

RESEARCH ARTICLE

Lung Diseases in Reverse Translation: Bedside to the Bench

Chitinase 3-like-1 protects airway function despite promoting type 2 inflammation during fungal-associated allergic airway inflammation

Joseph J. Mackel,^{1*} Jaleesa M. Garth,^{1*} MaryJane Jones,⁵ Diandra A. Ellis,⁵ Jonathan P. Blackburn,¹ Zhihong Yu,¹ Sadis Matalon,¹ Miranda Curtiss,^{1,2} Frances E. Lund,²  Annette T. Hastie,³ Deborah A. Meyers,⁴ and Chad Steele⁵

¹Department of Medicine, University of Alabama Birmingham, Birmingham, Alabama; ²Department of Microbiology, University of Alabama Birmingham, Birmingham, Alabama; ³Department of Medicine, Wake Forest University, Winston-Salem, North Carolina; ⁴Department of Medicine, University of Arizona, Tucson, Arizona; and ⁵Department of Microbiology and Immunology, Tulane University, New Orleans, Louisiana

Abstract

Exposure to fungi can result in a wide range of comorbidities depending on the immune status of the host. Chronic exposure and reactivity to fungi such as *Aspergillus fumigatus* can result in conditions such as severe asthma with fungal sensitization (SAFS) or allergic bronchopulmonary aspergillosis (ABPA). However, the pathophysiology of SAFS and ABPA is not well understood. Here, we report that the chitinase-like protein YKL-40 is elevated in lung lavage fluid from humans with asthma that are sensitized to fungi. Initial studies demonstrated that mice deficient in the murine ortholog of YKL-40, breast regression protein-39 (BRP-39, chitinase-3-like 1, *Chi3l1*), were not more susceptible to acute infection with *A. fumigatus*. However, in an experimental model of fungal-associated allergic airway inflammation (fungal asthma), *Chi3l1*^{−/−} mice had significantly increased airway hyperresponsiveness (AHR). Surprisingly, increased AHR in *Chi3l1*^{−/−} mice occurred in the presence of significantly lower type 2 responses (decreased eosinophil numbers and decreased IL-4, IL-5, IL-33, CCL17, and CCL22 levels), although type 1 and type 17 responses were not different. Increased AHR was not associated with differences in periodic acid-Schiff staining of lung tissue, differences in the expression of *Muc5ac* and *Clca3*, nor differences in lung edema. Bone marrow chimera studies revealed that the presence of BRP-39 in either the hematopoietic or nonhematopoietic compartment was sufficient for controlling AHR during fungal asthma. Collectively, these results indicate that BRP-39 protects against AHR during fungal asthma despite contributing to type 2 inflammation, thus highlighting an unexpected protective role for BRP-39 in allergic fungal asthma.

asthma; fungal; inflammation

INTRODUCTION

Asthma, the most common chronic respiratory condition worldwide, afflicts more than 300 million people and is characterized by wheezing, coughing, and chest tightness as a result of aberrant inflammation and airway remodeling processes such as airway smooth muscle hyperplasia and mucus production (1). Symptoms are well controlled in many patients with inhaled corticosteroids, which reduce inflammation, and long-acting β_2 adrenergic receptor agonists, which relax airway smooth muscle. Unfortunately, available treatments are not effective for all patients, and a subset of disease termed severe asthma is refractory to current therapies and therefore of intense research interest (2). Further, severe asthma with fungal sensitization (SAFS) is a phenotype of allergic asthma in which patients are sensitized to environmental fungi such as

Aspergillus fumigatus, which is associated with increased severity of disease (3–5). Individuals with SAFS often have uncontrolled asthma, more steroid usage, higher IgE levels, and increased intensive care unit admissions (3–5).

Activation of host immunity via recognition of cell wall components of *A. fumigatus* is critical for clearance of the organisms from the lung, yet can also contribute to pathogenesis of fungal asthma. Along these lines, our laboratory has previously reported such a role for β -glucan recognition via the C-type lectin receptor Dectin-1, which is protective during acute exposure to *A. fumigatus*, but contributes to asthma severity during chronic exposure (6). Similarly, we have recently studied the role of chitin degradation in acute and chronic *A. fumigatus* exposure via investigation of acidic mammalian chitinase (AMCase), which is capable of binding and cleaving chitin, and which we found to contribute to

* J. J. Mackel and J. M. Garth contributed equally to this work.

Correspondence: C. Steele (csteele4@tulane.edu).

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disease severity in *A. fumigatus*-associated allergic asthma (7). Chitin, the second most abundant polysaccharide in nature, is a critical component of the fungal cell wall (8) and the exoskeletons of aeroallergens such as house dust mite and cockroach (9). Early studies demonstrated that chitin induces the accumulation of IL-4-expressing innate immune cells, including eosinophils and basophils (10). Fungal chitin derived from home environments has been shown to induce acute eosinophilic inflammation in the lung (11). Other studies have shown that acute chitin exposure elicits patterns of innate cytokines (IL-25, IL-33, and thymic stromal lymphopoietin [TSLP] as well as IL-1 β and IL-23) that target distinct populations of resident lymphoid cells, revealing divergent pathways underlying the tissue accumulation of specific types of inflammatory myeloid cells (12). Despite these observations, a knowledge gap exists regarding immunoresponsiveness to chitin in a chronic exposure setting and the impact on allergic asthma. This is primarily the result of studies often employing models that administer shellfish-derived chitin (or beads coated with chitin) acutely (i.e., once or twice) and examine responses a day later (10–12). Other studies have employed the model antigen ovalbumin (13) or a commercial fungal hyphal extract (14) co-administered with shellfish-derived chitin to infer causality/pathology associated with chitin exposure. Collectively, prior research on a role for chitin in asthmatic responses has employed models that may only be tangentially related to responses observed in allergic asthma.

As a continuation of our previous study on chitin and associated host pathways (7), we report on the role of chitinase 3-like-1/BRP-39 (Chi3l1). Chitinase 3-like-1/BRP-39 is a chitinase-like protein that can bind but not cleave chitin and that has been associated with increased asthma risk and severity in humans (15). Study of chitinase 3-like-1/BRP-39 in ovalbumin and house dust mite mouse models of asthma revealed a role in promotion of Th2 inflammatory responses and airway hyperresponsiveness (16). In the current report, we sought to define a role for chitinase 3-like-1/BRP-39 in fungal asthma severity. Unexpectedly, our data indicate that chitinase 3-like-1/BRP-39 maintains lung function during chronic fungal exposure, which outweighs its contribution to lung inflammation in relation to its effect on AHR.

MATERIALS AND METHODS

Subjects, BALF Collection, and Processing, Luminex Analysis

Patients with mild-to-severe asthma were comprehensively characterized according to the National Heart, Lung, and Blood Institute (NHLBI) SARP phenotype protocol at Wake Forest School of Medicine as previously described (17), and a description of this cohort was recently reported (18). The sputum induction method was adopted from the NHLBI Asthma Clinical Research Network and used in SARP (17). Bronchoalveolar lavage fluid (BALF) and sputum samples were derived from both the SARP 1 and 2 cohorts. Per SARP protocol, BALF and sputum were not collected within a week of each other. Sputum was processed immediately after collection, and cell cytopins were stained for differential counts of at least 500 nonsquamous cells/subject slide. Total white blood cell (WBC) count in sputum was determined via trypan

blue staining and enumeration using a hemacytometer. Cell-free supernatants were aliquoted and stored at -80°C before use in Luminex analyses (see *Whole Lung and BALF Cytokine and Chemokine Analysis*). Biospecimens were randomly selected without a priori selection based on asthma severity, rather than based on whether they were skin test-positive or test-negative for *Alternaria*, *Aspergillus*, and/or *Cladosporium*. Sputum supernatants were assayed for different inflammatory cytokine, chemokine, and growth factor protein concentrations using Milliplex Human Cytokine/Chemokine Panels I, II, III, and IV (catalogs HCYTOMAG-60K-PX41, HCYP2MAG-62K-PX23, HCYP3MAG-63K, and HCY4MG-64K-PX21, respectively; MilliporeSigma). Standards for determination of linear curve plus two control samples representing high and low levels of each cytokine/chemokine were included in each assay. Human subjects were enrolled with written informed consent under approved Wake Forest School of Medicine IRB BG01-425 (Wake Forest University IRB). Human samples were analyzed under UAB IRB X130827009 (UAB IRB for Human Use).

Mice

Male and female, age-matched WT Balb/c mice were obtained from Charles River (Wilmington, MA). *Chi3l1*^{-/-} mice were a kind gift from Dr. Alison Humbles, MedImmune (Gaithersburg, MD), and bred at Tulane University. All animals were housed in a specific pathogen-free, Association for Assessment and Accreditation of Laboratory Animal Care-certified facility and handled according to Public Health Service Office of Laboratory Animal Welfare policies after review by the Tulane Institutional Animal Care and Use Committee.

Preparation of *A. fumigatus*, Acute and Chronic in Vivo Challenge, Lung Fungal Burden Assessment

A. fumigatus isolate 13073 (ATCC, Manassas, VA) was maintained on potato dextrose agar for 5–7 days at 37°C . Conidia were harvested by washing the culture flask with 50 mL of sterile phosphate-buffered saline supplemented with 0.1% Tween-20. The conidia were then passed through a sterile 40- μm nylon membrane to remove hyphal fragments and enumerated on a hemacytometer. The invasive aspergillosis model consisted of one intratracheal administration of 7×10^7 conidia. For lung fungal burden analysis, left lungs were collected and homogenized in 1 mL of PBS. Total RNA was extracted from 0.1 mL of unclarified lung homogenate using the MasterPure yeast RNA purification kit (Lucigen, Madison, WI), which includes a DNase treatment step to eliminate genomic DNA as previously reported because DNA is not predictive of organism viability in this assay (19). Lung *A. fumigatus* burden was analyzed with real-time PCR measurement of the *A. fumigatus* 18S rRNA (GenBank accession number AB008401) and quantified using a standard curve of *A. fumigatus* 18S rRNA produced in a bacterial plasmid and copy number calculated based on spectrophotometry. The repeated *A. fumigatus*-associated allergic airway inflammation model was performed as we have extensively reported (7, 18, 20, 21). Briefly, mice were lightly anesthetized with isoflurane and administered 1×10^7 live *A. fumigatus* conidia in a volume of 50 μL of PBS intratracheally (it). After resting for 7 days, mice were challenged intratracheally with 1×10^6 live *A. fumigatus* conidia in 50 μL of PBS daily for 5 consecutive

days (*days 7, 8, 9, 10, and 11*), allowed to rest for 2 consecutive days (*days 12 and 13*), and then challenged intratracheally with 1×10^6 live *A. fumigatus* conidia in 50 μ L of PBS daily for 3 consecutive days (*days 14, 15, and 16*). Twenty-four hours after the last *A. fumigatus* challenge (*day 17*), mice were euthanized for analysis via ketamine/xylazine overdose and aortic exsanguination.

Whole Lung and BALF Cytokine and Chemokine Analysis

Following euthanasia, the right lung was homogenized in PBS supplemented with Complete, Mini Protease Inhibitor Tablets (Roche Diagnostics), clarified by centrifugation (12,000 *g* for 10 min at 4°C), and stored at –80°C. From lung homogenate supernatants, IL-33, IL-38, CCL17, and CCL22 levels were quantified by ELISA (R&D Systems). For lung BALF cytokine analysis, a bronchoalveolar lavage was performed on individual mice as previously described (6, 22). Clarified lavage supernatants were analyzed for the protein levels of 32 cytokines and chemokines using the Luminex-based Milliplex multiplex suspension cytokine array (MilliporeSigma), according to the manufacturer's instructions. The data were analyzed using Bio-Plex Manager software (Bio-Rad). IL-22 and TGF- β 1 levels in lavage fluid were quantified by ELISA (R&D Systems). The TGF- β 1 assay included an acid activation step to convert latent TGF- β 1 to its detectable, immunoreactive form.

Lung Cell Flow Cytometry

Lung cells were isolated via BAL as previously described (6). Cells were washed, and Fc receptors were blocked with Mouse BD Fc Block (BD Biosciences) at 4°C for 20 min. Thereafter, cells were stained with a single-color LIVE/DEAD Fixable Dead Cell Stain (Invitrogen), followed by labeling with specific immune cell surface markers. The following staining parameters were employed: eosinophils as CD45⁺ CD11b⁺ Siglec F⁺ Ly-6G⁺ using anti-CD45 (BioLegend, catalog 103115, clone 30-F11), anti-CD11b (BioLegend, catalog 101237, clone M1/70), anti-Siglec F (BioLegend, catalog 155507, clone S17007L), and anti-Ly-6G (BioLegend, catalog 127621, clone 1A8); neutrophils as CD45⁺ CD11b⁺ Ly6G⁺ Siglec F⁺ using anti-CD45, anti-CD11b, anti-Ly-6G, and anti-Siglec F; samples were acquired using a 4-laser, 20-parameter analytic BD LSRFortessa, and data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR). Unstained lung leukocytes served as a control for background fluorescence and gating. Appropriately stained UltraComp eBeads (Thermo Fisher Scientific, Waltham, MA) served as single-color controls.

Pulmonary Function Assessment

Individual anesthetized *A. fumigatus*-exposed mice were intubated, and each animal was attached to a computer-controlled volume ventilator (flexiVent; SCIREQ). Regular breathing was set at 150 breaths per minute, with volume and pressure controlled by the flexiVent system based on individual animal weights. Positive end-expiratory pressure was set to 2 cm H₂O and measured during each breath stroke. The single-frequency forced oscillation technique was employed to measure total/dynamic lung resistance (Rrs). The low-frequency/broadband forced oscillation technique

was employed to measure Newtonian resistance (Rn; also known as airway hyperreactivity). All measurements were collected at baseline and after a linear dose response with methacholine challenge (10–40 mg/mL), as previously described (6, 7, 18, 21). Lung function was also assessed in naive WT and mutant mice, which confirmed no baseline anomalies and no differences between groups (data not shown).

Lung Edema and Mucin Analysis

Lungs were collected, weighed, dried for 24 h at 60°C, and then reweighed. For real-time PCR analysis of mucin gene expression, left lungs were collected into TRIzol Reagent (Life Technologies, Carlsbad, CA), homogenized, and RNA isolated according to the manufacturer's protocol. mRNA levels were then measured via TaqMan assay for *Muc5ac* and *Gob5* and normalized to *Hprt* (Applied Biosystems, Waltham, MA).

Bone Marrow Chimeras

WT and *Chi3l1*^{–/–} reciprocal chimeric mice were generated by irradiating the indicated recipient mice with 950 Rads from an X-ray source delivered in 2 equal doses administered 4–5 h apart (23). Following irradiation, mice were intravenously injected with 5 million total bone marrow cells (100% from either WT or *Chi3l1*^{–/–} mice) and were allowed to reconstitute for 8–10 wk before being subjected to experimental fungal asthma.

Histology

Lungs were inflated and fixed in 4% formalin. The fixed lungs were paraffin embedded and then processed and stained by GNO Histology Consultants (New Orleans, LA). Imaging was performed using a Swift Optical Instruments M10T-P Trinocular LED Microscope equipped with a Motic Moticam 5 + 5-megapixel digital camera.

Statistics

Data were analyzed using GraphPad Prism Version 7.0 statistical software (GraphPad Software). Comparisons between groups when data were normally distributed were made with the two-tailed unpaired Student's *t* test or two-way ANOVA. Significance was accepted at a value of *P* < 0.05.

RESULTS

Chitinase 3-like-1/BRP-39 Is Dispensable for Lung Fungal Clearance During Acute *A. fumigatus* Exposure

Chitin comprises up to 20% of the *A. fumigatus* cell wall depending on the morphology, i.e., conidia (lower percentage) versus hyphae (higher percentage) (24). Mammalian chitinases, which include acidic mammalian chitinase (AMCase) and chitotriosidase, were initially described as having antifungal properties against clinically relevant pathogens such as *Cryptococcus neoformans* and *Candida albicans* (25). We recently reported, however, that mice deficient in AMCase clear *A. fumigatus* from the lungs more efficiently than WT control mice (7), suggesting that AMCase-mediated degradation of chitin does not function in a traditional host defense capacity against *A. fumigatus*. We therefore asked the question whether binding of chitin by chitinase-3-like-1 affected

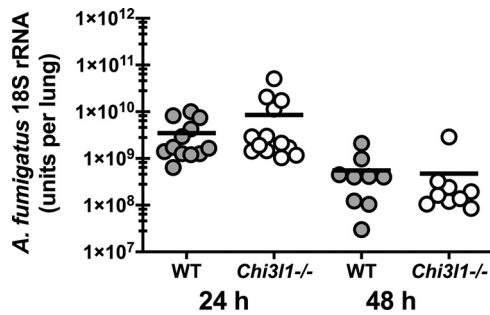


Figure 1. Chitinase 3-like-1/BRP-39 is dispensable for lung fungal clearance during acute *A. fumigatus* exposure. Balb/c wild-type (WT) and BRP-39-deficient (*Chi3l1*^{-/-}) mice were challenged intratracheally with 7×10^7 *A. fumigatus* conidia, and 24 or 48 h after exposure, lung fungal burden was assessed by real-time PCR analysis of *A. fumigatus* 18S rRNA levels. The figure represents cumulative data from three independent studies per time point ($n = 4$ or 6 mice per group, per study). Each symbol represents an individual mouse. Line within a given group represents the mean.

host defense against *A. fumigatus*. Results in Fig. 1 show that mice deficient in chitinase 3-like-1/BRP-39 (*Chi3l1*^{-/-}) had no impairments in clearing *A. fumigatus* from the lung. Thus, BRP-39 is not required for lung clearance of *A. fumigatus*.

Chitinase 3-like-1 (YKL-40/BRP-39) Is Elevated in Humans With Asthma Sensitized to Fungi but Protects Against AHR in an Experimental Animal Model of Fungal Asthma

In previous reports, we identified immune mediators that were increased in humans with asthma with allergic sensitization to fungi and subsequently pursued mechanistic investigation of these mediators in an experimental animal model of *A. fumigatus*-associated allergic airway inflammation (fungal asthma) (18, 21). This work uncovered immunopathogenic roles for mediators such as IL-7 (18) and IL-1 α and IL-1 β (21) and an immunoprotective role for CX3CL1/fractalkine (26) during fungal asthma. The chitinase-like protein YKL-40 (chitinase 3-like-1, BRP-39 in mice) is elevated in humans with asthma and positively correlates with more severe asthma (27, 28). Further, specific *Chi3l1* SNPs are associated with increased levels of YKL-40 in sputum of patients with asthma, which correlated with decreased lung function (29).

Analysis of bronchoalveolar lavage fluid from humans with asthma revealed that YKL-40 levels were significantly higher in fungal-sensitized versus nonsensitized asthmatics (Fig. 2A). This association suggests that YKL-40 may be induced as part of the immune response during fungal sensitization and led us to question whether it played a causal role in fungal asthma pathogenesis. Unexpectedly, mice deficient in chitinase 3-like-1 (*Chi3l1*^{-/-}, BRP-39) demonstrated significantly worse airway hyperresponsiveness (AHR) during *A. fumigatus*-associated allergic airway inflammation (Fig. 2B). There were no differences between naïve WT and *Chi3l1*^{-/-} mice (Fig. 2B). Thus, BRP-39 is required for optimal lung function during experimental fungal asthma.

Chitinase 3-like-1/BRP-39 Promotes Increased Lung Cellularity During Experimental Fungal Asthma

We have previously reported that differences in AHR often correlated with the magnitude of lung inflammation and cellularity, specifically increased neutrophil numbers, and, in some instances, increased eosinophil numbers (18, 21). Histological analysis of lung sections from WT and *Chi3l1*^{-/-} mice revealed an increase in inflammatory cells surrounding the blood vessels and the airways as well as infiltration into the alveolar spaces in WT mice (Fig. 3A) over that observed in *Chi3l1*^{-/-} mice (Fig. 3A). There were no differences between naïve WT and *Chi3l1*^{-/-} mice (Fig. 3A). Quantifying these differences by flow cytometric analysis of cells isolated via bronchoalveolar lavage demonstrated that *Chi3l1*^{-/-} mice had significantly lower eosinophil (Fig. 3B) and CD4 T-cell (Fig. 3C), but not neutrophil (Fig. 3D), numbers. Thus, despite worse AHR in *Chi3l1*^{-/-} mice, cells that often contribute to AHR, i.e., eosinophils and CD4 T cells, were surprisingly lower.

Chitinase 3-like-1/BRP-39 Promotes Type 2, but Not Type 1 or Type 17, Responses During Experimental Fungal Asthma

We and others have previously reported that type 1/Th1 and type 17/Th17 responses correlate with and contribute to disease severity during experimental fungal asthma (7, 18, 21). However, type 2/Th2 responses are also robustly induced during experimental fungal asthma and have been shown by others to also contribute to severity (30, 31). As there was a

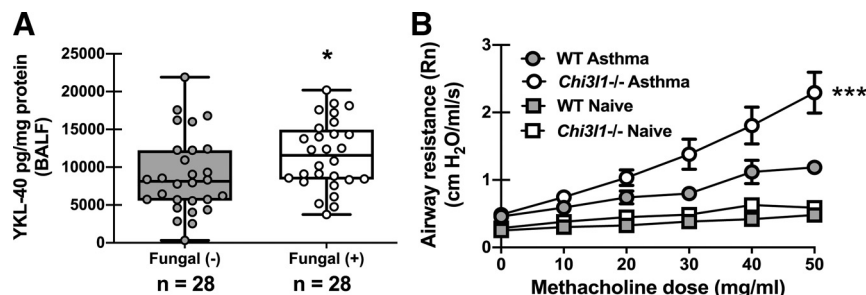


Figure 2. Chitinase 3-like-1 (YKL-40/BRP-39) is elevated in humans with asthma sensitized to fungi but protects against AHR in an experimental animal model of fungal asthma. A: bronchoalveolar lavage fluid (BALF) was collected from subjects with atopic asthma who were sensitized ($n = 28$) or were not sensitized ($n = 28$; sputum) to fungi. YKL-40 levels were quantified in clarified BALF supernatants by Milliplex. Data were normalized to the total protein content of each sample and expressed as mean pg/mg protein (each symbol represents a single subject). * P value < 0.05 (two-tailed Student's t test). B: Balb/c wild-type (WT) and BRP-39-deficient (*Chi3l1*^{-/-}) mice were chronically exposed to *A. fumigatus* as described in the MATERIALS AND METHODS. Twenty-four hours after the last organism challenge, airway (Newtonian) resistance was analyzed via mechanical ventilation using the flexiVent pulmonary function system. Naïve mice served as controls for baseline lung function. The figures illustrate cumulative data from two independent studies ($n = 6$ mice per group per study). Data are expressed as means $\pm$ SE. *** P value < 0.001 (two-way ANOVA).

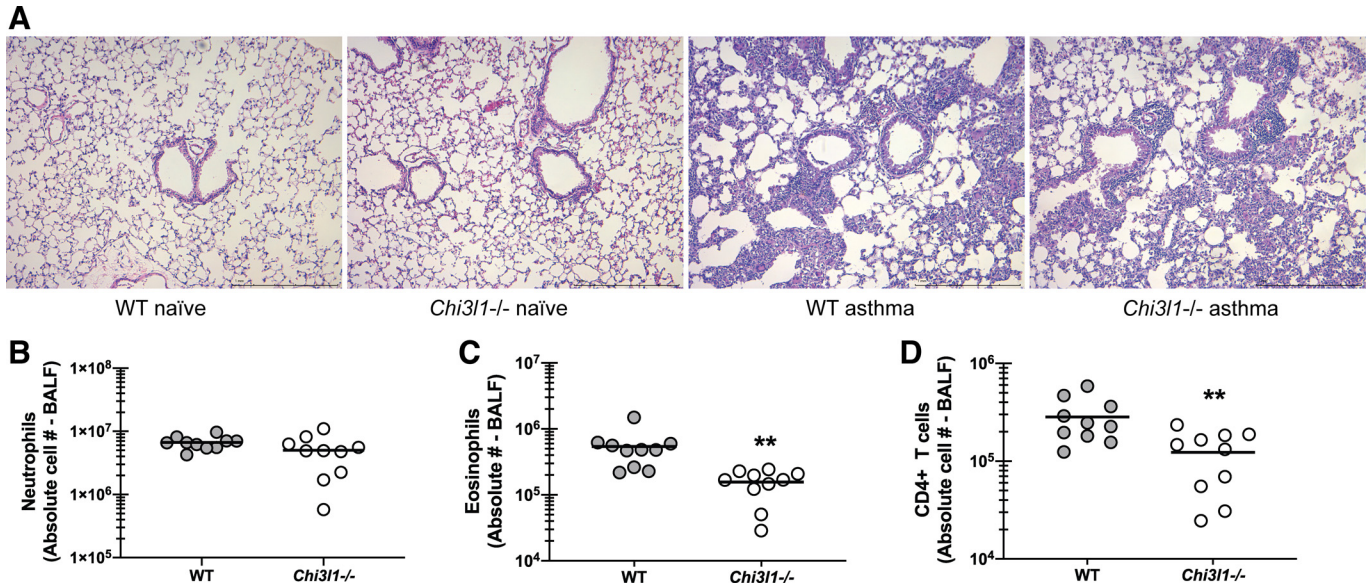


Figure 3. Chitinase 3-like-1/BRP-39 promotes increased lung cellularity during experimental fungal asthma. **A:** Balb/c wild-type (WT) and BRP-39-deficient (*Chi3l1*^{-/-}) mice were chronically exposed to *A. fumigatus* as described in the MATERIALS AND METHODS. Representative hematoxylin and eosin (H&E)-stained lung sections from naïve and asthmatic WT and *Chi3l1*^{-/-} mice. Original magnification $\times 10$. Bar = 1 mm. **B:** Balb/c wild-type (WT) and BRP-39-deficient (*Chi3l1*^{-/-}) mice were chronically exposed to *A. fumigatus* as described in the MATERIALS AND METHODS. Twenty-four hours after last challenge, lung cells from WT and *Chi3l1*^{-/-} mice were isolated by bronchoalveolar lavage, enumerated, Fc-blocked, stained with a live/dead staining kit, then stained for neutrophils (CD45⁺ CD11b⁺ Ly-6G⁺; **B**), eosinophils (CD45⁺ CD11b⁺ SiglecF⁺; **C**), and CD4 T cells (CD45⁺ CD3⁺ CD4⁺; **D**), and quantified via flow cytometry. The figure illustrates cumulative data from two independent studies ($n = 5$ mice per group, per study). Each dot presents a single mouse. The middle line represents the mean. Data are expressed as absolute cell number in lavage fluid. ** P value < 0.01 (two-tailed Student's t test).

specific change in lung cellularity during experimental fungal asthma, i.e., decreased eosinophils and CD4 T cells, but not neutrophils, we questioned whether these changes correlated with changes in various pro- and anti-inflammatory responses. Results in Fig. 4A show that the type 2 cytokines IL-4 and IL-5 were significantly lower in *Chi3l1*^{-/-} mice during fungal asthma, whereas IL-13 levels were unchanged. This correlated with reductions in the pro-type 2 cytokine IL-33 as well as the type 2 associated chemokines CCL17 and CCL22 (Fig. 4B). In contrast, the type 1 cytokine IFN- γ and the associated type 1 chemokines CXCL9 and CXCL10 were not different between WT and *Chi3l1*^{-/-} mice (Fig. 4C). Likewise, the type 17 cytokines IL-17A and IL-22 were not different (Fig. 4D) nor was the anti-inflammatory cytokine IL-10 (Fig. 4E). The immunoregulatory cytokine TGF- β 1 can drive multiple aspects of airway remodeling during asthma including airway smooth muscle proliferation and synthesis of extracellular matrix proteins (32). An increase in TGF- β 1 could potentially contribute to increased AHR in *Chi3l1*^{-/-} mice; however, we found that TGF- β 1 levels were decreased in the absence of BRP-39 (Fig. 4E). In contrast, the IL-1 family cytokine IL-38, which functions in an antagonistic manner after receptor binding (33), was found to be increased in *Chi3l1*^{-/-} mice (Fig. 4F). Thus, despite worse AHR in *Chi3l1*^{-/-} mice, cytokines that often contribute to AHR were either lower or not different in the absence of BRP-39.

Chitinase 3-like-1/BRP-39 Deficiency Does Not Affect Production of Mucins but Decreases Lung Edema During Fungal Asthma

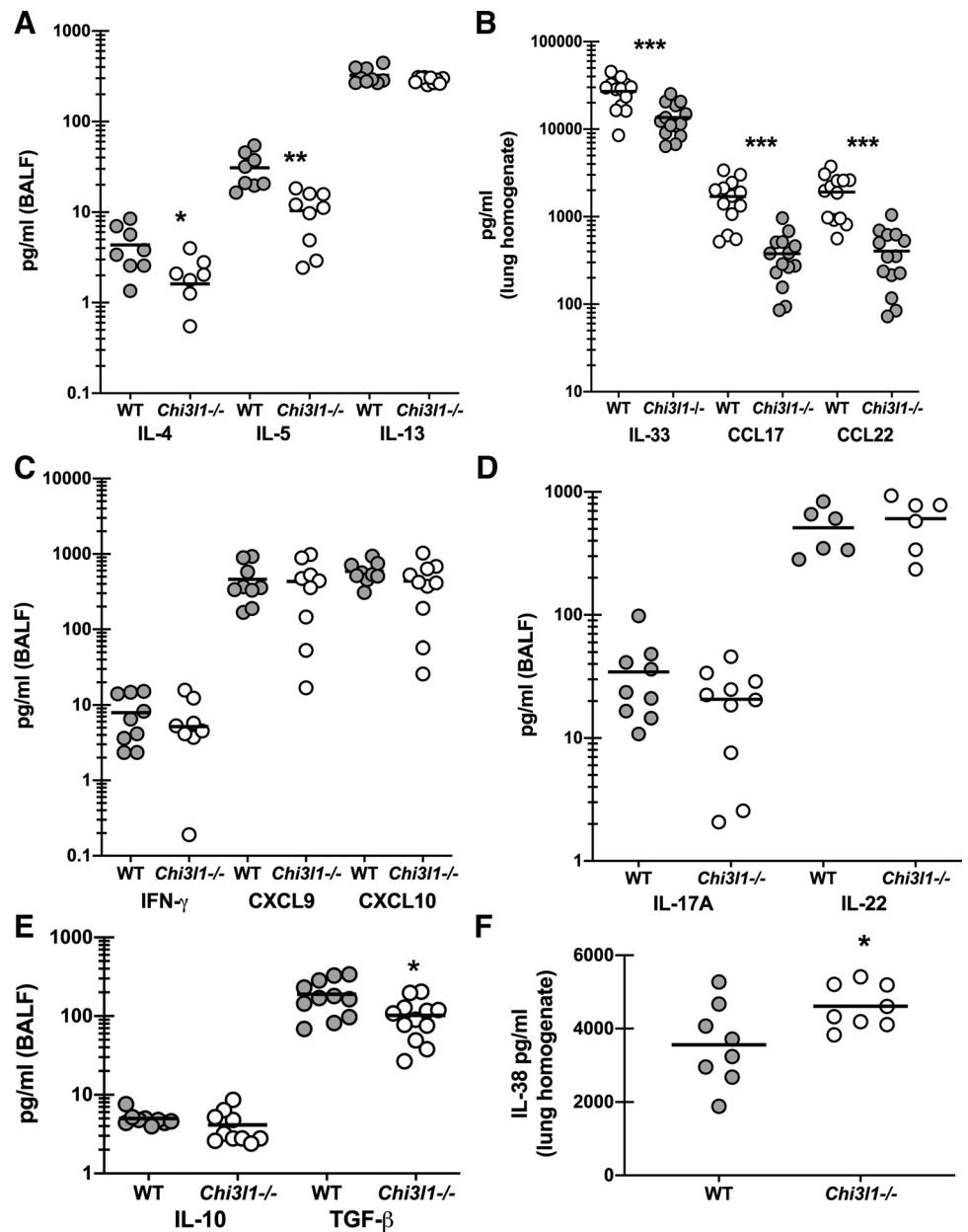
As differences in inflammation in terms of cell recruitment and cytokine profiles failed to explain increased AHR

in *Chi3l1*^{-/-} mice, we assessed mucus production by examining periodic acid-Schiff (PAS)-stained lung sections. This analysis revealed no differences in goblet cell hyperplasia and airway mucus between WT control (Fig. 5A) and *Chi3l1*^{-/-} mice (Fig. 5A). There were also no differences between naïve WT and *Chi3l1*^{-/-} mice (Fig. 5A). This was further supported by real-time PCR assessment of the mucus-associated genes *Muc5ac* (Fig. 5B) and *Gob5* (Fig. 5C), which showed no differences in mRNA expression between WT and *Chi3l1*^{-/-} mice. Though more commonly measured in acute lung injury, pulmonary edema can also contribute to AHR in animal models (34) and is present in very severe clinical cases of asthma (35). We analyzed wet/dry ratios of WT and *Chi3l1*^{-/-} mice and observed decreased ratios in the latter during fungal asthma. This decreased wet/dry ratio implies decreased edema, or potentially decreased blood volume, in the absence of BRP-39. Thus, despite worse AHR in *Chi3l1*^{-/-} mice, there were no differences in mucus gene expression or mucus appearance in the lung. Surprisingly, lung wet/dry ratio was lower in the absence of BRP-39, thus eliminating lung edema as a possible cause of increased AHR.

Presence of Chitinase 3-like-1/BRP-39 in Either the Hematopoietic or Nonhematopoietic Compartment Is Sufficient to Regulate Increased AHR during Fungal Asthma

BRP-39 is broadly expressed in both hematopoietic cells, including neutrophils and macrophages, and nonhematopoietic cells, including fibroblasts and epithelial cells (36). As a step toward understanding the discordant airway function and inflammatory features of *Chi3l1*^{-/-} mice, we determined

Figure 4. Chitinase 3-like-1/BRP-39 promotes type 2, but not type 1 or type 17, responses during experimental fungal asthma. Balb/c wild-type (WT) and BRP-39-deficient (*Chi3l1*^{-/-}) mice were chronically exposed to *A. fumigatus* as described in the MATERIALS AND METHODS. Twenty-four hours after last challenge, a bronchoalveolar lavage was performed and BAL fluid was clarified by centrifugation. **A:** IL-4, IL-5, and IL-13 levels were quantified in clarified BALF by Milliplex. **B:** IL-33, CCL17, and CCL22 levels were quantified in clarified lung homogenates by ELISA. **C:** IFN- γ , CXCL9, and CXCL10 were quantified in clarified BALF by Milliplex. **D:** IL-17A and IL-22 levels were quantified in clarified BALF by Milliplex and ELISA, respectively. **E:** IL-10 and TGF- β were quantified in clarified BALF by Milliplex and ELISA, respectively. **F:** IL-38 levels were quantified in clarified lung homogenates by ELISA. The figures illustrate cumulative data from two independent studies ($n=5$ mice per group, per study). Each dot presents a single mouse. The middle line represents the mean. Data are expressed as pg/mL in lavage fluid or lung homogenate. * P value < 0.05; ** P value < 0.01; *** P value < 0.001 (two-tailed Student's t test).



in which group of cells BRP-39 expression was protective. To answer this question, we created reciprocal bone marrow chimeras in which either the hematopoietic or nonhematopoietic compartment expressed BRP-39, as well as control chimeras in which both compartments (analogous to WT) or neither compartment (analogous to *Chi3l1*^{-/-} mice) expressed BRP-39. Following reconstitution, these chimeras were subjected to experimental fungal asthma and airway function was assessed. We found that expression of BRP-39 in either the hematopoietic compartment (WT $\rightarrow$ KO) or the nonhematopoietic compartment (KO $\rightarrow$ WT) was sufficient to regulate the magnitude of AHR (Fig. 6), indicating that BRP-39 expression in either compartment is sufficient to control AHR.

DISCUSSION

Studies have shown that YKL-40/chitinase 3-like-1/BRP-39 levels correlate with thickening of the epithelial basal membrane (37) and may function in airway smooth muscle remodeling in human asthma (38). Experimentally, although somewhat limited, mice deficient in BRP-39 (chitinase-3-like protein 1; *Chi3l1*) have attenuated type 2 responses and better lung function during chitin-negative ovalbumin-associated asthma and chitin-containing house dust mite-associated asthma (16, 39). Although chitooligosaccharides are the dominant ligands for the glycoside hydrolase 18 family (chitinases and chitinase-like proteins) (28, 40), IL-13R α 2 has been identified as a receptor for BRP-39 (23). Via IL-13R α 2, BRP-39

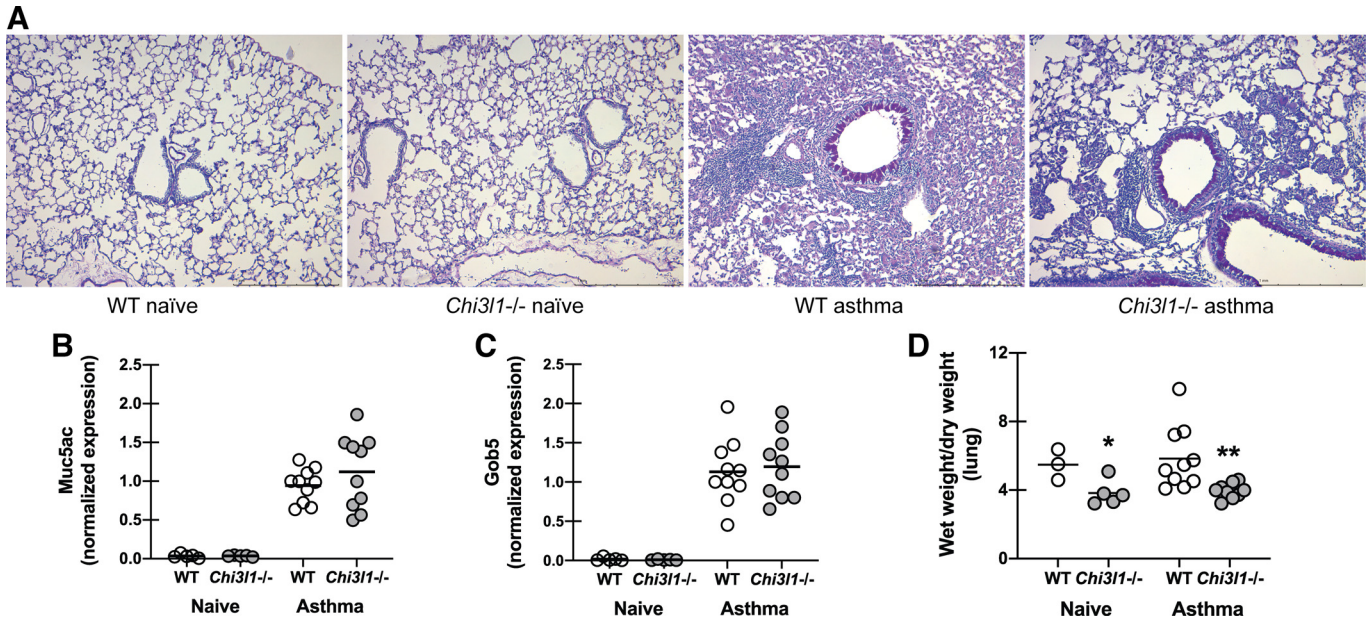


Figure 5. Chitinase 3-like-1/BRP-39 deficiency does not affect production of mucins but decreases lung edema during fungal asthma. Balb/c wild-type (WT) and BRP-39-deficient (*Chi3l1*^{-/-}) mice were chronically exposed to *A. fumigatus* as described in the MATERIALS AND METHODS. **A:** representative periodic acid-Schiff-stained lung sections from naïve and asthmatic WT and *Chi3l1*^{-/-} mice. Original magnification $\times 10$. Bar = 1 mm. **B** and **C:** twenty-four hours after the last challenge, the left lungs were collected and *Muc5ac* (**B**) and *Gob5* (**C**) gene expression was quantified by real-time PCR and normalized to HPRT. Data are expressed as $2^{-\Delta\Delta C_t}$. The figure illustrates cumulative data from two independent studies ($n = 2-3$ mice per group per study for naïve mice and $n = 5$ mice per group per study for asthmatic mice). Each dot presents a single mouse. The middle line represents the mean. **D:** twenty-four hours after the last challenge, lungs were harvested, weighed, dried for 24 h, and then reweighed. Data are expressed as wet weight/dry weight. The figure illustrates cumulative data from two independent studies ($n = 2$ or 3 mice per group per study for naïve mice and $n = 5$ mice per group per study for asthmatic mice). Each dot presents a single mouse. The middle line represents the mean. * P value < 0.05 ; ** P value < 0.01 (two-tailed Student's t test).

has been shown to promote resistance to hyperoxia-induced apoptosis, *S. pneumoniae*-induced pyroptosis and inflammatory activation, and lung melanoma metastasis (41). However, a previous study has reported that IL-13R $\alpha 2$ is not expressed in the lung during experimental *A. fumigatus*-

associated asthma (42), suggesting that IL-13R $\alpha 2$ is not functioning as ligand for BRP-39 during fungal asthma. Other studies have shown that BRP-39 induces inflammatory mediator production during lung metastasis, which can be reversed by treatment with chitin (43), whereas BRP-39 expressed on colonic epithelial cells binds chitin particles, which can be simultaneously bound by chitin-binding protein-expressing bacteria, leading to enhanced bacterial adhesion (44). Collectively, these studies suggest that chitin binding is a critical factor in the function of BRP-39. However, BRP-39 binding to fungal chitin and how this influences responses or severity of allergic fungal asthma has not been examined. Here, we sought to fill this gap in knowledge by elucidating the role of BRP-39 in an experimental animal model of fungal-associated allergic airway inflammation. Firstly however, data suggest that BRP-39 may play a role in lung host defense. Previous work has demonstrated that BRP-39 promotes clearance of *Streptococcus pneumoniae* from the lung by augmenting bactericidal activity of macrophages and limiting excessive inflammation (27). During *Pseudomonas aeruginosa* pneumonia, BRP-39 also functions to limit excessive inflammation and improve survival (45). In contrast, we confirmed that BRP-39 was not required for host defense against *A. fumigatus* as evidenced by similar lung fungal burden at both 24 and 48 h following infection. We did not assess inflammatory responses in these experiments, as any effect was not sufficient to affect fungal burden or survival. A noteworthy difference between the reported bacterial models

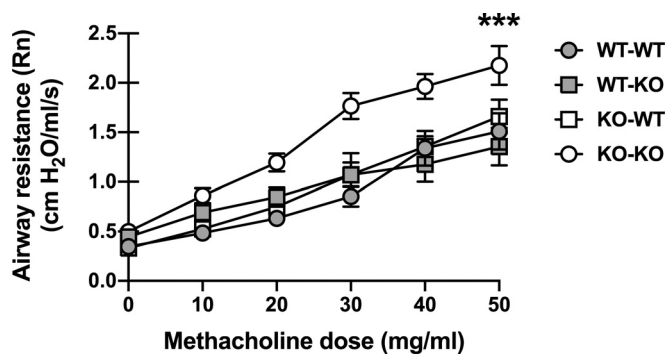


Figure 6. Presence of chitinase 3-like-1/BRP-39 in either the hematopoietic or nonhematopoietic compartment is sufficient to regulate increased AHR during fungal asthma. Bone marrow chimeras were generated in which mice expressed BRP-39 in either only the hematopoietic compartment (C57BL/6-*Chi3l1*^{-/-}; WT-KO), only the nonhematopoietic compartment (KO-WT), both compartments (WT-WT) or neither compartment (KO-KO) as described in the MATERIALS AND METHODS. Eight to ten weeks following reconstitution, chimeras were chronically exposed to *A. fumigatus* as described in the MATERIALS AND METHODS. Airway (Newtonian) resistance was analyzed via flexiVent. The figure illustrates cumulative data from two independent studies ($n = 5$ mice per group, per study). Data are expressed as means $\pm$ SE. *** P value < 0.001 (two-way ANOVA). KO, knockout; WT, wild-type.

and the *A. fumigatus* infection model employed here is much higher lethality in WT control mice in the bacterial infections compared to fungal infection. This difference also suggests that BRP-39 may be primarily required to regulate inflammation during lung infections that causes significant mortality due to hyperinflammatory responses. Nevertheless, BRP-39 is dispensable for fungal clearance from the lung during acute *A. fumigatus* challenge.

It is increasingly appreciated that the underlying pathogenic processes that result in asthma phenotypes are extremely diverse and that development of more effective and targeted treatments will likely require precise knowledge of these processes. To this end, we have sought to define pathways that contribute to fungal asthma pathogenesis by identifying factors that are modulated in humans with fungal (+) asthma and exploring the roles of these factors in experimental models. Our work has identified IL-7 (18) and IL-1R signaling (21) as immunopathogenic factors in fungal asthma. In contrast, we demonstrated that IL-1RA was an attractive and efficacious immunoprotective therapeutic for improving fungal asthma severity (21). Ongoing work in our laboratory has also identified CX3CL1 as an immunoprotective mediator during fungal asthma (26). YKL-40 has been implicated in non-type 2 asthma due to its relationship with mediators associated with IL-17-mediated inflammation (15, 46). Indeed, higher circulating YKL-40 consistently associates with more severe asthma, with clusters of patients exhibiting features of non-type 2-driven inflammation (47, 48). Our finding of higher YKL-40 levels in fungal-sensitized asthmatics may be intuitive based on YKL-40's known ability to bind chitin (28) as well as the fact that fungal asthma is often characterized by induction of type 17 responses (21, 49, 50). We therefore applied our model of fungal asthma to WT and *Chi3l1*^{-/-} mice and measured airway resistance to understand the effect of BRP-39 on lung function. While the role of BRP-39 in fungal asthma has not been previously investigated, study of *Chi3l1*^{-/-} mice in ovalbumin (OVA) and house dust mite (HDM) models of asthma reported decreased airway resistance (improved lung function) (16). In an alternative study design where bone marrow-derived dendritic cells overexpressing BRP-39 were transferred into mice that subsequently underwent the OVA asthma model, BRP-39 was also found to drive AHR (51). OVA and HDM models of asthma primarily drive a Th2 asthma phenotype, whereas the fungal asthma model employed here attempts to recapitulate a particular endotype of human asthma (severe asthma with fungal sensitization). Through the use of live *A. fumigatus* conidia, our model generates a complex and broad host response in addition to the typical Th2 asthmatic responses (6, 18). Thus, it is somewhat surprising that, in contrast to the findings in the OVA and HDM models described above, our experiment revealed a contrasting, protective/beneficial role for BRP-39 during fungal asthma. YKL-40 generally correlates with worse disease in human asthma (15, 47, 52, 53). Indeed, we show that YKL-40 levels were elevated in fungal-sensitized asthmatics (Fig. 2A), a cohort that we have reported to have more severe disease (18). Although the data are limited, in addition to the bacterial pneumonia studies mentioned previously, other studies also support a beneficial role for BRP-39 in some experimental lung disease models. In hypoxia-associated acute lung injury,

BRP-39 is required for survival in conjunction with regulating the magnitude of neutrophil recruitment and inflammation (54). In a murine model of bleomycin-induced fibrosis, BRP-39 ameliorated inflammation and cell death as well as played a profibrotic role in the repair phase by augmenting alternative macrophage activation, fibroblast proliferation, and matrix deposition (55). Therefore, there is a precedence for BRP-39 functioning in a protective capacity. The contrast between the pathogenic effect of BRP-39 in Th2-centric models of asthma and the protective effect of BRP-39 in our model of fungal asthma raises intriguing and potentially therapeutically important questions about the function of this molecule in different allergic and lung disease contexts.

BRP-39 promotes type 2/Th2 responses in the aforementioned OVA and HDM asthma models (16), and our data further support this finding during fungal asthma. We further found that numbers of eosinophils and CD4 T cells, classic contributors to Th2 asthma, were decreased. In addition to decreased IL-4 and IL-5 in BALF, our data expand the role of BRP-39 in the induction of type 2/Th2 responses by showing that the absence of BRP-39 resulted in lower levels of the pro-type 2/Th2 mediator IL-33. We further show that the levels of CCL17 and CCL22, chemokines that we have previously found to be associated with disease severity in our model (6, 7, 18), were also reduced in the absence of BRP-39. Therefore, our data support a central role of BRP-39 in both the initiation of type 2/Th2 responses via IL-33 induction and the recruitment of eosinophils (56) and CD4 T cells (57), which express the receptor for CCL17 and CCL22 (CCR4). BRP-39 has been shown to suppress differentiation of naïve T cells into Th1 cells (58) and to support IL-17A production by $\gamma\delta$ T cells (59). We have reported that type 1/Th1 and type 17/Th17 responses correlate with neutrophil levels and the severity of fungal asthma (7, 18, 21). Enhanced type 1/Th1 or type 17/Th17 responses could explain the exacerbation observed in the absence of BRP-39. However, levels of IFN- γ and IL-17A were unaffected by the absence of BRP-39 during fungal asthma as were the levels of neutrophils in the lung. The lone mediator we observed to be increased in the absence of BRP-39 was IL-38. IL-38 is not well studied in allergic asthma, although reports suggest it may regulate the production of some inflammatory cytokines as well as induction of Th2 cytokines and eosinophil recruitment (60). Our data support IL-38 functioning in the latter during fungal asthma; however, we cannot exclude an immunopathogenic role for IL-38, which will be the subject of future studies. Together, these data indicate that BRP-39 plays a role in the induction of select Th2 responses during fungal asthma, as has been reported in other models, but does not affect potentially immunopathogenic type 1/Th1 or type 17/Th17 responses. Overall, our findings are in accordance with BRP-39 as a driver of allergic inflammation, but paradoxical considering the phenotype of impaired lung function in our model.

Though the mechanism of increased AHR in *Chi3l1*^{-/-} mice during fungal asthma remains to be determined, we have excluded numerous potential drivers of immunopathogenesis. In fact, all the potentially immunopathogenic mediators that were modulated in the absence of BRP-39 were either not different or decreased, making increased AHR in this context all the more compelling. Therefore, we investigated nonimmune functions of BRP-39 that might explain

the increase in AHR in its absence. Excessive production of mucins is an additional feature of asthma pathogenesis that we considered as a possible correlate of increased AHR in *Chi3l1*^{-/-} mice. We assessed mucus production via qPCR analysis of *Muc5ac* and *Gob5* and via histological periodic acid-Schiff staining. These analyses indicated the transcription and presence of mucins in the airways of WT and *Chi3l1*^{-/-} during fungal asthma compared to naïve controls, but no difference between WT and *Chi3l1*^{-/-} mice. We further excluded increased lung edema as a driver of AHR in the absence of BRP-39 via analysis of lung wet weight/dry weight ratios. Although lung wet:dry weight did not correlate with AHR, it did correlate with lower inflammatory cell numbers (eosinophils and CD4 T cells) and lower cytokine values (in our model, lower type 2 cytokines). This finding is similar to recent data showing that *Chi3l1*^{-/-} mice exposed to RSV also have lower inflammatory cell numbers in the lung and lower type 2 cytokines (61). Therefore, lung injury is often a direct correlate of the level of inflammation. In regard to *Chi3l1*^{-/-} mice, this appears to be pathogen/model-specific, as bacterial infection contributes to elevated lung injury whereas fungal and viral exposure/infection does not.

We next utilized reciprocal bone marrow chimeras to discriminate between the functions of BRP-39 in relevant hematopoietic cells (leukocytes) versus nonhematopoietic cells (lung epithelial cells, airway smooth muscle cells), in hopes of guiding future studies into how BRP-39 suppresses AHR during fungal asthma. We found that chimeras expressing BRP-39 in either the hematopoietic or nonhematopoietic compartments exhibited lung function similar to chimeras expressing BRP-39 in both compartments. Only chimeras lacking BRP-39 entirely exhibited impaired lung function. BRP-39 is a secreted protein, and these results indicate that any effect of the source of BRP-39 secretion (hematopoietic or nonhematopoietic cells) is secondary to its downstream effects in terms of AHR. Other groups have recapitulated BRP-39 phenotypes with administration of BRP-39, also supporting its function as a secreted molecule (27, 41). BRP-39 can also bind to collagen (2) and other endogenous ligands including hyaluronan (62), leaving open the possibility of multiple potential nonimmune functions of secreted BRP-39. Recent studies suggest that low molecular weight hyaluronan fragments can drive AHR, whereas high molecular weight hyaluronan fragments can reduce AHR (63, 64). Although speculative, the absence of BRP-39 may result in hyaluronan-mediated events during fungal asthma that negatively affect AHR. Future studies will determine if during fungal asthma, there is an imbalance between low and high molecular weight hyaluronan fragments in *Chi3l1*^{-/-} mice. Further, a key point of interest in our pursuit of the role of BRP-39 in fungal asthma is to unravel the complex role of chitin during fungal asthma. In terms of the function of BRP-39, one possibility is its binding to chitin during exposure to a chitin-rich pathogen potentially, which inhibits binding to other endogenous ligands and thus interrupts protective or pathogenic processes. More broadly, mammals express multiple chitinases and chitinase-like proteins including BRP-39, all of which may affect either the amount of chitin in the lung or how it interacts with the immune system. Along these lines, acidic mammalian chitinase has been shown to prevent long-term damage caused by

accumulation of environmental chitin in the lung (65) and to promote chitin-dependent eosinophilia following exposure to *Aspergillus niger* preparations (11). Chitin cleavage by another true chitinase, chitotriosidase, supports development of Th2 responses during infection with the chitin-rich fungal pathogen, *Cryptococcus neoformans* (66). BRP-39 is reported to be constitutively expressed at higher levels in the lung than either chitotriosidase or acidic mammalian chitinase (67), and though it lacks chitinolytic activity, it retains the ability to bind chitin (28). As already discussed, during fungal asthma *Chi3l1*^{-/-} mice phenocopy selects aspects of other asthma models, including some containing chitin (primarily the induction of Th2 responses) but has the opposite effect on AHR. Given the complexity of the effects of chitin, particularly fungal chitin, on immune responses, we are currently undertaking studies to understand if BRP-39, in concert with active chitinases, modulates chitin levels or exposure of chitin to other recognition factors during fungal asthma.

In conclusion, we report that increased levels of chitinase 3-like-1/BRP-39 in humans with asthma are associated with allergic sensitization to fungi. Interrogating of the role of BRP-39 in a mouse model of *Aspergillus*-associated allergic airway inflammation revealed that BRP-39 is protective for lung function, yet paradoxically also drives type 2/Th2 inflammation. This correlation has not been observed in other models; thus, our data add another important aspect to our knowledge of the biology of this molecule. This effect occurs via a yet to be determined mechanism but likely occurs via a function of BRP-39 as a secreted molecule, which potentially binds multiple endogenous substrates as well as exogenous chitin, to currently unknown effect. These results add to our previous work on acidic mammalian chitinase to expand our understanding of the role of chitin-associated proteins and set the stage for further study of the role of fungal chitin in allergic asthma.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.J.M., A.T.H., D.A.M. and C.S. conceived and designed research; J.J.M., A.T.H., D.A.M., C.S., J.M.G., M.J., D.A.E., J.P.B., Z.Y., S.M. and M.C. performed experiments; J.J.M., A.T.H., D.A.M., C.S., J.M.G., S.M., and F.E.L. analyzed data; J.J.M., A.T.H., D.A.M., C.S., J.M.G., and F.E.L. interpreted results of experiments; J.J.M. and C.S. prepared figures; J.J.M., A.T.H., D.A.M. and C.S. drafted manuscript; J.J.M., A.T.H., D.A.M. and C.S. edited and revised manuscript; J.J.M., A.T.H., D.A.M. and C.S. approved final version of manuscript.

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