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Alternaria Induces STAT6-Dependent Acute Airway Eosinophilia and Epithelial FIZZ1 Expression That Promotes Airway Fibrosis and Epithelial Thickness

Taylor A. Doherty,*,† Naseem Khorram,* Kotaro Sugimoto,‡ Dean Sheppard,‡ Peter Rosenthal,* Jae Youn Cho,* Alexa Pham,* Marina Miller,* Michael Croft,† and David H. Broide*

The fungal allergen, *Alternaria*, is specifically associated with severe asthma, including life-threatening exacerbations. To better understand the acute innate airway response to *Alternaria,* naive wild-type (WT) mice were challenged once intranasally with *Alternaria.* Naive WT mice developed significant bronchoalveolar lavage eosinophilia following *Alternaria* challenge when analyzed 24 h later. In contrast to *Alternaria*, neither *Aspergillus* nor *Candida* induced bronchoalveolar lavage eosinophilia. Gene microarray analysis of airway epithelial cell brushings demonstrated that *Alternaria*-challenged naive WT mice had a >20-fold increase in the level of expression of found in inflammatory zone 1 (FIZZ1/Retnla), a resistin-like molecule. Lung immunostaining confirmed strong airway epithelial FIZZ1 expression as early as 3 h after a single *Alternaria* challenge that persisted for ≥5 d and was significantly reduced in STAT6-deficient, but not protease-activated receptor 2-deficient mice. Bone marrow chimera studies revealed that STAT6 expressed in lung cells was required for epithelial FIZZ1 expression, whereas STAT6 present in bone marrow-derived cells contributed to airway eosinophilia. Studies investigating which cells in the nonchallenged lung bind FIZZ1 demonstrated that $CD45^+CD11c^+$ cells (macrophages and dendritic cells), as well as collagen-1–producing $CD45^-$ cells (fibroblasts), can bind to FIZZ1. Importantly, direct administration of recombinant FIZZ1 to naive WT mice led to airway eosinophilia, peribronchial fibrosis, and increased thickness of the airway epithelium. Thus, *Alternaria* induces STAT6–dependent acute airway eosinophilia and epithelial FIZZ1 expression that promotes airway fibrosis and epithelial thickness. This may provide some insight into the uniquely pathogenic aspects of *Alternaria*-associated asthma. ***The Journal of Immunology*, 2012, 188: 000–000.**

Although asthma has many clinical, physiologic, and immunologic phenotypes, the majority of asthmatics have environmental allergic triggers, including dust mite, cockroach, and mold. *Alternaria* is an example of a common fungal allergen that is associated with the development of asthma (1). Sensitization to *Alternaria alternata* is a risk factor for persistence of asthma and fatal/near-fatal asthma (2–8). The spores of *Alternaria* are known to be a source of outdoor allergens for sensitized individuals, and they were recently detected at high levels indoors (9). Dispersion of the spores occurs during periods of warm, dry weather, especially in late summer/early fall, and has been associated with epidemic, severe asthma symptoms (2–8). Such clinical associations with *Alternaria* and asthma are intriguing, but the mechanisms contributing to the pathologic airway responses are still incompletely understood.

Allergic disease, including asthma, has largely been characterized by dysregulation of adaptive immunity in response to allergens, including Th2 cell differentiation and IgE sensitization. More recently, it has become clear that innate immune responses to allergens in the airway help to shape subsequent adaptive responses (10, 11). For example, recent reports have suggested that allergens with high protease activity, such as cockroach and fungal allergens, induce innate inflammatory events and allergen sensitization through a protease-activated receptor 2 (PAR-2)–mediated pathway in the bronchial epithelium (12–14). Investigations into such innate epithelial responses to inhaled allergens may provide important clues to the pathogenesis of asthma. In this study, we investigated whether *Alternaria* is able to induce an acute Th2-like airway inflammatory response in naive wild-type (WT) mice via activation of innate epithelial genes. We demonstrate that *Alternaria* induces a significant acute airway eosinophil response in naive WT mice that is mediated by innate immune mechanisms distinct from those triggered by protease allergens through PAR-2 on the epithelium. This innate proeosinophil inflammatory and proremodeling effect of *Alternaria* in naive WT mice is not shared with other common fungal allergens, such as *Aspergillus* and *Candida*, suggesting that different allergens trigger distinct innate airway epithelial pathways that contribute to asthma.

Materials and Methods

Mice and airway challenges

Six- to eight-week-old female naive C57BL/6 WT mice were administered 100 μg *A. alternata* (lot 130656), *Candida albicans* (lot 111797), or *As-*

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Microarray data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE34764.

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Abbreviations used in this article: BAL, bronchoalveolar lavage; FIZZ1, found in inflammatory zone 1; PAR-2, protease-activated receptor 2; rFIZZ1, recombinant found in inflammatory zone 1; TSLP, thymic stromal lymphopoietin; WT, wild-type.

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pergillus fumigatus (lot 118033) extracts (Greer, Lenoir, NC) intranasally in 80 μl PBS; they were killed 24 h later, at which time bronchoalveolar lavage (BAL) and lung specimens were processed. For selected experiments, naive WT mice were analyzed 3 h and 5 d after challenge. Control groups of naive WT mice were given intranasal challenges of 80 μl PBS. In selected experiments, PAR-2–deficient or STAT6-deficient mice (Jackson Laboratories) on a B6 background were administered 100 μg *A. alternata* extracts intranasally with WT controls, as described above. Collagen-1 GFP reporter mice were a gift from Dr. David Brenner (University of California San Diego, La Jolla, CA) and were described previously (15).

In some experiments, 5 μg recombinant FIZZ1 (rFIZZ1; PeproTech) or vehicle (PBS) was given intranasally to naive WT mice every day for 5 d, and mice were killed on day 8. The endotoxin level detected in rFIZZ1 was 0.0051 ng/ml by *Limulus* assay (Lonza). All experiments were approved by the University of California San Diego Institutional Animal Care and Use Committee.

BAL cellular analysis, lung processing, and FACS

BAL and lung processing were performed as previously described (16). BAL fluid was obtained by intratracheal insertion of a catheter and five lavages with 0.8 ml 2% filtered BSA (Sigma). The right lung was tied off, removed, and snap-frozen in liquid nitrogen for RNA isolation or ELISA. The left lung was instilled with 0.4 ml 4% paraformaldehyde and placed in paraformaldehyde for paraffin embedding and staining. To obtain lung single-cell suspensions, lungs were minced and shaken vigorously in RPMI 1640 with 2 mg/ml collagenase and 1 mg/ml DNAse I for 40 min. Lung cells were isolated using a 70-μm cell strainer.

BAL cells were incubated with a mAb to CD16/CD32 (24G.2) for 10 min to block FcRs and then stained with PE-conjugated Siglec-F, FITC-conjugated CD11c, and allophycocyanin-conjugated Gr-1 (eBiosciences) for 30 min. BAL cells were washed with FACS buffer, and eosinophils were identified as the Siglec-F^+ $CD11c^-$ population (17). FACS was performed using an Accuri C6 flow cytometer and analyzed with FlowJo software (Tree Star, Ashland, OR).

ELISA for cytokines and total IgE

ELISA of lung homogenate IL-5 and IL-13 (R&D Systems) was performed, as previously described (16). ELISA for IL-33 (R&D Systems) was performed on BAL supernatant. Serum total IgE was performed with an IgE ELISA kit (BD Biosciences), according to the manufacturer's instructions, and all ELISA plates were read with a BioRad Model 680 microplate reader.

Isolation of airway epithelial cells

To study which epithelial genes were induced by *Alternaria*, we adapted a technique first developed by two of the authors (K.S. and D.S.) for obtaining airway epithelial cells in mice by bronchial brushing (18). Prior to performing epithelial brushing, BAL was performed to remove BAL cells. The bronchial brushing was performed using a sterile plastic feeding tube (Solomon Scientific) 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).

Microarray and real-time RT-PCR analysis

Airway epithelial cells obtained by bronchial brushing were lysed by multiple passages through an 18G needle. RNA was then extracted, according to the manufacturer's protocol (Qiagen). Isolated epithelial RNA with sufficient purity (A260/280 = 1.97–2.12) and yield (8.99–12.66 ng/μl) was used for microarray analysis (GeneChip Mouse Gene 1.0 ST; Affymetrix). Gene chip results were confirmed by quantitative real-time PCR and analyzed with Vampire software. Microarray data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34764) and given accession number GSE34764. For whole-lung RNA extraction, the lung was homogenized in TRIzol reagent (Invitrogen), and RNA was extracted according to the manufacturer's protocol (Qiagen). RNA yield and purity were measured with the Nanodrop 1000 (Thermo Scientific). Single-strand cDNA was prepared by reverse transcription of 1 μg total RNA with the SuperScript III kit (Invitrogen). FIZZ1 was quantitated 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′ and reverse, 5′-CAG TAG CAG TCA TCC CAG CA-3′. Triplicates of samples were run with the mean value used for quantification.

WT and $STAT6^{-/-}$ bone marrow chimeras

WT CD45.1 and $STAT6^{-/-}$ ($CD45.2^+$) mice, purchased from Jackson Laboratories, received Sulfatrim antibiotic (5 ml/200 ml; Med Vet International) for 1 wk prior and 2 wk after receiving donor bone marrow. Recipient mice were irradiated twice with 450 rad, separated by 2 h. Donor bone marrow was isolated from both tibias and fibulas, and 15 million cells were injected to recipient via tail veins. Mice were rested for 6 wk before *Alternaria* challenge. Efficiency of chimerism was assessed by FACS for congenic markers CD45.1 and CD45.2 on BAL cells.

FIZZ1 lung-binding assay

The FIZZ1 lung-binding assay was adapted from a previously described method to detect FIZZ1 binding to mouse splenocytes (19). The strategy involved incubating naive WT lung cells with rFIZZ1, followed by the addition of primary and secondary Abs. Negative control lung samples were not incubated with rFIZZ1 but were identically processed otherwise.

To determine whether FIZZ1 binding colocalized with lung fibroblasts, single-cell suspensions from lungs of naive WT mice and collagen-1 GFP reporter mice were incubated with 0.5 μg rFIZZ1 or FACS buffer alone for 60 min at 4°C. Cells were washed and incubated with Fc blocking Ab for 30 min and stained with allophycocyanin-CD45 and polyclonal rabbit anti-FIZZ1 (PeproTech) for 30 min, followed by the addition of PE-conjugated anti-rabbit Fab fragment (eBiosciences). Cells from WT mice were also stained with FITC-CD11c.

Immunofluorescence

For collagen-1 and FIZZ1 staining, lung samples were deparaffinized by sequential placement in xylene and ethanol (16). Staining for collagen-1 was performed with a polyclonal Ab (Millipore) at 1:500 concentration, and staining for FIZZ1 was performed with rabbit polyclonal Ab (PeproTech) at 1:1000 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

Paraffinized 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 (16). All slides were blinded, and results are expressed as the area of positive staining per micrometer length of bronchiole basement membrane. H&E-stained lung sections were used to evaluate the thickness of the epithelium in micrometers was measured from the bottom of the basement membrane to the mucosal surface of the bronchial epithelium. Six individual areas per airway were measured, and a minimum of four airways was analyzed per slide. All measurements were done with Image-Pro Plus software.

Airway hyperresponsiveness

In some experiments, invasive pulmonary-function testing was performed using the Flexivent system (Scireq, Montreal, Canada), and airway resistance was analyzed by Scireq Flexivent 5.1 software, as previously described (16). Briefly, mice were anesthetized, cannulated via the trachea, and administered increasing doses of methacholine, and airway resistance was measured.

Statistical analysis

Statistical analysis was performed using Prism Software (GraphPad). The Mann–Whitney U test or Student t test was used, as indicated.

Results

Alternaria *induces acute BAL airway eosinophilia and lung Th2 cytokine production in naive WT mice*

We examined the acute airway inflammatory response to fungal allergens *Alternaria*, *Aspergillus*, and *Candida* in naive WT mice. One day after a single intranasal administration of these allergen extracts, only mice receiving *Alternaria* developed significant airway eosinophilia (Fig. 1A). More than 30% of the BAL cells were eosinophils (Siglec-F^+ $CD11c^-$ cells) after acute *Alternaria* exposure compared with <1% found after instillation of the same dose of *Aspergillus*, *Candida,* or control PBS (Fig. 1A). The total number of BAL eosinophils was significantly elevated only in the *Alternaria*-treated mice (Fig. 1A). *Alternaria* exposure induced acute airway eosinophilia in a dose-response manner. Naive WT mice challenged with increasing doses of *Alternaria* (10, 50, and 100 μg) had increases in both the percentage and total airway

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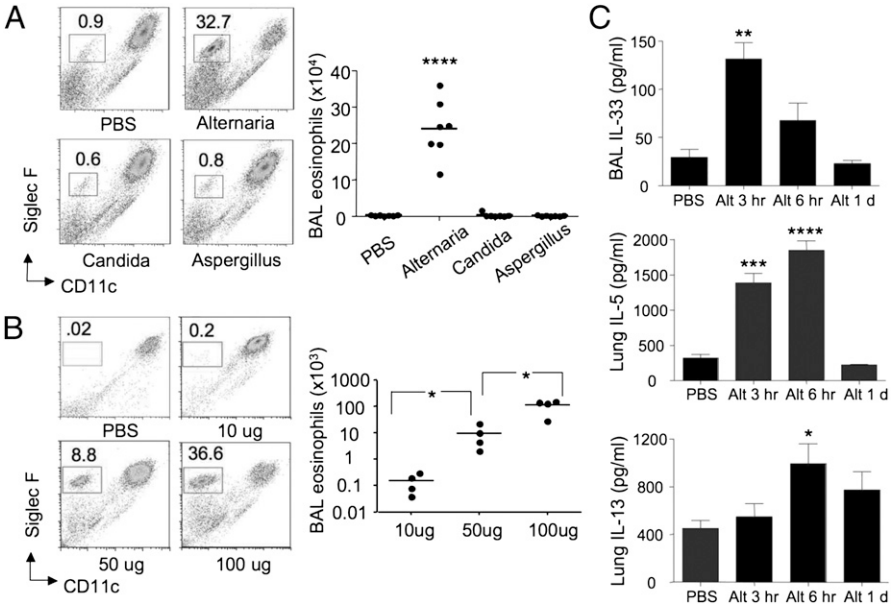

FIGURE 1. Single acute airway exposure of *Alternaria* in naive WT mice induces early eosinophilia and Th2 cytokine production. Naive mice received a single intranasal challenge with 100 μg of *Alternaria*, *Candida*, or *Aspergillus* extract or PBS. (**A**) BAL was performed 24 h later for eosinophil percentage by FACS (*left panels*) and absolute eosinophil counts (*right panels*). Eosinophils were defined as Siglec-F$^+$ CD11c$^-$ cells. (**B**) Naive mice were given intranasal administrations of 10, 50, or 100 μg *Alternaria* extract, and BAL was performed 1 d later. BAL eosinophil percentage (*left panels*) and total numbers (*right panels*) were analyzed at the given dose. FACS plots are representative of four to seven mice per group and dose. Eosinophil numbers for individual mice are shown. (**C**) ELISA for BAL IL-33 (*top panel*), lung IL-5 (*middle panel*), and lung IL-13 (*bottom panel*) at 3 h, 6 h, and 1d after *Alternaria* challenge (n = 4 mice/group). PBS data are from the 3-h time point. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$, compared with PBS, t test.

eosinophilia with escalating doses (Fig. 1B). Thus, *Alternaria* specifically induces airway eosinophilia within 24 h of a single exposure in naive WT mice in a dose-dependent manner.

Cytokines, including IL-33, IL-5, and IL-13, contribute to Th2-type responses. We measured levels of these cytokines in the BAL and lung at 3, 6, and 24 h after *Alternaria* challenge (Fig. 1C). BAL levels of IL-33 and lung levels of IL-5 and IL-13 were increased after *Alternaria* challenge compared with PBS challenge. Levels of Il-33 peaked at 3 h and trended down by 24 h. Lung levels of IL-5 and IL-13 were increased maximally at 6 h. Total serum IgE levels were not significantly different between *Alternaria*- and PBS-challenged mice (mean, 27.9 and 17.5 ng/ml, respectively; n = 3–4 mice/group). Overall, this suggests that *Alternaria* induces rapid Th2 cytokine production in addition to innate eosinophilia.

Bronchial brushing to identify Alternaria*-induced epithelial mediators*

To identify genes that may be upregulated in the epithelium during the acute response to a single challenge with *Alternaria*, we utilized a technique using a bronchial brush to obtain cells from the airway (18). Cytospun cells from bronchial brushings had the ultrastructural appearance of ciliated airway epithelial cells and immunostained positive for the epithelial marker E-cadherin (Fig. 2A). Compared with whole-lung cell suspensions that contained both CD45$^+$ and CD45$^-$ cells, the cells from the airway brushing were nearly all CD45$^-$ by FACS (Fig. 2B). Additionally, nearly all of the live airway brushing CD45$^-$ cell population expressed E-cadherin compared with control Ab staining (Fig. 2B). These bronchial brushing airway epithelial cells were used to identify genes induced by acute exposure of naive WT mice to *Alternaria.*

FIZZ1 is highly induced in the airway epithelium in naive WT mice after acute Alternaria *exposure*

Using the technique from Fig. 2, we isolated epithelial RNA from *Alternaria*- and PBS-challenged mice for microarray analysis. As shown in Fig. 3A, FIZZ1 (Retnla) was induced ~20-fold after *Alternaria* exposure in the expression array and was subsequently confirmed by quantitative PCR to be induced >200-fold compared with PBS (Fig. 3A, 3B). Other genes with highly elevated transcript levels included some critically involved in mucus production, including *MUC5AC* (8-fold) and *Clca3* (9-fold). Because mucus production occurs exclusively in the epithelium, the fact that these genes were highly induced in *Alternaria*-challenged mice supports our airway brushing characterizing an epithelial transcriptome. Chitinase genes *Ym1* (*Chi3l3*) and *Chi3l1* are members of a family of proteins increasingly associated with severe asthma, and they were induced 5-fold (20, 21). Interestingly, many other genes significantly upregulated in the epithelium of *Alternaria*-exposed mice, compared with PBS-treated mice, included muscle-related genes and structural proteins that may represent epithelial–mesenchymal transition proteins, such as MYO18b, a marker for myocyte differentiation (22).

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 of *Alternaria*-challenged mice compared with PBS-treated mice (Fig. 3C). Some subepithelial cells also expressed FIZZ1, although at a significantly lower frequency compared with epithelial

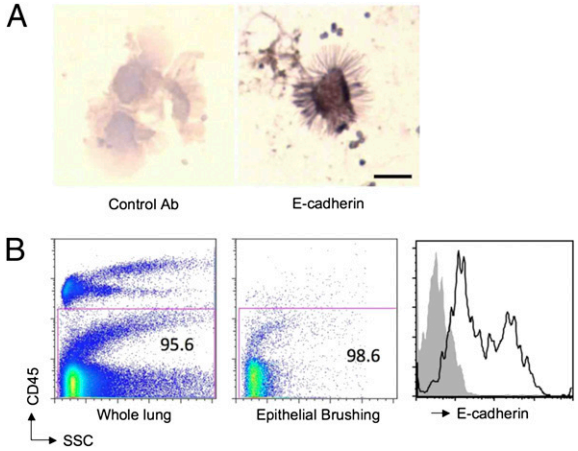

FIGURE 2. Airway epithelial cells obtained by bronchial brushing. (**A**) Cells collected by brushing of large airways in naive WT mice were analyzed by morphology and immunostained for E-cadherin. Scale bar, 10 μm. (**B**) Airway brushing cells pooled from six mice were analyzed by FACS (*middle panel*) and compared with lung cells derived from naive WT mouse lung (*left panel*) and stained for E-cadherin (*right panel*). Shaded graph represents isotype control.

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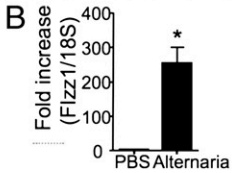
Locus	Description	Fold Change	p
Retnla	resistin like alpha	20.515	2.04E-34
Myo18b	myosin XVIIIb	11.859	6.78E-32
Clca3	chloride channel calcium activated 3	9.101	3.92E-30
Muc5ac	mucin 5, subtypes A and C, tracheobronchial/gastric	8.319	2.30E-29
Acta1	actin, alpha 1, skeletal muscle	8.121	3.22E-29
Xirp2	xin actin-binding repeat containing 2	8.051	4.70E-29
Myh4	myosin, heavy polypeptide 4, skeletal muscle	7.967	4.86E-29
Actn3	actinin alpha 3	7.573	1.48E-28
Mybpc1	myosin binding protein C, slow-type	7.489	1.92E-28
Neb	nebulin	7.432	2.67E-28
Fitm1	fat storage-inducing transmembrane protein 1	7.064	6.62E-28
Pvalb	parvalbumin	7.053	6.26E-28
Casq1	calsequestrin 1	6.572	4.17E-27
Ttn	titin	6.487	4.54E-27
Atp2a1	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	6.425	5.05E-27
Tnnc2	troponin C2, fast	6.245	1.02E-26
Pygm	muscle glycogen phosphorylase	6.212	1.19E-26
Myoz1	myozenin 1	6.151	1.66E-26
Mrgprg	MAS-related GPR, member G	5.807	8.04E-26
Ryr1	ryanodine receptor 1, skeletal muscle	5.745	1.28E-25
Ckm	creatine kinase, muscle	5.721	9.62E-26
Myot	myotilin	5.593	1.60E-24
Dcpp2	demilune cell and parotid protein 2	5.56	1.94E-25
Chi3l3	chitinase 3-like 3	5.553	2.46E-25
Tnnt3	troponin T3, skeletal, fast	5.47	2.97E-25
Myl1	myosin, light polypeptide 1	5.171	1.96E-24
Myh2	myosin, heavy polypeptide 2, skeletal muscle, adult	5.056	4.68E-24
Sprr2a1	small proline-rich protein 2A1	5.034	3.56E-24
Chi3l1	chitinase 3-like 1	5.01	3.99E-24
Sprr2a3	small proline-rich protein 2A3	4.844	1.39E-23



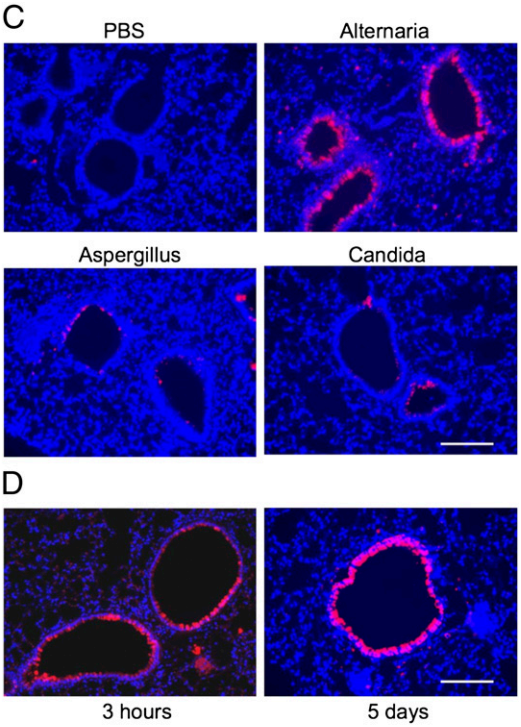


FIGURE 3. Epithelial brushing microarray and lung FIZZ1 expression after acute *Alternaria* airway challenge. (**A**) Mice received *Alternaria* or PBS one time and underwent bronchial brushing 24 h later and subsequent RNA isolation for microarray analysis (n = 3 mice/group). (**B**) Confirmatory quantitative PCR performed on bronchial brush samples for FIZZ1 (n = 3–4 mice/group). (**C** and **D**) Lung sections from mice challenged with *Alternaria*, *Candida*, or *Aspergillus* extracts or PBS. Immunofluorescent staining was performed for FIZZ1 at 24 h after challenge (C) and 3 h (D, *left panel*) or 5 d (D, *right panel*) after one challenge with *Alternaria*. Scale bars, 100 μm. $*p < 0.01$

expression. FIZZ1 expression was detected in the epithelium of lung sections as early as 3 h after a single *Alternaria* challenge and remained elevated for 5 d (Fig. 3D). In contrast, we detected minimal expression of epithelial FIZZ1 in lung sections from WT mice challenged one time with fungal allergens other than *Alternaria*, such as *Aspergillus* and *Candida.* 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 epithelial FIZZ1 expression and BAL eosinophilia are STAT6 dependent*

The transcription factor STAT6 is critical to IL-4/IL-13 signaling and Th2 cell development (23) but little is known about its acute role in airway disease in naive WT mice. To investigate this, WT and STAT6-deficient (STAT6$^{-/-}$) mice were challenged with a single dose of *Alternaria*. Dramatically, FIZZ1 expression was nearly absent in the epithelium of STAT6$^{-/-}$ mice (Fig. 4A). Lung FIZZ1 mRNA levels were also significantly reduced in acute *Alternaria*-challenged STAT6$^{-/-}$ mice (Fig. 4B). Additionally, BAL eosinophilia was strongly reduced in STAT6$^{-/-}$ mice compared with WT mice (Fig. 4C). This suggests that both FIZZ1 expression and the acute airway inflammatory events 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 the addition of protease inhibitors (12, 24). Additionally, *Alternaria* was shown in vitro to induce the proallergic cytokine thymic stromal lymphopoietin (TSLP) in epithelial cells through PAR-2, suggesting that other epithelial-derived factors, such as FIZZ1, may be regulated by PAR-2 (14). 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 of stained lung sections and no difference at the lung transcript level (Fig. 4B). Additionally, the BAL eosinophilia was unchanged in PAR-2–deficient mice compared with WT mice. This suggests that PAR-2 is not critical for FIZZ1 epithelial expression or acute airway eosinophilia after *Alternaria* challenge.

Bone marrow-derived STAT6 contributes to Alternaria-*induced airway eosinophilia*

To address whether STAT6 expressed in lung structural cells or bone marrow-derived cells contributed to airway eosinophilia and FIZZ1 expression after *Alternaria* challenge, we performed bone marrow chimera studies with WT and STAT6$^{-/-}$ mice. Six weeks after WT mice and STAT6$^{-/-}$ mice were irradiated and received either WT or STAT6$^{-/-}$ bone marrow transplantation, mice were challenged once with *Alternaria* and analyzed 24 h later. We used congenic WT mice (CD45.1) to determine the efficiency of chimerism in the lung (Fig. 5A). FACS analysis of BAL cells revealed 94–98% efficiency of chimerism in the lung in all groups. We next determined the level of BAL eosinophils in these mice (Fig. 5B). WT CD45.2 mice that received WT CD45.1 bone marrow developed significant airway eosinophilia when measured 24 h after one *Alternaria* challenge. STAT6$^{-/-}$ recipients that received WT bone marrow had similar levels of airway eosinophilia compared with WT mice that received WT bone marrow. In contrast, WT mice that received STAT6$^{-/-}$ bone marrow had significantly reduced eosinophilia after *Alternaria* challenge. This suggests that STAT6 expressed in hematopoietic cells contributes to the innate eosinophilia after a single *Alternaria* challenge.

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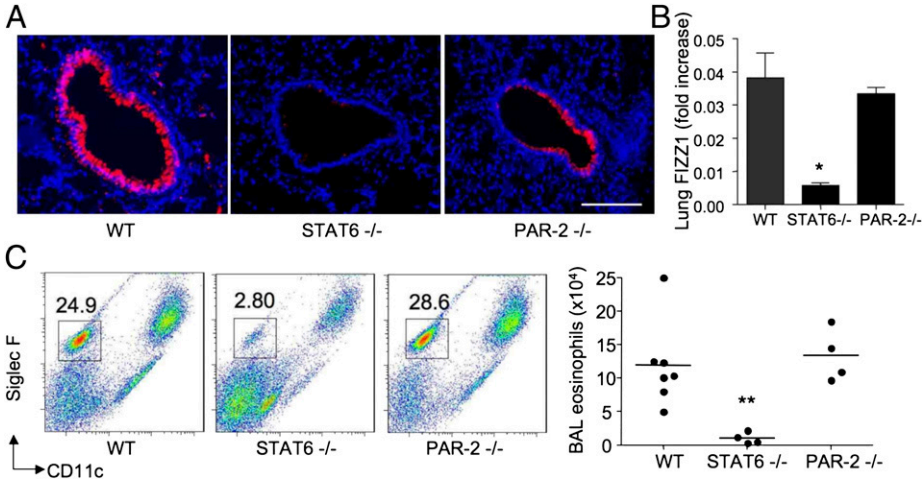

FIGURE 4. *Alternaria*-induced lung FIZZ1 expression and early eosinophilia are dependent on STAT6 but not PAR-2. (**A**) WT, STAT6$^{-/-}$, or PAR-2$^{-/-}$ mice were challenged with *Alternaria*, and FIZZ1 immunofluorescent staining was performed on lung sections. Scale bar, 100 μm. (**B**) FIZZ1 mRNA levels measured in whole-lung samples from WT, STAT6$^{-/-}$, or PAR-2$^{-/-}$ mice. (**C**) Percentage of BAL eosinophils by FACS (*left panel*) and total numbers (*right panel*) for WT, STAT6$^{-/-}$, or PAR-2$^{-/-}$ mice. n = 4–6 mice/group. *$p < 0.005$, **$p < 0.01$, compared with WT mice, t test.

Alternaria-*induced epithelial FIZZ1 is dependent on STAT6 expression in lung cells*

We performed immunofluorescent staining for FIZZ1 in lung sections from WT and STAT6$^{-/-}$ bone marrow chimeras that received a single challenge with *Alternaria*. Stained lung sections from WT CD45.2 mice that received WT CD45.1 bone marrow revealed strong FIZZ1 staining in the airway epithelium (Fig. 5C). Further, WT recipients that received STAT6$^{-/-}$ bone marrow had similar levels of FIZZ1 staining in the airway epithelium compared with WT recipients that received WT bone marrow. However, STAT6$^{-/-}$ mice that received WT bone marrow had significantly reduced FIZZ1 staining in the airway epithelium after *Alternaria* challenge. This suggests that STAT6 in lung cells is required for *Alternaria*-induced FIZZ1 expression.

FIZZ1 binds to inflammatory and structural cells in the lung

The receptor for FIZZ1 is unknown, so we used a FIZZ1 lung-binding assay (19) to identify cell types in the lung that bind to FIZZ1 (Fig. 6A). Single-cell suspensions from digested lung were gated on either leukocytes (CD45$^+$) or structural cells (CD45$^-$). Both lung CD45$^+$ cells (Fig. 6B) and lung CD45$^-$ cells (Fig. 6C) bound to FIZZ1. In the lung CD45$^+$ cell population, CD45$^+$ CD11c$^+$ cells (consisting of both macrophages and dendritic cells) displayed significant FIZZ1 binding (Fig. 6B). In contrast, analysis of CD45$^+$CD11c$^-$ cells revealed a smaller population that bound FIZZ1 (Fig. 6B). The CD45$^-$ population also displayed FIZZ1 binding (Fig. 6C). Because the lung CD45$^-$ population is composed of structural cells, including fibroblasts, we then performed the same FIZZ1-binding assay using single-cell suspensions from digested lungs from collagen-1 GFP reporter mice in which lung fibroblasts strongly express GFP (15); we determined that the CD45$^-$collagen-1$^+$ population bound to FIZZ1 (Fig. 6D). These studies suggest that several cell types (macrophages, dendritic cells, fibroblasts) within the naive lung may bind and respond to FIZZ1. However, not all cell types in the lung bind FIZZ1, because only a small population of lung CD45$^+$CD11c$^-$ cells bound FIZZ1.

Intranasal rFIZZ1 administration leads to airway eosinophilia, increased epithelial thickness, and fibrosis

To evaluate possible roles of FIZZ1 in the airway, we administered rFIZZ1 to naive WT mice for 5 d and performed BAL and histologic analysis 3 d later. Mice that received rFIZZ1 had elevated levels of eosinophils in the airways compared with mice that received only PBS (Fig. 7A). These mice also had evidence of increased epithelial thickness (Fig. 7B), a feature associated with severe asthma (25). 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. 7C). To determine whether these changes were associated with increased airway reactivity, we performed invasive pulmonary testing in mice receiving rFIZZ1 or PBS, but we did not detect a difference in airway resistance after increasing doses of meth-

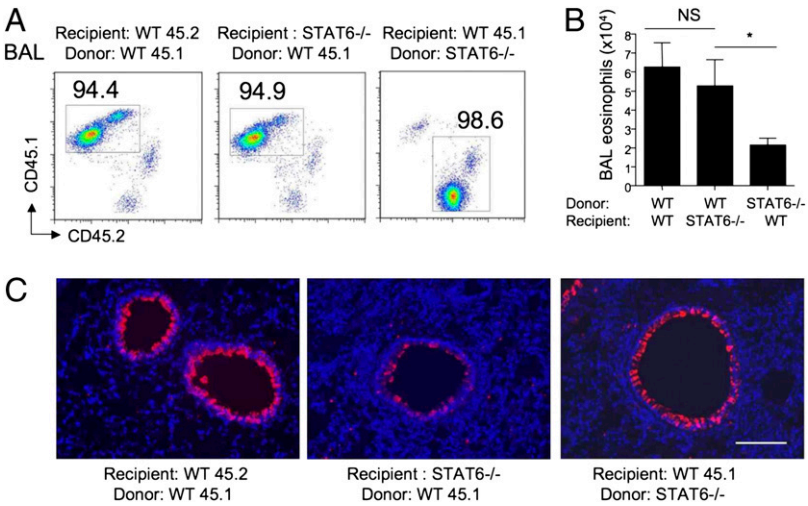

FIGURE 5. Bone marrow STAT6 contributes to *Alternaria*-induced eosinophilia, but FIZZ1 expression is dependent on STAT6 in lung structural cells. (**A**) WT (CD45.1 and CD45.2) and STAT6$^{-/-}$ mice were irradiated and injected i.v. with WT or STAT6$^{-/-}$ bone marrow and challenged once with *Alternaria* 6 wk later. FACS analysis of BAL cells for percentage of donor bone marrow cells in airway. (**B**) Total BAL eosinophils in WT and STAT6$^{-/-}$ bone marrow chimeric mice 24 h after single *Alternaria* challenge. (**C**) FIZZ1 immunofluorescent staining of lung sections; scale bar, 100 μm. Data are representative of 8–10 mice/group (A, C). *$p < 0.05$, Mann–Whitney test (n = 8–10 mice/group).

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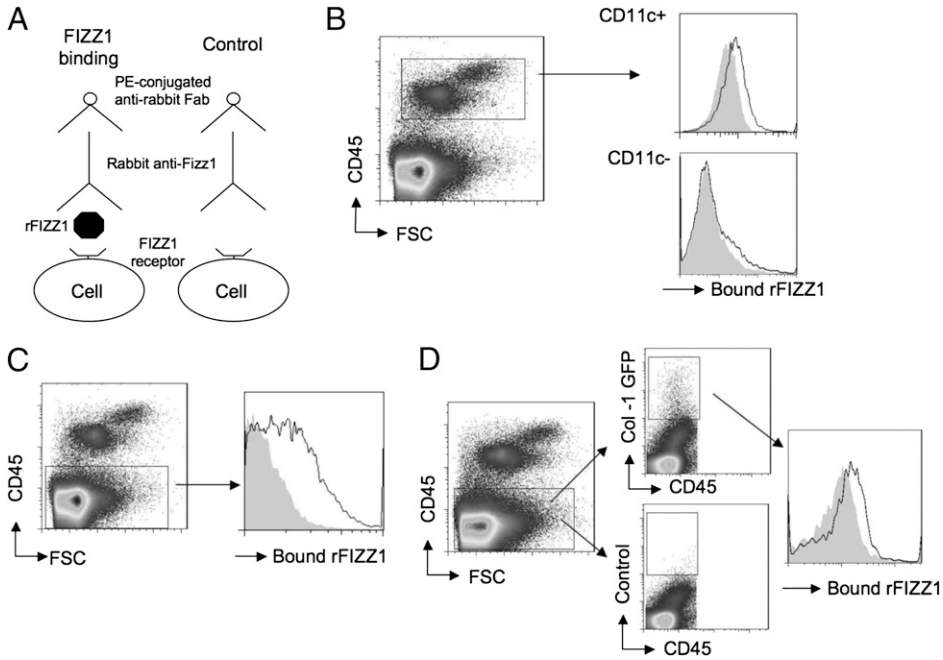

FIGURE 6. rFIZZ1 binds to leukocytes and structural cells isolated from naive WT mouse lung. (**A**) Single-cell suspensions from two naive WT mouse lungs were incubated with rFIZZ1, followed by staining. (**B**) Lung cells were stained with CD45 and CD11c. $CD45^+$ cells (*left panel*) were gated, and $CD11c^+$ and $CD11c^-$ populations (*right panels*) were analyzed for FIZZ1 binding. (**C**) $CD45^-$ cells were gated and analyzed for FIZZ1 binding. (**D**) $CD45^-$ cells from Col-1 GFP mice were gated on collagen-1^+ cells and analyzed for FIZZ1 binding. Lung cells were analyzed from pooled samples from two mice and are the results of two independent experiments.

acholine (Fig. 7D). These data suggest that FIZZ1 may have many roles in the airway, including promoting eosinophilia, epithelial changes, and peribronchial fibrosis.

Discussion

Alternaria has been associated with the development, persistence, and severity of asthma (4). Little is known about the reasons for the unique pathogenicity of *Alternaria*. In this study, we demonstrate that naive WT mice developed significant BAL eosinophilia following *Alternaria* challenge. In contrast to *Alternaria*, neither *Aspergillus* nor *Candida* induced BAL eosinophilia. Gene microarray analysis of airway epithelial cell brushings demonstrated that *Alternaria*-challenged naive WT mice had a 20-fold increase in the expression of FIZZ1/Retnla, a resistin-like molecule whose increased expression in airway epithelium was confirmed by quantitative PCR. Additional genes that were highly induced by *Alternaria* in airway epithelium included those involved in mucus expression (*MUC5AC*, *Clca3*), as well as chitinase genes (*Yim1* and *Chi3l1*). Lung immunostaining confirmed strong airway epithelial FIZZ1 expression present as early as 3 h

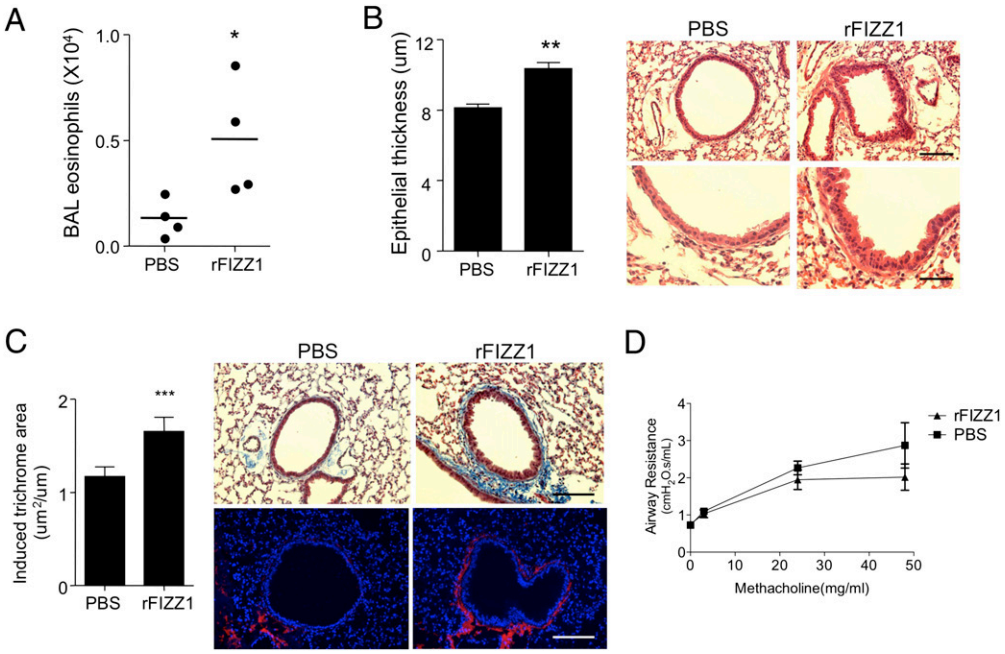

FIGURE 7. Exogenous FIZZ1 induces airway eosinophilia, epithelial thickening, and fibrosis. (**A**) Naive WT mice received intranasal rFIZZ1 or vehicle (PBS) for five consecutive days, and BAL and lung were analyzed 3 d later. Total BAL eosinophils were enumerated (n = 4 mice/group). (**B**) Epithelial thickness (*left panel*) was measured in H&E-stained lung sections from mice receiving rFIZZ1 (*right panels*) or PBS (*middle panels*) (n = 19–25 airways/group). Scale bar, *top row*: 100 μm, *bottom row*: 50 μm. (**C**) Lung sections stained with trichrome (*top row*) and scored (*left panel*) and stained for collagen-1 (*bottom row*) (n = 23–25 airways/group). Scale bars, 100 μm. (**D**) BALB(c) mice were challenged with intranasal rFIZZ1 or PBS for 5 d, and invasive airway resistance was measured after increasing doses of methacholine (n = 4 mice/group). *$p < 0.05$, t test; **$p < 0.0001$, ***$p < 0.001$, Mann–Whitney test.

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after a single *Alternaria* challenge that persisted for ≥5 d and was significantly reduced in STAT6-deficient, but not PAR-2–deficient, mice. Bone marrow chimera studies revealed that STAT6 expressed in lung cells was required for epithelial FIZZ1 expression. In contrast, STAT6 present in bone marrow-derived cells contributed to airway eosinophilia. Direct administration of rFIZZ1 to naive mice led to airway eosinophilia, peribronchial fibrosis, and increased thickness of the airway epithelium. Studies investigating which cells in the lung bind FIZZ1 demonstrated that both fibroblasts ($CD45^-$collagen-1^+) and leukocytes ($CD45^+$) in the lung, in particular $CD45^+CD11c^+$ macrophages and dendritic cells, bound to FIZZ1. Thus, *Alternaria* induces STAT6-dependent acute airway eosinophilia and epithelial FIZZ1 expression that promotes airway fibrosis and epithelial thickness. This may provide some insight into the uniquely pathogenic aspects of *Alternaria*-associated asthma.

Our studies identified that the innate acute eosinophilic response to *Alternaria* is mediated by STAT6 and not PAR-2. This is in contrast to a report of mice challenged with intranasal purified protease from a different fungal allergen (*Aspergillus*), which did not require STAT6 for the development of early eosinophilic airway inflammation at 18 h but did require intact allergen protease activity (24). In our studies, we used whole allergen extracts from *Alternaria* and *Aspergillus* instead of purified protease from *Aspergillus*, and this may have contributed to the different results. Other investigators suggested that a PAR-2–mediated pathway may drive inflammatory events induced by protease allergens, including *Alternaria* (12–14, 26). We did not detect a reduction in eosinophilic inflammation in PAR-2–deficient mice in response to *Alternaria* exposure; instead, our data suggest a STAT6-mediated pathway. In vitro studies showed that PAR-2 is important in *Alternaria*-induced bronchial epithelial cell activation and production of TSLP (12, 14); however, as demonstrated in our study, in vivo *Alternaria*-challenged PAR-2–deficient mice may use alternate STAT6-dependent pathways. Studies also examined the role of PAR-2 in the adaptive immune response in mice challenged with a nonfungal cockroach allergen. These studies demonstrated that cockroach challenge did not induce an acute innate airway eosinophilia in WT mice (26). However, cockroach challenge in PAR-2–deficient mice resulted in reductions in airway inflammation in response to cockroach after three challenges over 17 d (13). Thus, differences between the results of our studies (which do not demonstrate dependence on PAR-2) and those of other investigators may be related to our use of in vivo models (as opposed to in vitro studies), the time points studied (acute innate 24 h versus late adaptive-immune response), and the allergen used (*Alternaria* versus cockroach).

We detected early increases in IL-33, IL-5, and IL-13 in the lung after one *Alternaria* challenge. This is consistent with a recent report showing that *Alternaria* induces the release of the pro-Th2 cytokine IL-33 within a few hours in vivo and was dependent upon extracellular ATP inducing calcium influx in epithelial cells (27). The same study showed that mice receiving a single challenge with *Alternaria* extract had elevated lung levels of Th2 cytokines IL-5 and IL-13 that were nearly absent in MyD88 and ST2 (IL-33R)–deficient mice when measured 12 h later. This work highlights the complexity of the innate response to *Alternaria* and the multiple pathways involved, including TLR/IL-1 family signaling. We chose a 24 h time point following *Alternaria* challenge for gene microarray analysis, because we were interested in determining which genes expressed at this time point might be responsible for persistent airway inflammation and remodeling. Indeed, we identified both FIZZ1 and members of the chitinase gene family as being expressed at this time point, both candidate genes for remodeling. Given the very early rise (within a few hours) of Th2 cytokines detected in the lung in our studies, as well as those of other investigators (27), the 24-h time point that we used for microarray analysis was likely not optimal for detection of cytokine and chemokine genes induced in the initial few hours following *Alternaria* challenge (e.g., TSLP, IL-25, IL-33).

To identify genes that are upregulated in the airway epithelium in vivo after a single *Alternaria* challenge, we obtained airway epithelial cells using bronchial brushings, an adapted method initially developed by two of the authors (K.S. and D.S.) (18). The airway epithelial cell morphology, positive E-cadherin staining, absence of CD45, and transcript signature that includes mucus genes strongly suggest that the predominant cell type obtained by brushing was airway epithelium. The most highly expressed transcript, FIZZ1, was also detected in airway epithelium using a systemic adaptive-immune sensitization protocol with OVA in alum and subsequent OVA challenge detecting FIZZ1 expression 2–3 wk later (28, 29), but FIZZ1 has not been reported to be induced following an innate stimulus with *Alternaria.* FIZZ1 is a resistin-like molecule that shares homology with human resistin and is known to be induced during Th2-mediated inflammation (30). FIZZ1 is expressed primarily in the inflamed airway epithelium, as well as by alternatively activated macrophages after allergen challenge or helminth infection (19, 28). The expression of FIZZ1 in the lung is regulated by STAT6 following one exposure to *Alternaria* in naive mice at 24 h, as demonstrated in this study, as well as at later time points following exposure to allergen (2 wk) or bleomycin (7 d) (29, 31). The receptors for FIZZ1 remain elusive, although reports suggested that signaling occurs through Bruton's tyrosine kinase and Notch1 (32, 33). Using a previously published FIZZ1-capture assay (19), we identified that both lung $CD11^+$ cells (including macrophages and dendritic cells) and lung fibroblasts bound to FIZZ1. This is consistent with a previous report that found that rFIZZ1 could bind to splenic macrophages and dendritic cells (19), and it also extends the observation to lung fibroblasts, which have not been previously reported to bind to FIZZ1. Although the FIZZ1-binding assay has limitations in terms of sensitivity, the levels of FIZZ1 binding that we detected in the lung are similar to those reported in splenic macrophages and dendritic cells. Prior reports showed that FIZZ1 can induce myofibroblast differentiation, including collagen-1 and α-smooth muscle actin production, in vitro, suggesting that structural cells can respond to FIZZ1 directly (34, 35).

We found that rFIZZ1 administered intranasally to the airways of naive mice leads to eosinophil accumulation. This is consistent with previous reports suggesting that FIZZ1 regulates eosinophil chemotaxis in the gastrointestinal tract (36, 37). It is possible that FIZZ1 participates in the early recruitment of eosinophils after *Alternaria* challenge; however, given the relatively low magnitude of the eosinophilic response induced by rFIZZ1 compared with that induced by *Alternaria*, it is likely that other mediators, such as IL-5 and eotaxin, play larger roles. Previous reports suggested that FIZZ1 dampens helminth-induced Th2-type inflammation (19, 38) but that it may promote features of remodeling (34, 35, 39). Airway remodeling is an important feature of asthma, and previous reports suggested that FIZZ1 can induce lung collagen deposition and myofibroblast differentiation (34, 35, 39). Additionally, repetitive intranasal administration of rFIZZ1 was noted to induce fibrotic changes in a lung granuloma model (40). Thus, FIZZ1 may have several roles in the lung, depending on the cell types involved and stage of the inflammatory response. Our data, as well as those from other investigators, suggested an early proinflammatory and proremodeling role for FIZZ1 (35, 37, 39, 40); however, other investigators suggested an anti-inflammatory role during more

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chronic Th2 responses (19, 38). Further work will be required to fully elucidate the multiple functions of FIZZ1 in the lung during chronic inflammatory responses.

In summary, we characterized a unique acute eosinophilic airway response to *Alternaria* that is STAT6 dependent and associated with significant upregulation of FIZZ1 in airway epithelium. Further, exogenous FIZZ1 induced airway eosinophilia, epithelial changes, and airway fibrosis. This underscores the potential importance of FIZZ1 in asthma and airway remodeling and might translate to a role for related human resistin molecules in human asthma.

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Disclosures

The authors have no financial conflicts of interest.

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