+}$, $n = 6$; IL-13- $C3aR^{-/-}$, $n = 6$; PBS-RAG-1 $^{-/-}$ - $C3aR^{+/+}$, $n = 2$; IL-13-RAG-1 $^{-/-}$ - $C3aR^{+/+}$, $n = 6$; IL-13-RAG-1 $^{-/-}$ - $C3aR^{-/-}$), and no significant differences between IL-13 ($C3aR^{+/+}$) and IL-13 ($C3aR^{-/-}$) mice or IL-13 (RAG-1 $^{-/-}$ - $C3aR^{+/+}$) and IL-13 (RAG-1 $^{-/-}$ - $C3aR^{-/-}$) mice were observed. PBS controls shown were $C3aR^{+/+}$ and RAG-1 $^{-/-}$ - $C3aR^{+/+}$ mice that were stimulated with PBS. PBS = phosphate-buffered saline.

To further ascertain if C3aR^{-/-} mice had a defect resulting from genetic deletion of the C3aR locus that prevented the airway epithelium from differentiating into goblet cells, the aforementioned strains of mice were also stimulated intranasally with IL-13. As shown in Figure 3, stimulation with IL-13 increased airway epithelial PAFS-positive staining in lungs from all strains of mice relative to the PBS controls (Figures 3A and 3B). Furthermore, levels of PAFS staining from the lungs of mice exposed to IL-13 were not significantly different whether mice were C3aR sufficient or C3aR deficient (Figure 3C), and indicate that C3aR deficiency does not impact the ability of airway epithelial cells to produce mucus upon exposure to a known regulator of goblet cells (29, 31, 32).

Airway Epithelial Clara Cells Express the Receptor for C3a

Under normal conditions, airway epithelial Clara cells express low levels of mucins in the proximal airways, but, with exposure to inflammatory stimuli, they can differentiate into goblet cells, which store and secrete elevated levels of mucins (25). Although C3aR expression has been previously described on human and mouse airway epithelial cells under conditions of allergic airway inflammation (11, 12), the expression of C3aR on Clara cells has not been fully characterized. To determine if C3aR is present on Clara cells and to evaluate the possibility that C3a and C3aR regulate the expression of Muc5ac by these cells, C3aR expression was examined on Clara cells isolated from C3aR^{+/+} and C3aR^{-/-} mice (Figure 4). Analysis of total mRNA by RT-PCR

revealed C3aR-specific RNA in Clara cells isolated from wild-type but not C3aR^{-/-} animals (Figure 4A). Western blot analysis of lysates from Clara cells obtained from C3aR^{+/+} mice and stained for C3aR protein revealed a band migrating at approximately 54 kD that was not present in lysates from C3aR^{-/-} animals (Figure 4A). FACS analysis using the same anti-C3aR antibody demonstrated a peak shift in Clara cells isolated from C3aR^{+/+} mice not seen with C3aR^{-/-} control mice (Figure 4B). These findings were further verified by immunofluorescence using Clara cells grown on coverslips (Figure 4C). Positive staining for C3aR was detected on isolated Clara cells from the C3aR^{+/+}, but not from the C3aR^{-/-}, mice.

C3a Regulation of Muc5ac Expression by Cultured Airway Epithelial Cells Is Dependent on C3aR

To determine if Clara cells isolated from the airways of C3aR^{+/+} and C3aR^{-/-} mice express Muc5ac in response to C3a, cells were isolated, cultured, and stimulated with 10 nM C3a for 24 hours. Cells were then stained for Muc5ac and visualized by fluorescent microscopy (Figure 5A). Clara cells isolated from C3aR^{+/+} mice and stimulated with C3a showed increased production of Muc5ac relative to Clara cells isolated from C3aR^{-/-} mice, which displayed a signal comparable to the media controls. Furthermore, stimulation of cells from C3aR^{+/+} mice with C3a(desArg) did not show a detectable increase in Muc5ac expression (Figure 5A). Cell culture supernatants in this experiment were also assayed for the presence of IL-13. Similar to results obtained *in vivo* when C3aR^{+/+} and C3aR^{-/-} mice were challenged intranasally with C3a, no IL-13 was detected in cultures of Clara cells stimulated with C3a *in vitro* (data not shown), indicating that the regulation of Muc5ac in this system can occur independently of IL-13.

Many studies have used cultures of polarized human and rodent airway epithelial cells to study mucin expression and goblet cell formation *in vitro*. Isolated airway epithelial cells are grown at an air-liquid interface; once differentiated, they are morphologically and functionally similar to airway epithelial cells observed *in vivo*. To determine if the interaction of C3a with C3aR stimulates mucin expression and goblet cell formation, tracheal epithelial cells isolated from mice and differentiated as described (23) were examined by RT-PCR and immunofluorescence for C3aR expression, and it was found that these cells express C3aR-specific mRNA and protein (data not shown). Polarized tracheal epithelial cells from C3aR^{+/+} and C3aR^{-/-} mice were then incubated with 10 nM C3a for 24 hours and stained by immunofluorescence for Muc5ac (Figure 5B). Consistent with data obtained using Clara cells, only cells isolated from C3aR^{+/+} mice showed an increase in Muc5ac signal in response to C3a, whereas cells from C3aR^{-/-} tracheal epithelia revealed no apparent increase in Muc5ac levels relative to media controls. Moreover, incubating C3a-stimulated polarized tracheal epithelial cells with increasing amounts of the C3aR antagonist SB 290157 (24) dramatically reduced Muc5ac expression in a dose-dependent manner (Figures 6A and 6B). Finally, C3a-stimulated cells were examined by transmission electron microscopy to assess if these cultured cells morphologically resembled airway epithelial goblet cells (Figure 5C). Analysis of C3a-stimulated wild-type epithelium revealed the presence of cells with densely packed granules organized at the luminal surface of the epithelial cells, which are structural characteristics of goblet cells. In contrast, these cellular structures were not observed in C3a-stimulated epithelium from C3aR^{-/-} mice.

DISCUSSION

These data establish a novel role for the complement activation product C3a and its receptor in the hypersecretion of mucus in

Figure 4. C3aR expression on isolated airway epithelial Clara cells. Analysis of C3aR mRNA by reverse transcriptase–polymerase chain reaction and protein by Western blot from lysates of airway epithelial Clara cells isolated from C3aR^{+/+} and C3aR^{-/-} mice (A). Analysis of C3aR protein expression on airway epithelial Clara cells isolated from C3aR^{+/+} (solid line) and C3aR^{-/-} (dashed line) mice by fluorescence-activated cell sorter (B) and by immunofluorescence ($\times 630$ original magnification) (C). Results shown are representative of three separate experiments.

Figure 5. Muc5ac expression and goblet cell formation in airway epithelial cells stimulated with C3a *in vitro*. Immunofluorescence for Muc5ac expression by airway epithelial Clara cells (A) or on cross sections of filters of polarized tracheal epithelial cells (B) isolated from C3aR^{+/+} and C3aR^{-/-} mice and stimulated with 10 nM C3a for 24 hours ($\times 630$ original magnification). Transmission electron microscopy for goblet cell formation on sectioned filters containing polarized tracheal epithelial cells isolated from C3aR^{+/+} and C3aR^{-/-} mice and stimulated with C3a as described above (ASL, aqueous surface layer; C, ciliated cell; G, goblet cell; S, secretory cell) (C). Media controls shown were cells isolated from wild-type mice that were stimulated with media alone, and samples shown are representative of at least two separate experiments conducted in triplicate.

asthma. This is supported by the observations that mice deficient in C3 or C3aR lack goblet cells and Muc5ac production in a model of allergic airway disease. Stimulation with C3a alone, but not C3a(desarg), was shown to induce goblet cells in the airway epithelia of mice and elevate Muc5ac expression in cultures of airway epithelial cells. C3a-induced mucin production was not observed in the absence of C3aR or in the presence of the C3aR antagonist SB 290157 demonstrating that binding of C3a to its receptor on airway epithelium was critical. Furthermore, this study demonstrates for the first time that C3aR is present on epithelial Clara cells, a cell type identified as the main producer of mucins in the proximal airways and the precursor to goblet cells during inflammation (25).

Current evidence supports the paradigm that allergens such as *A. fumigatus* can promote Th2 responses with the release of cytokines that contribute to much of the pathology in allergic airway disease (21). Much attention has focused on the Th2 cytokine IL-13 because blocking, genetic deletion, or overexpression of this mediator demonstrates its ability to trigger airway inflammatory responses and induce airway epithelial mucin production (29, 31, 32). The data here, however, demonstrate that C3a induces goblet cell formation and mucin production in T-cell-deficient mice and in the absence of IL-13 (Figure 2). In addition to promoting Th2 responses, reports have documented that *A. fumigatus* (33) as well as other allergens (8,9,34,35) are also efficient activators of the complement system. In the model of allergic airway disease used here, exposure of mice to the *A. fumigatus* antigen results in generation of C3a (unpublished observations) and production of IL-13 (14), both of which could have an additive impact on epithelial differentiation into mucus-producing goblet cells. This hypothesis is supported by the observations that intranasal stimulation of mice with either C3a or IL-13 results in a comparable increase in mucus indices (Figures 2C and 3C). However, neither mediator alone had the magnitude

of goblet cell development compared with challenging mice with the *A. fumigatus* antigen preparation (Figure 1D).

Furthermore, even though C3a regulation of mucus production required epithelial expression of C3aR (Figures 2 and 5), the fact that IL-13 could stimulate production of epithelial mucin in the presence or absence of C3aR (Figure 3) suggests that deficiency of C3aR has no apparent impact on the ability of IL-13 to regulate these responses. It is plausible that signaling by IL-13 and C3aR could converge on a common intracellular pathway. Activation of the epidermal growth factor receptor (EGFR) by IL-13, as well as many other inflammatory mediators, has been shown to promote goblet cell metaplasia in mice (36). Although little is known on how C3a and its receptor might interact with the EGFR, C3aR has been shown to activate members of the extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK) pathways (37–39), both of which are used by the EGFR to induce mucin expression (40–42). Further experimentation will delineate if C3a requires EGFR activation to regulate mucin synthesis.

The data presented here not only establish an important role for C3a and its receptor in regulating airway epithelial cell responses but also suggest a mechanism by which C3a could alter lung function during allergic airway inflammation. After exposure to airborne allergens, release of biologically active fragments during complement activation could initiate deleterious epithelial responses that promote airway obstruction. Previous reports have demonstrated that C3a and its receptor contribute to airway hyperresponsiveness in mouse models of allergic airway disease (8, 14). Given that C3a stimulates airway smooth muscle contraction (43, 44) and that airway smooth muscle cells express C3aR (11,12), it is likely that C3a and its receptor mediate airway hyperresponsiveness through airway smooth muscle contraction. However, C3a could also increase the airway resistance

Figure 6. Inhibition of C3a-mediated expression of Muc5ac by airway epithelial cells with the C3aR antagonist SB 290157. Immunofluorescence for Muc5ac expression by polarized tracheal epithelial cells isolated from C3aR^{+/+} and C3aR^{-/-} mice and stimulated incubated with 10 nM C3a and increasing doses of SB 290157 for 24 hours (A). Samples shown ($\times 400$ original magnification) are representative of at least two separate experiments conducted in duplicate. Muc5ac production from C3a-stimulated mice was quantified by measuring Muc5ac staining as described in METHODS (B). Values are presented as mean index $\pm$ SEM (media–C3aR^{+/+}, n = 2; SB 290157 [2 µg/ml]–C3aR^{+/+}, n = 4; C3a–C3aR^{+/+}, n = 4; C3a–C3aR^{-/-}, n = 4; C3a+SB 290157 [0.5 µg/ml]–C3aR^{+/+}, n = 4; C3a+SB 290157 [1 µg/ml]–C3aR^{+/+}, n = 4; C3a+SB 290157 [2 µg/ml]–C3aR^{+/+}, n = 4), and significant differences between C3a (C3aR^{+/+})– and C3a+SB 290157 (C3aR^{+/+})–treated cells are indicated as *p < 0.05 as determined by Student's *t* test. Media controls shown were cells isolated from wild-type mice that were stimulated with media alone.

observed in these models by obstructing the airway lumen with elevated epithelial production of mucus.

In addition to the results described here, additional reports have suggested a role for C3a and epithelial expression of the C3aR in airway obstruction using mice deficient in other complement components. Challenging mice deficient in factor B, a complement component required for alternative pathway activation, resulted in reduced airway C3a levels and periodic acid Schiff (PAS)-positive airway epithelial cells compared with their controls (45). Moreover, emphasis has been placed on the C5a anaphylatoxin and its receptor in allergic airway disease (46–49). Although challenge of C5-deficient (B10.D2/oSn) or C5aR-deficient mice does not significantly alter the number of goblet cells compared with controls in our hands (data not shown), recent studies examining respiratory syncytial virus infection in C5-deficient (B10.D2/oSn) mice demonstrated that the development of PAS-positive epithelium could be blocked through the use of the C3aR antagonist SB 290157, suggesting that C3a, and not C5a, was responsible for goblet cell formation in this model (50). In contrast, a report by Kohl and coworkers (46) described reduced goblet cell numbers in antigen-challenged C5aR^{-/-} mice. These opposing observations may result from the complex regulation of airway epithelial cells by C3a and C5a in these models.

Collectively, these studies identify a novel C3aR-dependent mechanism for regulating goblet cells and Muc5ac production and implicate C3a as a major mediator in airway obstruction in asthma. Considering the important contribution of airway epithelium to the pathology in asthma, further study of the role

of complement activation, C3a, and its receptor in these complex mechanisms will delineate the overlapping contributions leading to airway inflammation and obstruction in this disease.

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