Title
Asthma-associated allergen Alternaria induces STAT6 dependent epithelial FIZZ1 that promotes airway fibrosis

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Asthma-associated allergen *Alternaria* induces STAT6 dependent epithelial FIZZ1 that promotes airway fibrosis

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology by Naseem Melissa Khorram

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Professor Elina Zuñiga

2011
The Thesis of Naseem Melissa Khorram is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2011
I dedicate this to my father who showed me the art and beauty in science and my mother without whose undying love and affection this would not be possible.
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Acknowledgements

I would first like to express my extreme gratitude to Dr. Doherty for his guidance, support, and wonderful mentorship. Not only has Dr. Doherty taught me about Immunology, he has also served as a wonderful role model for the type of physician scientist I would like to become. He has taught me a lifetime of lessons that extend far beyond experimental design.

I would also like to thank Dr. Broide for giving me the opportunity to join his lab. His belief in my success as a scientist has provided me with great motivation. He has been a wonderful mentor and his guidance and instruction are forever appreciated.

Next, I would like to thank Dr. Dean Sheppard, Dr. Kotaro Sugimoto, Peter Rosenthal, Alexa Pham, Dr. Jae Youn Cho, Dr. Marina Miller, and Dr. Michael Croft for their various technical and intellectual contributions.

Finally, I would like to thank my family and friends, especially Jon Haynes for spending countless hours listening to me discuss my research and always encouraging and supporting me.
ABSTRACT OF THE THESIS

Asthma-associated allergen *Alternaria* induces STAT6 dependent epithelial FIZZ1 that promotes airway fibrosis

by

Naseem Melissa Khorram

Master of Science in Biology

University of California, San Diego, 2011

Professor David H. Broide, Chair

Professor Cornelis Murre, Co-Chair

*Alternaria* is a fungal allergen whereby sensitization to it serves as a risk factor for the development, persistence, and severity of asthma.

Naïve WT C57/B6 mice received one intranasal challenge with *Alternaria*, *Candida*, or *Aspergillus* allergen extracts and airway eosinophil numbers analyzed 24 hours later. RNA from airway epithelial cells was processed for gene microarray analysis. Lung cells from naïve WT and
collagen-1 GFP mice were incubated with rFIZZ1, stained for cell type, and analyzed by FACS. Mice received rFIZZ1 repetitively and airway eosinophils and lung histology were analyzed. Finally, WT and STAT6−/− bone marrow chimeric mice were challenged with Alternaria and airway eosinophil levels determined.

Only naïve mice that received one Alternaria challenge developed significant airway eosinophilia. Gene microarray analysis of airway epithelial cells demonstrated that Alternaria-challenged WT mice had an over 20-fold increase in expression of “Found in Inflammatory Zone 1” (FIZZ1/Retnla). Epithelial FIZZ1 and BAL eosinophils were reduced in STAT6-deficient, but not PAR-2-deficient mice. rFIZZ1 displayed binding to CD45+CD11c+ (macrophages and dendritic cells) and collagen-1 producing CD45− cells (fibroblasts). Administration of rFIZZ1 to naïve WT mice led to airway eosinophilia, peribronchial fibrosis, and increased thickness of the airway epithelium. Irradiated WT mice that received STAT6-deficient bone marrow showed reduced airway eosinophils after Alternaria challenge compared with irradiated STAT6-deficient mice that received WT bone marrow.

Alternaria induces acute airway eosinophilia dependent on STAT6 expressed in hematopoetic cells. Epithelial FIZZ1 expression is induced by Alternaria that binds to collagen-1 producing cells and promotes airway fibrosis and epithelial thickness.
I:

Introduction
An estimated 300 million people worldwide are afflicted by asthma (1). Asthma is characterized by chronic inflammation of the airways that can be life-threatening when severe and uncontrolled (1). Environmental allergens can serve as triggers for asthmatics and exacerbate symptoms. Despite the abundance of environmental allergens, very few are associated with the development and severity of asthma. Sensitization to the fungal allergen Alternaria alternata is a risk factor for the persistence and fatality of asthma (2, 4). Alternaria spores disperse during warm, dry weather predominantly in the late summer/early fall and have recently been detected at high levels indoors as well (3,4). The clinical associations with Alternaria and asthma are compelling but the mechanisms contributing to the airway responses remain a mystery.

Allergens, by definition, are environmental agents that are able to elicit T helper lymphocyte type 2 responses (5). Allergic inflammation mediated by adaptive immunity is initiated by the uptake of allergens by antigen-presenting cells (APCs) that present peptides on major histocompatibility complex II (MHC) molecules to naïve T cells. In a Th2 polarizing environment, naïve T cells differentiate into Th2 cells that produce IL-4, IL-5, and IL-13 and have pleiotropic effects in asthma (6). The precise mechanisms by which environmental allergens drive Th2 responses remain unknown, but increasing evidence is mounting that allergens can induce pathogenic innate immune responses (5, 7).
The innate immune system has evolved to recognize pathogen associated molecular patterns (PAMPS) through germ-line encoded pattern recognition receptors (PRR) (5). One such PRR family known as the toll-like receptor family (TLR) are known to play a role in allergic inflammation. For instance, stimulation of TLR2 and TLR4 pathways has been shown to both drive (8) and inhibit allergic inflammation (9). Additionally, a recent study suggests that the house dust mite allergen can activate TLR4 on the bronchial epithelium thereby driving a Th2 inflammatory response (10). Thus, evaluating the early epithelial responses to allergens may provide insight to asthma pathogenesis.

The airway epithelium serves as a barrier from the inhaled environment and when functioning normally, acts as the first line of defense (11). The structural integrity of the epithelium is maintained through the formation of tight junctions that are made up of various interacting proteins that allow cellular communication and regulate cellular transport (12). There is evidence that the airway epithelium in asthma is impaired and thus compromising its barrier activity (13). Furthermore, abnormal functioning of the epithelium is a source of cytokine and chemokine release that can drive Th2 inflammation (14). Thus, the epithelium may be central to the inflammatory response in asthma.

In addition to airway inflammation, remodeling of the airways occurs in asthma and is characterized by increases in subepithelial fibrosis, smooth muscle mass, mucus producing cells, and angiogenesis. The epithelium is
thought to play a role in airway remodeling through epithelial-mesenchymal transition as well as through production of pro-remodeling factors (11). Thickening of the airway walls through deposition of proteins such as fibronectin and collagen-I as well as an increase in smooth muscle are associated with chronic and severe asthma (15). Notably, airway remodeling is thought to be responsible for the progressive decline in lung function in asthma for which current corticosteroid therapies have little effect on reducing subepithelial fibrosis (16-18). The role of pro-remodeling mediators released very early from the epithelium after initial allergen exposure are largely unexplored.
II:

Results
Alternaria induces early airway eosinophilia

We examined the innate airway inflammatory response to fungal allergens Alternaria, Aspergillus, and Candida. Twenty-four hours after a single intranasal administration of these allergen extracts, only mice receiving Alternaria developed significant airway eosinophilia (Fig. 1A). Over 30% of the BAL cells were eosinophils (Siglec-F+CD11c- cells) after Alternaria exposure compared with less than 1% found after instillation of the same dose of Aspergillus, Candida, or control PBS (Fig. 1A). The total numbers of eosinophils were significantly elevated only in the Alternaria-treated mice (Fig. 1B). Thus, Alternaria specifically induces early airway eosinophilia when compared with two other fungal allergen extracts.

Cells obtained by bronchial brushing

In order to identify genes that may be upregulated in the epithelium during the initial response to Alternaria, we used a catheter brush to obtain cells from the airway as described in Materials and Methods. After several BAL washes to remove airway hematopoetic cells, cells from bronchial brushings appeared to resemble ciliated airway epithelial cells by morphology and immuno-stained positive for the epithelial marker E-cadherin (Fig. 2A). Compared with whole lung cell suspensions, the cells from the brushing were nearly all CD45 negative by FACS (Fig. 2B). Furthermore, of the CD45 negative population, nearly all cells expressed E-cadherin compared with
control antibody staining (Fig. 2B). Thus, we have utilized a method to obtain structural cells from the airway that are predominately epithelial cells.

Found in inflammatory Zone 1 (FIZZ1) is highly induced in the epithelium after Alternaria exposure

Utilizing the technique from Fig. 2, we isolated epithelial RNA from *Alternaria* and PBS challenged mice for microarray analysis. FIZZ1 (Retnla) was induced approximately 20-fold after *Alternaria* exposure in the expression array and subsequently confirmed by PCR to be induced 200 fold compared with PBS (Fig. 3A & B). Other genes with highly elevated transcript levels included some critically involved in mucus production including MUC5AC (8-fold) and C1ca3 (9-fold). Because mucus production occurs exclusively in the epithelium, the fact that these genes were highly induced in *Alternaria*-challenged mice supports the presence of an epithelial transcriptome.

Chitinase genes Ym1 (Chi3l3) and Chi3l1 and are members of a family of proteins increasingly associated with severe asthma and were induced five fold (30, 34). Interestingly, many other genes significantly upregulated in the epithelium of *Alternaria* exposed mice compared with PBS included muscle-related genes and structural proteins that may represent epithelial-mesenchymal transition proteins such as MYO18b, a marker for myocyte differentiation (31).
To visualize FIZZ1 expression in the *Alternaria* challenged airway, we performed immunofluorescent staining of lung sections. As expected, FIZZ1 was highly expressed in the epithelium in *Alternaria*-challenged mice compared with PBS (Fig. 3C). In contrast, we detected minimal expression of epithelial FIZZ1 in lung sections from mice challenged one time with fungal allergens *Aspergillus* and *Candida* (Fig. 3C). FIZZ1 expression was detected in the epithelium as early as 3 hr after a single *Alternaria* challenge and remained elevated for five days (Fig. 3D). Thus, airway epithelial FIZZ1 is specifically and highly induced early after exposure of mice to *Alternaria* and persists for days after one challenge.

*Alternaria*-induced FIZZ1 expression and eosinophilia is STAT-6 dependent but PAR-2 independent

The transcription factor STAT6 is critical to IL-4/IL-13 signaling and Th2 cell development (32), but little is known about an early role for STAT6 in airway disease. Previous reports have suggested that FIZZ1 is induced in a STAT6 dependent manner in other model systems (33, 35) suggesting that *Alternaria*-induced FIZZ1 expression may be regulated by STAT-6. To investigate this, WT and STAT6-deficient (STAT6−/−) mice were challenged with a single dose of *Alternaria*. FIZZ1 expression was nearly absent in the epithelium of STAT6−/− mice (Fig. 4A). Lung FIZZ1 mRNA levels were also significantly reduced in *Alternaria* challenged STAT6−/− mice (Fig. 4B).
Additionally, BAL eosinophilia was strongly reduced in STAT6\(-/-\) mice compared with WT mice (Fig 4D). This suggests that both FIZZ1 expression and the early airway eosinophilia induced by *Alternaria* are dependent on STAT6.

Fungal allergens are known to possess significant protease activity and early inflammatory responses in the airway can be impaired by addition of protease inhibitors (36). Additionally, *Alternaria* has been shown in-vitro to induce the pro-allergic cytokine TSLP in epithelial cells through protease activated receptor 2 (PAR-2) suggesting that other epithelial derived factors such as FIZZ1 may be regulated by PAR-2 (21). To test this, we challenged WT and PAR-2-deficient mice with a single dose of *Alternaria*. In contrast to STAT6\(-/-\) mice, PAR-2 deficient mice had only a slight reduction in the level of FIZZ1 expression in the epithelium and no difference in mRNA FIZZ1 levels (Fig. 4B). Furthermore, the BAL eosinophilia was unchanged in PAR-2 deficient mice compared with WT mice (Fig 4C and 4D). This suggests that both FIZZ1 epithelial expression and early airway eosinophilia after *Alternaria* challenge is not PAR-2 dependent.

FIZZ1 binds to hematopoetic and structural cells in the lung

The receptor for FIZZ1 is currently unknown, so we used a FIZZ1 capture assay (29) to identify cell types in the lung that bind to FIZZ1. This assay involved incubating single cell suspensions of naïve lung cells with
recombinant FIZZ1 (rFIZZ1) followed by the addition of primary and secondary antibodies (Fig 5A). The control samples did not contain rFIZZ1, but were identically processed otherwise. Lung cells were gated on hematopoetic (CD45+) or structural cells (CD45-). Lung CD45+CD11c+ cells, comprised of both macrophages and dendritic cells (27), displayed significant FIZZ1 binding after capture assay was performed (Fig. 5B). Analysis of CD45+CD11c- cells revealed a smaller population that bound FIZZ1. The non-hematopoetic compartment (CD45-) also displayed binding to FIZZ1 (Fig. 5C), suggesting that multiple cell types within the naïve lung may respond to FIZZ1. Collagen-1 CD45- cells (fibroblasts) also showed FIZZ1 binding (Fig. 5D).

Intranasal rFIZZ1 administration leads to airway eosinophilia and fibrosis

To evaluate possible roles of FIZZ1 in the airway, we administered recombinant FIZZ1 (rFIZZ1) to naïve mice for 5d and performed BAL and histologic analysis 3 days later. Mice that received rFIZZ1 had elevated levels of eosinophils in the airways compared with mice that received only PBS (Fig. 6A). These mice also had evidence of increased epithelial thickness (Fig. 6B), a feature associated with severe asthma (22).

Airway remodeling is an important feature of asthma and previous reports have suggested that FIZZ1 may promote remodeling changes such as collagen deposition and myofibroblast differentiation (23-25). Additionally, repetitive intranasal administration of recombinant FIZZ1 was found to induce
fibrotic changes in a lung granuloma model (26). Thus, we determined levels of airway fibrosis in lung sections from mice that received intranasal rFIZZ or PBS. Elevated levels of peribronchial fibrosis detected by trichrome staining and increased collagen-1 immunofluorescent staining were present in lung sections from mice that received rFIZZ1 compared with those that received PBS (Fig. 6C). These data suggest that FIZZ1 may have several roles in the airway including promoting eosinophilia, epithelial changes, and peribronchial fibrosis.

Hematopoietic STAT6 expression contributes to early eosinophilia induced by *Alternaria*

To evaluate whether the dependence of STAT6 for early eosinophilia was due to structural cells in the lung or bone marrow derived lung cells, WT and STAT6\(^{-/-}\) chimeric mice were generated as in *Materials and Methods*. Donor bone marrow cells were tracked using a surface congenic marker (CD45.1 or CD45.2). Airway cells from mice six weeks after irradiation and bone marrow transfer revealed successful (90-95%) chimerism (Fig 7A). Twenty-four hours after a single *Alternaria* challenge, WT mice that received STAT6\(^{-/-}\) bone marrow donor had significantly reduced eosinophilia when compared to WT or STAT6\(^{-/-}\) mice that received WT bone marrow (Fig 7B). Thus, hematopoietic expression of STAT6 contributes to *Alternaria* induced early eosinophilia.
Dr. Dean Sheppard and Dr. Kotaro Sugimoto contributed to the development of the bronchial brushing technique that was optimized by Alexa Pham and Dr. Marina Miller (Fig. 2). Dr. Jae Youn Cho offered technical support for the immunofluorescent FIZZ1 staining (Fig. 3C, 3D, 4A). Peter Rosenthal provided technical support for generating chimeric mice. Dr. Taylor Doherty contributed to the experimental design and data collection for all experiments. Dr. Croft and Dr. Broide provided intellectual insight.
Figure 1. Single airway exposure of *Alternaria* in naïve WT mice induces early eosinophilia.

A. Naïve mice received a single intranasal challenge with 100ug of *Alternaria*, *Candida*, or *Aspergillus* extract or PBS and BAL performed 24 hours later for eosinophil percent by FACS.

B. Absolute BAL eosinophil counts 24 hours after a single allergen challenge. Eosinophils were defined as Siglec-F+ CD11c- cells. FACS plots representative of 4-7 mice per group. Eosinophil numbers for individual mice shown, *p<0.05, **p<0.0001, t- test.
Figure 2. Cells obtained by bronchial brushing.

A. Cells collected by bronchial brushing of large airways in naïve mice were analyzed by morphology and immunostained for E-cadherin.
B. Live cells pooled from 6 mice obtained by bronchial brushing were analyzed by FACS (center) and compared with naïve whole lung (left) and stained for E-cadherin (right). Grey shaded region on histogram represents isotype control.
A. Control Ab  E-cadherin

B. CD45  SSC

Whole lung  Epithelial Brushing  E-cadherin

95.6  98.6
Figure 3. Epithelial gene microarray analysis and FIZZ1 expression after allergen challenge.

A. Microarray analysis on bronchial epithelial cells 24 hours after a single *Alternaria* or PBS exposure.
B. Confirmatory qPCR performed on bronchial brush samples for FIZZ1 (*Retnla*).
C. Lung sections from mice challenged with either *Alternaria*, *Candida*, *Aspergillus* extracts or PBS and immunofluorescent staining performed for FIZZ1 at 24 hours after challenge.
D. Lung sections with immunofluorescent staining for FIZZ1 from mice challenged with *Alternaria* 3 hours (left) or 5 days (right) before sacrifice. Scale bars 100 µm. *p < 0.01, 3 mice per group (A), 3-4 mice per group in (B).
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Figure 3 continued.
Figure 3 continued.
Figure 4. *Alternaria*-induced FIZZ1 expression and early eosinophilia is dependent on STAT6, but not PAR-2.

A. WT, STAT6−/−, or PAR-2−/− mice were challenged with *Alternaria* and FIZZ1 immunofluorescent staining performed on lung sections.
B. FIZZ1 mRNA levels measured in lung samples from WT, STAT6−/−, or PAR-2−/− mice.
C. Percent BAL eosinophils by FACS for WT, STAT6−/−, or PAR-2−/− mice 24 hours after *Alternaria* challenge.
D. Total BAL eosinophils for WT, STAT6−/−, or PAR-2−/− mice 24 hours after *Alternaria* challenge.
4-6 mice in each group, Scale bars 100um, *p < 0.005 and **p < 0.01 compared with WT mice, t test.
A.

WT  STAT6 -/-  PAR-2 -/-

B.

Fold Increase (FIZZ1/18S)

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C.

Siglec F

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Figure 4 continued.
Figure 5. Naïve lung hematopoetic, structural cells, and collagen-1 CD45$^-$ cells can bind FIZZ1.

A. FIZZ1 staining technique.
B. Single cell suspensions from two naïve lungs were incubated with rFIZZ1 and stained with CD45 and CD11c. CD45 positive cells were gated on CD11c$^+$ and CD11c$^-$ populations (right) and analyzed for FIZZ1 binding.
C. CD45$^-$ cells were gated and analyzed for FIZZ1 binding.
D. Single cell suspensions from two naïve GFP collagen-1 mice were incubated with rFIZZ and gated on CD45$^-$ cells and analyzed for FIZZ1 binding.
C.

D.

Figure 5 continued.
Figure 6. Administration of exogenous rFIZZ1 induces airway eosinophilia and fibrosis in naïve WT mice.

A. Total BAL eosinophils after naïve WT mice received intranasal rFIZZ1 or vehicle (PBS) for five days in a row and BAL and lung were analyzed three days later. 4 mice per group, 19-25 airways.

B. Epithelial thickness measured in H&E lung sections from mice receiving rFIZZ (right) or PBS (middle). 4 mice per group, 19-25 airways.

C. Lung sections stained with trichrome and scored (top). Lung sections stained with collagen-1 (bottom row).

*p < 0.05, t test. **p < 0.0001, ***p < 0.001, Mann-Whitney. Scale bars, top row 100 µm; bottom row 50 µm.
Figure 6 continued.
Figure 7. Hematopoetic expression of STAT6 contributes to Alternaria-induced eosinophilia.

A. Percent chimerism in BAL. CD45.1 B6 mice received bone marrow from either CD45.2+ or STAT6−/− mice. CD45.2+ mice received bone marrow from CD45.1+ mice. STAT6−/− mice received bone marrow from CD45.1+ mice.

B. Total BAL eosinophils 24 hours after chimeric mice received intranasal Alternaria. *p<0.05.
A.

B. Total Eosinophils ($\times 10^4$)
III:

Discussion
Asthma is a complex disorder characterized by airway inflammation and remodeling. The majority of asthmatics have allergic triggers, and while there are many environmental allergens only a few including *Alternaria* have been associated with development, persistence, and severity of asthma. Exposure to *Alternaria* has been linked to a life-threatening degree of asthma exacerbation (4). The mechanisms controlling the unique pathogenicity of *Alternaria* remains largely unknown.

To identify highly expressed genes in the airway epithelium twenty-four hours after a single intranasal challenge of *Alternaria*, we used a bronchial brushing technique to isolate airway epithelial cells for microarray analysis. The most highly induced gene was the resistin-like molecule found in inflammatory zone 1 (FIZZ1), or Retnla/RELMα. FIZZ1 has been implicated in fibrosis (33), smooth muscle contraction (19) and associated with increased lung inflammation (20). We sought to determine epithelial regulation of FIZZ1 in-vivo as well as possible functions for this molecule in the airway. Compared with other fungal allergens *Aspergillus* and *Candida*, we found that only *Alternaria* induces airway eosinophilia 24 hours after a single exposure. Others have shown that PAR-2 is required in-vitro for epithelial cells and eosinophils to release pro-inflammatory mediators when exposed to *Alternaria* (21, 40). In contrast, we did not detect a reduction in airway eosinophilia after *Alternaria* exposure in PAR-2 deficient mice. Instead our data suggests requirement for the transcription factor STAT6 as mice deficient in STAT6
showed significantly reduced airway eosinophilia after *Alternaria* exposure (Fig 4C,D). In our studies, the whole allergen extract was utilized which contains large amounts of carbohydrates and lipids rather than a protease purified from *Aspergillus* that has been previously shown to act independently of STAT6 to induce early eosinophilia (36). Furthermore, we found similar protease activity (data not shown) between *Aspergillus* and *Alternaria* which supports a non-protease dependent mechanism.

While one recent report showed that *in vivo* primed WT CD4 OVA-specific T cells can induce allergic lung inflammation that is STAT6 dependent and STAT6 in the epithelium is required for FIZZ1 expression, the role of STAT6 in an innate eosinophilic model has not been characterized (42). In line with this, our data shows that STAT6 in the lung hematopoetic compartment is necessary for innate eosinophilia induction by *Alternaria*.

To identify genes that are upregulated in the airway epithelium after a single *Alternaria* challenge, we used a modified catheter to scrape cells from the left and right main bronchi. The ciliated morphology of the cells, absence of CD45 and presence of the epithelial factor E-cadherin, as well as upregulated mucus genes, suggests that we obtained an epithelial cell type although we are not able to exclude minor contamination from fibroblasts. Gene microarray analysis revealed the most highly expressed transcript to be FIZZ1. Our immunofluorescent staining for FIZZ1 confirmed its presence in the airway epithelium after *Alternaria* challenge. This is in accordance with the
initial description of FIZZ1 as being expressed by the airway epithelium after allergen exposure (41).

FIZZ1 is primarily expressed by the inflamed airway epithelium as well as alternatively activated macrophages during Th2 inflammatory responses (29). In support of our findings, FIZZ1 expression in various models of lung fibrosis was found to be dependent on STAT6 (33,35). FIZZ1 signaling is believed to occur through Bruton’s-tyrosine kinase (BTK) and Notch 1, but its receptors remain unknown (43,37). Utilizing a similar technique to the previously published FIZZ1 capture assay (29) we found that FIZZ1 binds both structural cells (CD45-) as well as hematopoetic cells (CD45+) namely CD11c+ dendritic cells and macrophages. Furthermore, we found that FIZZ1 also binds collagen-1 producing CD45- cells (fibroblasts). This data is consistent with previous findings that FIZZ1 can bind dendritic cells (29) as well as structural cells (23, 24) and plays a role in myofibroblast differentiation (20).

We found that FIZZ1 given repetitively to naïve mice can lead to airway eosinophilia. This is consistent with previous reports of FIZZ1 as a regulator of eosinophil chemotaxis in the gastrointestinal tract (38, 39). While our data suggests that FIZZ1 is likely playing an early pro-inflammatory role, others have suggested an anti-inflammatory role for FIZZ1 during more chronic Th2 inflammatory models (25, 26, 29). Further research is necessary to fully discern the multiple roles of FIZZ1 in lung inflammation.
In summary, we have identified a unique early airway eosinophilic response to *Alternaria* that is dependent on STAT6 in lung hematopoietic cells. Using an empiric approach to decipher what factors are released by the airway epithelium in response to *Alternaria*, gene microarray analysis revealed FIZZ1 as the most highly induced gene. We found that exogenous FIZZ1 administered to naïve mice results in the accumulation of eosinophils in the airway and airway fibrosis. While this highlights the potential importance of FIZZ1 in allergic asthma and airway remodeling further work will be required to fully elucidate the functions of resistin molecules in human asthma during chronic lung inflammation and whether human resistin homologues have similar functions in asthma.
IV:

Materials and Methods
Mice and airway challenges

Six to eight week-old female C57BL/6 mice (Jackson and Charles River Laboratories) were given intranasal challenges of 100µg of Alternaria (1.15 EU), Candida (0.08 EU), or Aspergillus (0.53 EU) (Greer, Lenoir NC) in 80µl and sacrificed 24 hours later. For some experiments mice were analyzed 3 hours and 5 days after challenge. Control groups of mice were given intranasal challenges of 80µL PBS. PAR-2 and STAT6 deficient mice were obtained from Jackson laboratories. In some experiments, 5µg of recombinant Fizz-1 (Peprotech) or vehicle (PBS) was given intranasally to mice every day for five days and mice killed on day 8. The University of California San Diego Institutional Animal Care and Use Committee approved all experiments.

Isolation of airway epithelial cells

Prior to performing epithelial brushing, bronchoalveolar lavage (BAL) was performed to remove airway hematopoietic cells. Sterile plastic feeding tubes (Solomon Scientific) were modified by removal of the rubber bulb, sanding to create roughness, and autoclaving. The tube was inserted into the right main and left main bronchus with gentle brushing and immediately placed in RNALater (Qiagen).

Airway Cellular Analysis and Lung Processing
BAL fluid was obtained by intratracheal insertion of a catheter and five lavages with 0.8 mL of 2% filtered bovine serum albumin (BSA) (Sigma). The right lung was tied off, removed, and snap-frozen in liquid nitrogen for RNA isolation. The left lung was instilled with 0.4 mL 4% paraformaldehyde (PFA) and placed in PFA for paraffin embedding and staining. To obtain lung single cells suspensions, lungs were manually minced and shaken in RPMI with 2 mg/ml collagenase and 1mg/ml DNAse I (Roche) for 35 minutes. Cells were purified using a 70-um cell strainer (BD Falcon). Live total BAL cells were counted using the Accuri C6 flow Cytometer. Cells were subsequently washed with FACS buffer and eosinophils were identified as the SiglecF⁺CD11c⁻ population.

Microarray and real time RT-PCR analysis

Airway epithelial cells were lysed by multiple passages through an 18G needle. RNA was then extracted according to manufacturer’s protocol (Qiagen). Isolated epithelial RNA with sufficient purity and yield was used for microarray analysis (GeneChip Mouse Gene 1.0 ST, Affymetrix). Gene chip results were analyzed with Vampire software and confirmed by quantitative real-time PCR. For whole lung RNA extraction, the lung was homogenized in TRIzol reagent (Invitrogen) and RNA was extracted according to manufacturer’s protocol (Qiagen). RNA yield and purity was measured with the Nanodrop 1000 (Thermo Scientific). Single-strand cDNA was prepared by
reverse transcription of 1 µg of total RNA with the SuperScript III kit (Invitrogen). Fizz-1 gene expression was quantified by amplification of cDNA in SYBR Green Supermix (Applied Biosystems) using the following primer pairs: forward, 5'-CCC TTC TCA TCT GCA TCT C-3', reverse, 5'-CAG TAG CAG TCA TCC CAG CA-3'. Triplicates of samples were run with the mean value used for quantification.

Flow Cytometry

BAL cells were incubated with a monoclonal antibody to CD16/CD32 (24G.2) for 10 minutes to block Fc receptors and then stained with PE-conjugated Siglec-F, FITC-conjugated CD11c, and APC-conjugated Gr-1 (eBioscience) for 30 minutes. FIZZ1 capture assay was performed similar to previously described (29). Briefly, single cell suspensions from lungs of naïve mice were incubated with 0.5 µg rFIZZ1, or FACS buffer alone, for 60 minutes at 4°C. Cells were washed and incubated with Fc blocking antibody for 30 minutes, stained with FITC-CD11c, APC-CD45, and polyclonal rabbit anti-FIZZ1 (Peprotech) for 30 minutes followed by addition of PE-conjugated anti-rabbit Fab fragment (eBioscience).

Immunofluorescence

For collagen-1 and Fizz-1 staining, lung samples were de-paraffinized by sequential placement in xylene and ethanol. Staining for collagen-1 was
performed with a polyclonal antibody (Millipore) diluted 1:500 and staining for Fizz-1 was performed with rabbit polyclonal antibody (PeproTech) at 1:1,000 concentration. Tyramide Signal Amplification Kit #41 (Invitrogen) was used for fluorescent signal amplification with subsequent DAPI staining (Vector Laboratories). Lung airways were visualized with a DM2500 microscope (Leica Microsystems).

Remodeling Analysis and Epithelial Thickness

Lung sections were stained with Masson’s Trichrome and the area of peribronchial fibrosis on trichrome-stained sections was quantified by analysis with Image-Pro Plus software (27). All slides were blinded and results are expressed as the area of positive staining per micrometer length of bronchiole basement membrane. Hematoxylin and eosin stained lung sections were used to evaluate the thickness of the epithelium. The length in micrometers was measured from the bottom of the basement membrane to the end of the epithelium. Six individual areas were measured per airway and a minimum of four airways was analyzed per slide. All measurements were done with the Image-Pro Plus software.

WT and STAT6−/− bone marrow chimeras

CD45.1+ and STAT6−/− (CD45.2+) (Jackson Laboratories) recipient mice received 5mL/200mL water of Sulfatrim antibiotic (Med Vet International) for
one week prior to receiving donor bone marrow and two weeks after receiving bone marrow. Recipient mice were irradiated twice with 450R separated by two hours. Donor bone marrow was isolated from both tibias and fibias and 15 million cells were injected to recipients via tail vein. Mice rested for six weeks to allow full chimerism before *Alternaria* challenge. Successful chimerism was assessed by FACS analysis on BAL cells for percent CD45.1+ and CD45.2+ cells.

Statistical Analysis

Statistical analysis was performed using Prism Software (Graphpad). The Mann-Whitney test or student’s t-test was used as indicated.
References


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