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IL-1RA regulates immunopathogenesis during fungal-associated allergic airway inflammation

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Abstract

Severe asthma with fungal sensitization (SAFS) defines a subset of human asthmatics with allergy to one or more fungal species and difficult to control asthma. We have reported that human asthmatics sensitized to fungi have worse lung function and a higher degree of atopy, which was associated with higher IL-1RA levels in bronchoalveolar lavage fluid. IL-1RA further demonstrated a significant negative association with bronchial hyperresponsiveness to methacholine. Here, we show that IL-1α and IL-1β are elevated in both bronchoalveolar lavage fluid and sputum from human asthmatics sensitized to fungi, implicating an association with IL-1α, IL-1β or IL-1RA in fungal asthma severity. In an experimental model of fungal-associated allergic airway inflammation, we demonstrate that IL-1R1 signaling promotes type 1 (IFN-γ, CXCL9, CXCL10) and type 17 (IL-17A, IL-22) responses that were associated with neutrophilic inflammation and increased airway hyperreactivity. Each of these were exacerbated in the absence of IL-1RA. Administration of human recombinant IL-1RA (Kineret®/Anakinra) during fungal-associated allergic airway inflammation improved airway hyperreactivity and lowered type 1 and type 17 responses. Taken together, these data suggest that IL-1 receptor signaling contributes to fungal asthma severity via immunopathogenic type 1 and type 17 responses and can be targeted for improving allergic asthma severity.
Introduction

Atopic asthma is a chronic allergic disease wherein exposure to allergens results in a heterogeneous mixture of symptoms including increased IgE, bronchiectasis, smooth muscle cell hypertrophy and hyperplasia, goblet cell hyperplasia, mucus hypersecretion, airway hyper-responsiveness and remodeling, neutrophilia, eosinophilia and lung fibrosis (1) (2). While there are numerous allergens associated with asthma exacerbations, fungi, including the mold Aspergillus fumigatus, are considered the most ubiquitous. Herein, a subset of asthma termed “severe asthma with fungal sensitization” (SAFS) has been described for individuals whose asthma is poorly-controlled and who are sensitized to the fungal organisms Alternaria, Aspergillus, Cladosporium and/or Penicillium (3). Reports indicate that the estimated prevalence of SAFS ranges from 17 to 46% of the estimated 5-10% asthmatics defined as severe (4) (5) (6) (7). As a result, individuals sensitive to fungi experience more frequent exacerbations, have higher incidences of uncontrolled asthma and use corticosteroids more frequently than non-sensitive asthmatics (4) (5) (6) (7).

A growing area of interest is the identification of factors that contribute to immunopathogenesis in allergic asthma and determining whether such factors could be viable therapeutic targets. Indeed, biologics targeting the type 2 cytokines IL-4, IL-5 and IL-13 have been successful in treating severe asthma (8). With regards to SAFS specifically, studies comparing genetic susceptibility in individuals with atopic asthma vs. individuals with SAFS have identified the pattern recognition receptors (PRRs) TLR3, TLR9 and Dectin-1 and the cytokines/chemokines IL-10, CCL2 and CCL17 as candidates for therapeutic development (9). We have also recently identified IL-7 as a potential immunopathogenic factor in human asthma and experimental fungal-associated allergic airway inflammation (10). In a study of pediatric asthmatics, those with SAFS demonstrated higher IgE levels and required higher usage of maintenance oral steroids to achieve similar clinical status as non-SAFS asthmatics (7). Intriguingly, pediatric SAFS was associated with elevated levels of the type 2 associated cytokine IL-
33 in bronchoalveolar lavage fluid. Likewise, a study of adult SAFS subjects also found elevated IL-33 in serum (6). IL-33 is a member of the IL-1 family of cytokines, which is comprised of eleven members, seven with pro-inflammatory characteristics (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β and IL-36γ) and four with putative anti-inflammatory characteristics (IL-1RA, IL-36Ra, IL-37 and IL-38). We have previously shown during experimental fungal-associated allergic airway inflammation that the beta-glucan receptor Dectin-1 (11), the pro-inflammatory cytokine IL-22 (11) and the common γ-chain cytokine IL-7 (10) contribute to disease severity, a component of which was the induction of the IL-1 family members IL-1α and IL-1β. Here, we observed higher levels of IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1RA) in lung samples from fungal-sensitized asthmatics compared to fungal-negative asthmatics, implicating a role for these factors in human fungal asthma pathogenesis. We therefore sought to clarify a role for these IL-1 family members in immunopathogenic responses during chronic exposure to A. fumigatus.
Results

Human asthmatics sensitized to fungi have elevated IL-1α and IL-1β levels in bronchoalveolar lavage and sputum

We have recently reported that human asthmatics sensitized to fungi (National Heart Lung and Blood Institute’s Severe Asthma Research Program (SARP) cohorts 1 and 2) have more severe disease as determined by multiple lung function (forced expiratory volume in one second, FEV1; concentration of methacholine producing 20% drop in FEV1, PC20) and atopic measurements (serum IgE, blood eosinophils numbers) (10). In that report, Luminex®-based analysis identified multiple cytokine, chemokine and growth factors that were significantly elevated in bronchoalveolar lavage fluid (BALF) from fungal-sensitized asthmatics (10). Among these was IL-1RA, with regression analysis revealing that IL-1RA had a negative correlation with PC20 (i.e. higher IL-1RA levels correlated with lower PC20), which may suggest a role for IL-1RA in immunopathogenesis during fungal asthma (10). Alternatively, elevated IL-1RA levels could serve as a biomarker of lung function decline during fungal asthma. Here, we extend these observations by showing that IL-1β was also significantly higher in BALF from fungal-sensitized asthmatics (Figure 1A), whereas IL-1α trended higher (Figure 1B). We also performed an additional analysis using the SARP 1 and 2 cohort, here using sputum from adult asthmatics who were atopic and sensitized to fungi vs. asthmatics who were atopic, but not sensitized to fungi. Differences between fungal-sensitized vs. non-sensitized human asthmatics include current age and age of asthma onset, both of which were younger in fungal-sensitized asthmatics (Table 1). There was also a trend towards lower FEV1/Forced Vital Capacity (FVC) (Table 1, P = 0.078) in fungal-sensitized asthmatics, as we have recently reported (10). Fungal-sensitized asthmatics demonstrated significantly higher IgE levels, percentage and number of blood eosinophils and usage of inhaled corticosteroids (Table 1). We observed significantly higher IL-1β (Figure 1C) and IL-1α (Figure 1D) levels in sputum from fungal (+) asthmatics. IL-1RA levels in sputum were not significantly different
There was no difference however in the ratio of IL-1RA to IL-1α or IL-1β in either fungal-sensitized or non-sensitized asthmatics (data not shown). Overall, these results suggest that IL-1 signaling may exist as a central pathway in determining the severity of fungal asthma.

**Signaling through the IL-1 receptor worsens lung function during experimental fungal-associated allergic airway inflammation**

As IL-1α and IL-1β levels were elevated in fungal (+) asthmatics, we sought to determine the impact of IL-1 signaling on the severity of fungal asthma. Employing an experimental model of fungal-associated allergic airway inflammation (10) (11), mice deficient in the receptor for IL-1α and IL-1β (Il1r1−/− mice) demonstrated a dramatic decrease in central airway resistance (airway hyperreactivity; Figure 2A) and total lung resistance (Figure 2B) compared to wild-type control mice. Hematoxylin and eosin staining of lung tissue sections revealed fewer inflammatory cells surrounding the airways and in alveolar spaces in Il1r1−/− mice (Figure 2C). Periodic acid-Schiff staining of lung tissue sections demonstrated higher mucus staining in the airways of wild-type control mice that was much lower in Il1r1−/− mice (Figure 2C). However, analysis of Muc5ac and Gob5 mRNA expression did not reveal any significant differences between WT and Il1r1−/− mice (Figure 2D). Thus, IL-1α and IL-1β signaling during fungal-associated allergic airway inflammation contributes to lung inflammation which negatively impacts lung function.

**IL-1 receptor antagonist regulates the severity of experimental fungal-associated allergic airway inflammation**

As mentioned previously, we recently reported that IL-1RA was elevated in BALF from fungal (+) asthmatics and negatively correlated with lung function (10). As these results suggested that high levels of IL-1RA may act in an immunopathogenic manner during fungal asthma, we sought to clarify the role of IL-1RA. In contrast to mice deficient in the IL-1 receptor, mice deficient in the IL-1 receptor
antagonist (IL-1RA; Il1rn-/- mice) subjected to fungal-associated allergic airway inflammation demonstrated a profound decrement in lung function, as evidenced by a significant increase in central airway resistance (Figure 3A) and total lung resistance (Figure 3B). Hematoxylin and eosin staining and Periodic acid-Schiff staining (Figure 3C) of lung tissue sections again demonstrated a low to moderate level of inflammatory cell recruitment and mucus staining in wild-type control mice. In direct contrast, Il1rn-/- mice surprisingly did not demonstrate mucus staining, rather there was evidence for a destructive inflammatory response leading to either the collapse or occlusion of airways as well as consolidation of the alveolar space. Based on these observations, we examined additional lung function measurements that reflect peripheral airway and parenchymal lung function. This analysis demonstrated that dynamic compliance, a measure of the ease with which the respiratory system can be extended, was significantly lower in Il1rn-/- mice (Figure 3D). In addition, tissue damping, a measure of resistance to air flow in the peripheral airways (Figure 3E), and tissue elastance, a measure of tissue stiffness (Figure 3F), were increased in Il1rn-/- mice. Despite these changes in lung function, analysis of Muc5ac and Gob5 mRNA expression demonstrated significantly lower levels in Il1rn-/- mice (Figure 3G). Thus, IL-1RA is required for regulating lung responses that contribute to lung function impairment during fungal-associated allergic airway inflammation.

**Neutrophils and their associated inflammatory mediators are differentially regulated by IL-1R1 and IL-1RA during experimental fungal-associated allergic airway inflammation**

Continued phenotyping of asthma subsets has led to the classification of allergic asthmatics into two broad inflammatory categories, type 2, eosinophilic-dominated asthma and type 17, neutrophilic-dominated asthma (12), the latter of which is often viewed as more severe and steroid-resistant. However, neutrophils are central effector cells against fungi, *A. fumigatus* in particular (13), and thus are an expected component of the immune response to inhaled fungi. We have previously reported that neutrophil levels in experimental fungal-associated allergic airway inflammation may be associated
with a lower inflammatory profile and improved lung function (11), although in some cases, experimental fungal-associated allergic airway inflammation may be severe in the absence of changes in the neutrophil population (10). Here, the absence of IL-1 receptor signaling was associated with decreased neutrophil recruitment during experimental fungal-associated allergic airway inflammation, as Il1r1/- mice had a 50% reduction in neutrophil recruitment after chronic fungal exposure (Figure 4A). In contrast, IL-1RA was required to regulate the magnitude of neutrophil recruitment, as Il1rn/- mice demonstrated a 3-fold increase in neutrophil numbers during chronic fungal exposure (Figure 4B). Representative flow plots are included in Supplemental Figure 1. The magnitude of neutrophil recruitment in these mice was directly associated with the level of IL-17A, with significantly lower levels produced by lung digest cells from mice deficient in IL-1 receptor signaling (Figure 4C) and significantly higher levels produced in the absence of IL-1RA (Figure 4D). In contrast, while IL-22 production was dependent on the IL-1 receptor (Figure 4C), IL-1RA was not required for IL-22 regulation (Figure 4D). Additional analyses showed that the pro-neutrophil survival factor G-CSF was dependent, at least in part, on IL-1 receptor signaling (Figure 4E) and IL-1RA was required for maintaining G-CSF at wild-type levels during chronic fungal exposure (Figure 4F). In contrast, the neutrophil chemoattractants CCL3 and CCL4 were not dependent on IL-1 receptor signaling (Figure 4E), but were regulated by IL-1RA (Figure 4F). We also observed decreased CXCL1 levels in Il1r1/- mice, although this was not regulated by IL-1RA (i.e. Il1rn/- mice did not demonstrate an increase in CXCL1; data not shown). Thus, the severity of experimental fungal-associated allergic airway inflammation is associated with neutrophil levels in the lung where IL-1 receptor signaling is required for the induction of pro-neutrophil survival factors and IL-1RA is required for controlling the level of these survival factors as well as neutrophil chemoattractants.
The absence of IL-1R1 and IL-1RA results in varied type 2 responses during experimental fungal-associated allergic airway inflammation

The dominant immune response in allergic asthma immunopathogenesis is often considered to be type 2 and eosinophil-centric. Similar to our findings with neutrophils detailed above, experimental fungal-associated allergic airway inflammation severity may (10) or may not (11) be associated with the level of eosinophils. IL-1 receptor deficiency resulted in a significant reduction in the number of eosinophils recruited to the lung during experimental fungal-associated allergic airway inflammation (Figure 5A). Somewhat surprisingly, IL-1RA deficiency also resulted in a trend towards lower eosinophil numbers ($P = 0.089$; Figure 5B). Representative flow plots are included in Supplemental Figure 1. Regarding type 2 responses, IL-4 was not different between $Il1r1^{-/-}$ mice and wild-type controls whereas IL-5 was significantly lower (Figure 5C). In contrast, both IL-4 and IL-5 were significantly lower in $Il1rn^{-/-}$ mice (Figure 5D). IL-13 was not consistently produced during fungal-associated allergic airway inflammation whereas IL-9 levels were not different between WT and $Il1r1^{-/-}$ mice or WT and $Il1rn^{-/-}$ mice (data not shown). The major eosinophil chemoattractant CCL11 was not modulated by IL-1 receptor deficiency (Figure 5E) but was significantly lower in the absence of IL-1RA (Figure 5F). The pro-allergic chemokines CCL17 and CCL22, which we have previously employed as biomarkers of fungal-associated allergic airway inflammation severity (10) (11) and are recognized biomarkers in human allergic bronchopulmonary aspergillosis (14) (15), were not affected by IL-1 receptor deficiency (Figure 5G) or IL-1RA deficiency (Figure 5H). Finally, deficiency in IL-1 receptor signaling did not impact the levels of the IL-1 family and pro-type 2 cytokine IL-33 (Figure 5I), while IL-33 was increased in the absence of IL-1RA (Figure 5J). Thus, analogous to neutrophils, the severity of experimental fungal-associated allergic airway inflammation in IL-1 receptor deficient mice is associated with eosinophil levels and pro-eosinophil survival factors. In contrast, severity of experimental fungal-associated allergic airway inflammation in IL-1RA deficient mice did not involve heightened type 2 or eosinophil-centric responses.
Cellular changes in whole lung during experimental fungal-associated allergic airway inflammation

As \( II1rn^{-/-} \) mice had histological and physiological evidence of peripheral airway and parenchymal lung dysfunction (Figure 3), we examined cellular changes in the whole lung to complement measurements made in the airway (assessed in lung lavage fluid, Figures 4 and 5). For this, we performed flow cytometry on cells collected from enzymatic digests of whole lungs. This analysis demonstrated that eosinophils, but not neutrophils, were significantly decreased in whole lungs from \( II1r1^{-/-} \) mice (Figure 6A), whereas both were decreased when assessed in lung lavage fluid (Figures 4A and 5A). Regarding \( II1rn^{-/-} \) mice, eosinophils were significantly decreased and neutrophils were significantly increased in whole lungs (Figure 6B), which is consistent with the lung lavage fluid data (Figures 4B and 5B). We have previously reported that IL-17A and IL-22 function in an immunopathogenic manner during experimental fungal-associated allergic airway inflammation (11) (16). We recently reported that CD4 T cells as well as the innate lymphocytes \( \gamma \delta \) T cells and invariant Natural Killer T cells (iNKT) cells were cellular sources of IL-22 (and likely IL-17A) in the lung during experimental fungal-associated allergic airway inflammation (10). As IL-17A and IL-22 production by lung cells were different between \( II1r1^{-/-} \) and \( II1rn^{-/-} \) mice (Figure 4), we determined the levels of CD4 T cells, \( \gamma \delta \) T cells and iNKT cells in whole lungs. Results showed that CD4 T cells and iNKT cells were not different between wild-type and \( II1r1^{-/-} \) mice, however there was a significant decrease in \( \gamma \delta \) T cells (Figure 6A). The decrease in \( \gamma \delta \) T cells was associated with lower IL-17A and IL-22 levels in \( II1r1^{-/-} \) mice (Figure 4C). Regarding \( II1rn^{-/-} \) mice, the opposite was true, in that \( \gamma \delta \) T cells were significantly increased but there was no changes in the levels of CD4 T cells and iNKT cells (Figure 6B). The increase in \( \gamma \delta \) T cells was associated with higher IL-17A levels in \( II1rn^{-/-} \) mice (Figure 4D). Representative flow plots are included in Supplemental Figure 2. Thus, analysis of cell populations in
whole lung generally replicate the findings in lung lavage fluid and was associated with aspects of the lung immune response during experimental fungal-associated allergic airway inflammation.

**Type 1 responses are differentially regulated by IL-1R1 and IL-1RA during experimental fungal-associated allergic airway inflammation**

Recent studies have implicated a role for IFN-γ associated type 1 responses in severe asthma pathogenesis and corticosteroid insensitivity (17) (18). Although some data exists for IL-1 receptor mediated regulation of type I IFNs (IFN-α, IFN-β) (19), it is not clear if IL-1α and/or IL-1β regulate type II IFNs (IFN-γ). However, after chronic exposure to *A. fumigatus*, production of IFN-γ by lung digest cells demonstrated a profound requirement for IL-1 receptor signaling (Figure 7A). Similarly, the IFN-γ inducible chemokines CXCL9 and CXCL10 showed significantly impaired induction in *Il1r1/-/-* mice compared to wild-type controls (Figure 7A). IL-1 mediated induction of IFN-γ was tightly controlled, as the absence of IL-1RA resulted in more than a 10-fold increase in IFN-γ production (Figure 7B). Similarly, this lack of IFN-γ regulation resulted in enhanced CXCL9 and CXCL10 production (Figure 7B). Thus, IL-1 receptor signaling drives IFN-γ mediated responses during experimental fungal-associated allergic airway inflammation while IL-1RA functions as a critical regulator of these responses.

**In vivo administration of human IL-1RA improves lung function during experimental fungal-associated allergic airway inflammation**

Human recombinant IL-1RA is used to treat many autoimmune and inflammatory diseases in which IL-1-induced responses are associated with immunopathology and poor outcomes (20) (21). As our results implicate IL-1 receptor signaling as a major immunopathogenic pathway in fungal asthma and describe IL-1RA as an important regulator of this pathway, we assessed the potential impact of human recombinant IL-1RA (Kineret®/Anakinra) on airway hyperresponsiveness. Administration of
Kineret®/Anakinra throughout the development of experimental fungal-associated allergic airway inflammation had a modest effect on central airway resistance at a dose of 10 mg/kg (which is 10-fold less than the recommended dose in humans) (Figure 8A). However, Kineret®/Anakinra at this dose demonstrated more potent efficacy in reducing dynamic lung resistance compared to vehicle-treated mice (Figure 8B). A more profound effect on central airway resistance was observed at a dose of 50 mg/kg (Figure 8C), although this dose did not further improve total lung resistance (Figure 8D). Improved lung function was associated with significantly lower production of the type 1 associated cytokine IFN-γ and associated chemokines CXCL9 and CXCL10 (Figure 8E) and lower production of the type 17 associated cytokine IL-17A, but not IL-22 (Figure 8F) in Kineret®/Anakinra mice. There was no effect of Kineret®/Anakinra treatment on the production of the type 2 cytokines IL-4 and IL-5 (Figure 8G). There was also no difference in Muc5ac and Gob5 mRNA expression between vehicle and Kineret®/Anakinra treated mice (Figure 8H) and no significant differences in any cell population in lung lavage fluid or whole lungs (Supplemental Figures 1 and 2). Thus, augmenting IL-1RA levels during experimental fungal-associated allergic airway inflammation improves the severity of disease concomitant with lowering type 1 and type 17 responses.
Discussion

While defining biomarkers of asthma severity, prognosis and therapeutic responses is a growing area of interest, immunopathogenic pathways contributing to allergic asthma are not well understood. Our previous report on biomarkers in lung lavage fluid identified the IL-1 family members IL-1β and IL-1RA as potential contributors to immunopathogenesis vs. immunoprotection during fungal-associated allergic airway inflammation (10). In the current report, analysis of biomarkers in sputum from fungal (+) asthmatics identified IL-1α and IL-1β as potential immunopathogenic contributors. Previous data from our lab has demonstrated that the level of IL-1α and IL-1β was associated with the severity of experimental fungal-associated allergic airway inflammation (11) (10). Much of what is known regarding the role of IL-1α, IL-1β and IL-1RA in allergic asthma has been derived from studying allergic responses to the model antigen ovalbumin (22) (23) rather than aeroallergens such as fungi or house dust mite. The IL-1 family members IL-1β and IL-18 have been posited as therapeutic targets in asthma (24) (25) as IL-1β may contribute to steroid resistant asthma (26) and the NLRP3 inflammasome may function in the generation of Th2 cells (27). Despite these observations, it is not well understood how signaling induced by IL-1α and IL-1β contributes to human fungal asthma, and other than observational data, this has not been addressed by our previous experimental studies. Likewise, the significance of elevated IL-1RA in fungal (+) asthmatics (10) and how IL-1RA affects responses induced by IL-1α and IL-1β signaling are also not clear. Although IL-1β was elevated in both lung lavage fluid and sputum from fungal (+) asthmatics, IL-1α was elevated in sputum only, whereas IL-1RA was elevated in lung lavage fluid only. While it is perplexing that IL-1α and IL-1RA were not consistently increased in both human specimens (as observed for IL-1β), we cannot exclude the possibility that sputum processing with a reducing agent (dithiothreitol) may affect (positively or negatively) the structural integrity and/or detection of some mediators. Nevertheless, as our analysis demonstrates increased levels in lung samples from fungal-sensitized asthmatics, we hypothesize that a balance between pathways induced
by IL-1α/IL-1β and the regulation of these pathways by IL-1RA determines the severity of fungal asthma.

We (11) (10) and others (28) (29) (30) (31) have employed a live organism, chronic exposure model to identify immunopathogenic processes induced during fungal-associated allergic airway inflammation. This model is relevant, because it replicates the daily exposure humans have to fungi such as Aspergillus fumigatus (32) and mimics persistent local antigenic stimulation in individuals incapable of adequate fungal clearance (individuals with SAFS, allergic bronchopulmonary aspergillosis (ABPA) etc.). Key features include induction of type 1, type 2 and type 17 responses, robust neutrophil and eosinophil recruitment, increased IgE production and airway hyperresponsiveness (AHR). The live organism, chronic fungal exposure model offers advantages over other models that employ adjuvants to skew immune responses (33) or those that employ commercially-available, yet uncharacterized fungal extract preparations, which have recently come under scrutiny due to their significant variability in allergen content (34). Our initial studies demonstrated that signaling through the IL-1 receptor (IL-1R1) had a profound effect on airway and total lung resistance. Better lung function in the absence of the IL-1 receptor was associated with histological evidence of decreased mucus in the airways and fewer inflammatory cells, specifically neutrophils and eosinophils. A surprising finding, however, was negligible impact of IL-1 receptor deficiency on type 2/Th2 responses. Indeed, the inflammasome component NLRP3 has been shown to bind the IL-4 promoter in CD4 T cells (27). Moreover, deficiency in caspase-8, which is required for IL-1β processing, is associated with defective Th2 responses during ovalbumin challenge (23). In contrast, our data demonstrated that although eosinophil numbers were significantly lower in IL-1R1 deficient mice, the absence of IL-1 receptor signaling had no effect on production of the type2/Th2 associated mediators IL-4, IL-33, CCL11, CCL17 and CCL22 and only a minor effect on IL-5. However, these mediators were measured in either lung homogenates or whole lung digest cell culture supernatants, therefore we cannot exclude a role for IL-1 receptor signaling driving type 2/Th2 responses specifically in CD4 T cells or ILC2s (despite each of these being present...
in the lung digest cell culture). In contrast, IL-1 receptor signaling was required for the neutrophilic component of fungal-associated allergic airway inflammation. We have reported that IL-1 signaling is critical for the induction of IL-17A and IL-22 after acute *A. fumigatus* challenge (35) and confirmed here that this pathway is also required for IL-17A and IL-22 induction during fungal-associated allergic airway inflammation. Our previous report has shown that IL-22, and likely IL-17A, function in an immunopathogenic capacity during experimental fungal-associated allergic airway inflammation (11). We have recently reported that IL-22 is primarily produced by γδ T cells, iNKT cells and ILC3s during experimental fungal-associated allergic airway inflammation (10) and we speculate these are lung cell sources of IL-17A as well. Thus, our data implies that IL-1 receptor signaling in these cells is required for their ability to produce IL-22 and likely IL-17A. IL-17A, and IL-22 to a lesser extent, is widely recognized as an inducer of mediators that function in neutrophil activation/expansion or recruitment via the induction of neutrophil survival factors and chemoattractants (36). Although we did not observe defective production of the chemoattractants CCL3 and CCL4, we did find a reduction in G-CSF, implying that IL-1 receptor signaling functions more in neutrophil activation/expansion rather than recruitment. While it would be of interest to interrogate the role of neutrophils in airway hyperreactivity during our live organism fungal asthma model, this is not feasible, as the experimental manipulation of neutrophil levels (e.g. depletion) results in the development of invasive fungal infection (37). Two recent studies demonstrated that expression of IL-1R1 in sputum samples predicted airway neutrophilic inflammation and airway obstruction in neutrophilic severe allergic asthma (38) (39). Moreover, fungal beta-glucan alone or in combination with LPS can drive a mixed Th2/Th17 response that culminates in a neutrophilic, steroid refractory asthma (40). This is supported by our previous work demonstrating that immunopathogenic inflammatory responses (such as IL-1β production and neutrophil recruitment) during fungal asthma is primarily driven by the fungal beta glucan receptor Dectin-1 (11). Collectively, our data leads us to hypothesize that a Dectin-1 → IL-1α/β → IL-1R1 → IL-17A → G-CSF axis supports neutrophilic inflammation during severe fungal-associated allergic airway inflammation.
High serum levels of IL-1RA have been shown to correlate with lower risk of worsening asthma control and attacks in adults (41). In contrast, serum IL-1RA may be elevated during acute asthma exacerbations in a pediatric cohort compared to those with stable asthma (42). Interestingly, this study also documented that IL-1RA decreased in response to corticosteroids. An additional study has shown that patients with neutrophilic asthma have an impairment in the ratio of IL-1RA/IL-1β when compared to eosinophilic asthma (43). Our previous study reported that IL-1RA was increased in human asthmatics sensitized to fungi and was negatively associated with the lung function measurement PC20 (10). Collectively, data in humans have not provided a clear insight into the role of IL-1RA in allergic asthma. In experimental studies, exposing mice deficient in IL-1RA to the ovalbumin asthma model resulted in worse lung function and enhanced type2/Th2 responses (22). In contrast to this observation, we did not observe enhanced type 2/Th2 responses when mice deficient in IL-1RA were subjected to fungal-associated allergic airway inflammation. In fact, despite observing higher IL-33 levels in lung homogenates, IL-4 and IL-5 production by lung digest cells were significantly reduced. Additionally, the major chemoattractant for eosinophils, CCL11, was also significantly reduced and the number of eosinophils trended lower. However, histological assessment demonstrated a lack of mucus production and lower Muc5ac and Gob5 mRNA expression, which was associated with the lower type 2 responses observed. Instead, histology revealed an unrestricted inflammatory process occurring in the absence of IL-1RA. Based on flow cytometry data, this inflammation was neutrophilic in nature. A component of IL-1RA mediated regulation was at the level of IL-17A, which was produced at 4-fold higher levels in IL-1RA deficient mice compared to wild-type controls. This elevation in IL-17A was further associated with heightened induction of G-CSF as well as CCL3 and CCL4. Although data exists that mice deficient in IL-1RA demonstrate baseline increases in inflammatory responses in models of arthritis and psoriasis (44) (45), this has been exclusively observed in IL-1RA deficiency on the Balb/c background and not in those on the C57BL/6 background that we employed here. Collectively, this data leads us to hypothesize that a primary function of IL-1RA is to restrict the magnitude of IL-17A-associated
responses that promote neutrophilic inflammation during fungal-associated allergic airway inflammation.

Growing evidence supports a role for a type1/Th1/IFN-γ signature in severe asthma associated with corticosteroid resistance. Type1/Th1/IFN-γ-associated immune responses are exacerbated in the airways of individuals with severe asthma (17). Modeling this in an experimental severe asthma model (albeit not fungal) shows that mice deficient in IFN-γ fail to exhibit airway hyperresponsiveness (17). More recently, severe asthmatics demonstrate high mRNA levels of the type1/Th1 associated chemokine CXCL10 in bronchoalveolar lavage cells (18). In an experimental asthma model, IFN-γ and CXCL10 were found to be refractory to corticosteroid treatment. Interestingly, high CXCL10 gene expression was also associated with a mast cell signature in both humans and mice (18). Our data provides further insight into this pathway, as IFN-γ and its associated chemokines CXCL9 and CXCL10 were dependent on IL-1 receptor signaling for optimal production during fungal-associated allergic airway inflammation. While IL-1R1 dependency for IL-17A/IL-22 responses was expected, IL-1R1 dependent IFN-γ production was a surprising finding. Although we have reported that iNKT cells produce IL-22 (and likely IL-17A) in an IL-1R1 dependent manner after acute and chronic A. fumigatus exposure (46) (10), iNKT cells have also been reported to be a source of IFN-γ after A. fumigatus exposure (47) (48). The dependency of IFN-γ production on IL-1R1 is not widely recognized, with only a few reports showing IL-1R1 dependent IFN-γ production by CD8 T cells during contact hypersensitivity (49) and cardiac transplantation (50). In contrast, IL-1RA functioned as a potent regulator of IFN-γ production during fungal-associated allergic airway inflammation. Moreover, IL-1RA mediated regulation of IFN-γ has been shown in models of Toxoplasma gondii-mediated arthritis (51) and experimental autoimmune encephalomyelitis (52), IL-1RA is required for the induction of IFN-γ during cutaneous Leishmania major infection (53). Other studies have shown that treatment of humans PBMCs with IL-1RA had no effect on Staphylococcus epidermidis-induced IFN-γ production (54).
Overall, these observations suggest that the interaction/relationship between IL-1RA and IFN-γ is diverse based on the model system employed.

The final question we asked was whether the IL-1/IL-1RA axis was a viable therapeutic target in fungal-associated allergic airway inflammation. Human recombinant IL-1RA, Kineret®/Anakinra, is FDA-approved for the treatment of rheumatoid arthritis and neonatal onset multisystem inflammatory disease (NOMID) (a subtype of cryopyrin-associated periodic syndromes, CAPS). We show that treating mice with Kineret®/Anakinra over the development of fungal-associated allergic airway inflammation was effective at improving lung function. Kineret®/Anakinra lowered both AHR (Newtonian resistance), which is the resistance of the central or conducting airways, and total lung resistance, which is the level of constriction in the lungs. A previous study has demonstrated that Kineret®/Anakinra administered during invasive aspergillosis resulted in reduced IL-17A production and increased IFN-γ production in p47phox−/− mice (i.e chronic granulomatous disease mice), while treatment of wild-type mice resulted in the opposite effect, decreased IFN-γ and no effect of IL-17A (55). A second study has demonstrated that treating mice deficient in CFTR with Kineret®/Anakinra lowered A. fumigatus lung burden. However, the only immunologic mechanism proposed in this study was lower IL-1β production (56). Finally, a study using PBMCs from patients with allergic bronchopulmonary aspergillosis stimulated with heat-killed A. fumigatus and cultured with Etanercept®, Adalimumab® or Kineret®/Anakinra demonstrated lower IL-5 and IL-13 production, although other mediators such as IL-17A and IFN-γ were not investigated (57). Our study is the first to document efficacy of blocking IL-1R1 signaling in vivo during experimental aeroallergen-associated allergic airway inflammation and show that this was effective at lowering both type 1 (IFN-γ) and type 17 responses (IL-17A), but had no effect on modulating type 2 responses or inflammatory cell recruitment.

In summary, we have identified IL-1 family members as putative biomarkers of disease severity in human asthmatics that were sensitized the fungi. Interrogating the role of IL-1 receptor signaling in experimental fungal-associated allergic airway inflammation revealed this pathway to drive the
induction of type 1 and type 17 responses that augmented neutrophilic responses leading to worse lung function. In turn, IL-1RA was required to regulate the magnitude of type 1 and type 17 responses, neutrophilic inflammation and lung function during experimental fungal-associated allergic airway inflammation (Figure 9). IL-1RA itself also demonstrated an ability to serve as a viable target for treating fungal-associated allergic airway inflammation. Surprisingly, IL-1R1/IL-1RA had varied or little impact on type 2 responses. Collectively, our results provide insight into the role IL-1 family members play in immunopathogenesis during severe allergic asthma.
Materials and Methods

Subjects, sputum induction and processing and Luminex® analysis

Patients with mild to severe asthma were comprehensively characterized according to the NHLBI Severe Asthma Research Program (SARP) phenotype protocol at Wake Forest School of Medicine as previously described (58) and a description of this cohort was recently reported (59). The sputum induction method was adopted from the NHLBI Asthma Clinical Research Network and used in SARP (60). BALF and sputum samples were derived from both the SARP 1 and 2 cohorts. The number of subjects that had both BALF and sputum collected in the current cohort was N = 21 (18% of the subjects in Table 1). Per SARP protocol, BALF and sputum were not collected within a week of each other. Sputum was processed immediately after collection and cell cytospins were stained for differential counts of at least 500 nonsquamous cells/subject slide. Total white blood cell count in sputum was determined via trypan blue staining and enumeration using a hemacytometer. Cell-free supernatants were aliquoted and stored at -80° before use in Luminex® analyses (see below). Biospecimens were randomly selected without a priori selection based on asthma severity, rather based on whether they were skin test positive or 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 (catalog numbers HCYTOMAG-60K-PX41, HCYP2MAG-62K-PX23, HCYP3MAG-63K and HCY4MG-64K-PX21 respectively, MilliporeSigma, Burlington, MA). Standards for determination of linear curve plus two control samples representing high and low levels of each cytokine/chemokine were included in each assay.
Mice

WT C57BL/6, Il1r1-/- (IL-1R1) and Il1rn-/- (IL-1RA) male mice, 6 to 8 weeks of age, were obtained from The Jackson Laboratory (Bangor, ME). All animals were housed in a specific pathogen-free, Association for Assessment and Accreditation of Laboratory Animal Care-certified facility and handled according to National Institutes of Health Public Health Service Office of Laboratory Animal Welfare Guide for Care and Use of Laboratory Animals: Eighth Edition after review by the UAB and Tulane Institutional Animal Care and Use Committees.

A. fumigatus preparation and fungal-associated allergic airway inflammation model

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 repeated A. fumigatus exposure model was employed as previously described (11). 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 (i.t.). After resting for 7 days, mice were challenged i.t. 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 i.t. with 1 × 10^6 live A. fumigatus conidia in 50 μl 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 sacrificed for analysis via ketamine/xylazine overdose and aortic exanguination.

Whole lung cytokine and chemokine analysis, lung cell isolation and culture

Following sacrifice, the right lung was homogenized in PBS supplemented with Complete Mini protease inhibitor tablets (Roche Diagnostics, Risch-Rotkreuz, Switzerland), clarified by centrifugation,
and stored at −80°C. From lung homogenate supernatants, IL-33, CCL17, and CCL22 levels were quantified by ELISA (R&D Systems, Minneapolis, MN). For lung cell isolation, the lungs were collected and minced in IMDM media (MilliporeSigma, Burlington, MA) supplemented with 1X penicillin-streptomycin-glutamine (Mediatech, Herndon, VA), 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA), and 0.4 mg/ml polymyxin B (Thermo-Fisher Scientific, Waltham, MA), followed by incubation for 60 min with tissue culture-grade type IV collagenase (1 mg/ml; MilliporeSigma Burlington, MA) in a 37°C orbital shaker at 100 rpm. The cell suspension was filtered through sterile 70-μm and 40-μm nylon filters and RBCs lysed with ACK buffer (Lonza, Walkersville, MD) to create single-cell preparations. One million cells in a volume of 200 μl were cultured overnight with one million *A. fumigatus* conidia (1:1) followed by collection and clarification of supernatants. Supernatants from lung digest cells were analyzed for protein levels of 32 cytokines and chemokines using the Luminex®-based Milliplex® multiplex suspension cytokine array (MilliporeSigma, Burlington, MA), according to the manufacturer’s instructions. The data were analyzed using Bio-Plex Manager software (Bio-Rad, Hercules, CA). Lung digest cell supernatants were also used to quantify levels of IL-22 by ELISA (R&D Systems, Minneapolis, MN).

**Lung cell flow cytometry**

Lung cells were isolated via bronchoalveolar lavage or by enzymatic digestion of whole lungs as previously described (11) (16). Cells were washed and Fc receptors were blocked with Mouse BD Fc Block (BD Biosciences, San Diego, CA) 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+ (BioLegend, San Diego, CA; Cat. #103115, clone 30-F11) CD11b+ (BioLegend Cat. #101237, clone M1/70) Siglec F+ (BioLegend Cat. #155507, clone S17007L) Ly-6G+, neutrophils as CD45+ CD11b+ Ly6G+ (BioLegend Cat. #127621, clone 1A8) Siglec F+, CD4 T cells as CD45+ CD4+
(BioLegend Cat. #100421, clone GK1.5) CD3+ (BioLegend Cat. #100216, clone 17A2), γδ T cells as CD45+ γδ TCR+ (BioLegend Cat. #118117, clone GL3) CD3+ and iNKT cells as TCRβ+ (BioLegend Cat. #109239, clone H57-597) PBS-57 CD1d tetramer+ (NIH Tetramer Core, Emory University). Samples were acquired using a four-laser, 20-parameter analytic BD LSRFortessa, and data were analyzed using FlowJo software (Tree Star, Ashland, OR). Unstained lung leukocytes served as a control for background fluorescence and gating as well as single color controls. Appropriately stained UltraComp eBeads (ThermoFisher Scientific, Waltham, MA) served as single color controls.

**Pulmonary function assessment**

Individual anesthetized *A. fumigatus*-exposed mice were intubated and each animal attached to a computer-controlled volume ventilator (flexiVent; SCIREQ Montreal, PQ, Canada). 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 (R) and compliance (C). The low-frequency/broadband forced oscillation technique was employed to measure Newtonian resistance (Rn; also known as airway hyperreactivity), tissue damping (G) and tissue elastance (H). All measurements were collected at baseline and after a linear dose response with methacholine challenge (10–40 mg/ml), as previously described (10) (16). Lung function was also assessed in naïve WT and mutant mice, which confirmed no baseline anomalies and no differences between groups as we have previously reported (11).

**In vivo treatment with Kineret®/Anakinra**

In specific experiments, WT BL/6 mice were treated with Kineret®/Anakinra (SOBI, Stockholm, Sweden), a recombinant, non-glycosylated form of human IL-1RA. Specifically, mice were sensitized and challenged with *A. fumigatus* as described above, and intraperitoneally injected with anakinra (10
mg/kg or 50 mg/kg in 200 μL 0.9% NaCl; R&D Systems) or vehicle daily on days 0-16. 24 h after final injection and *A. fumigatus* challenge, mice underwent pulmonary function assessment or cytokine/chemokine analysis as described above.

**Statistics**

Data were analyzed using GraphPad Prism® Version 7.0 statistical software (GraphPad Software, San Diego, CA). Comparisons between groups when data were normally distributed were made with the two-tailed unpaired Student’s t test or 2-way-ANOVA. Significance was accepted at a value of p < 0.05.

**Study Approval**

Human subjects were enrolled with informed consent under approved Wake Forest School of Medicine IRB #BG01-425 (Wake Forest University Institutional Review Board, Winston-Salem, NC). Human samples were analyzed under UAB IRB #X130827009 (UAB Institutional Review Board for Human Use, Birmingham, AL). All animal research was conducted under approved UAB IACUC protocols #10199 and #20236 (UAB Institutional Animal Care and Use Committee, Birmingham, AL) and approved Tulane IACUC protocols #217 and #412 (Tulane Institutional Animal Care and Use Committee, New Orleans, LA).
Author contributions

MSG and CS designed the research. MSG, KMR, JMG, JPB and MJ performed the research. ZY and SM performed the lung function analysis. MSG and CS analyzed the data. ATH and DAM conducted all human subjects recruitment and clinical analysis. MSG and CS wrote the manuscript. CS supervised all aspects of the project. All authors agreed to all of the content of the submitted manuscript.
Acknowledgements

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Figure 1. Human asthmatics sensitized to fungi have elevated IL-1α and IL-1β levels in bronchoalveolar lavage fluid and sputum. Bronchoalveolar lavage fluid (BALF) or sputum was collected from subjects with atopic asthma who were sensitized (BALF, n = 28-29; sputum, n = 32-33) or were not sensitized (BALF, n = 29; sputum, n = 61-62) to fungi. IL-1β and IL-1α were quantified in clarified BALF (A, B) and sputum (C, D) supernatants by MilliPlex. *Note: IL-1β data in BALF was previously reported in Table format in (10). Data were normalized to the total protein content (BALF) or total WBC (sputum) of each sample and expressed as mean pg/mg protein or mean pg/total WBC (each symbol represents a single subject). P values assessed using a two-tailed Student's t test.
Figure 2. Signaling through the IL-1 receptor worsens lung function during experimental fungal-associated allergic airway inflammation. C57BL/6 (WT) and IL-1 receptor deficient (Il1r1−/−) mice were chronically exposed to *A. fumigatus* as described in the Methods. Twenty-four hours after the last organism challenge, (A) airway (Newtonian) resistance and (B) total lung resistance was analyzed via mechanical ventilation using the flexiVent pulmonary function system. The Figures illustrate cumulative data from three independent studies (n = 4-5 mice per group per study). Data expressed as mean ± SEM. *** represents a p value of < 0.001 (2-way ANOVA). Representative (C) Hematoxylin and Eosin (H&E; top images) and Periodic acid-Schiff (PAS; bottom images)-stained lung sections from WT (left images) and Il1r1−/− (right images) mice. Original magnification 20x. Bar = 100 μm. (D) Twenty-four hours after the last challenge, the left lungs were collected and Muc5ac and Gob5 gene expression was quantified by real-time PCR and normalized to HPRT. Data expressed as $2^{-\Delta\Delta Ct}$. The Figure illustrates cumulative data from two independent studies (n = 4 mice per group per study). Quantitative data is represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, line representing the median, whiskers ranging from minimum to maximum values and “+” indicating the mean.
Figure 3. IL-1 receptor antagonist regulates the severity of experimental fungal-associated allergic airway inflammation. C57BL/6 wild-type (WT) and IL-1 receptor antagonist deficient (Il1rn−/−) mice were chronically exposed to A. fumigatus as described in the Methods. Twenty-four hours after the last organism challenge, (A) airway (Newtonian) resistance and (B) total lung resistance was analyzed via mechanical ventilation using the flexiVent pulmonary function system. The Figures illustrate cumulative data from two independent studies (n = 4-5 mice per group per study). Data are expressed as mean ± SEM. *** represents a p value of < 0.001 (2-way ANOVA). Representative (C) Hematoxylin and Eosin (H&E; top images) and Periodic acid-Schiff (PAS; bottom images)-stained lung sections from WT (left images) and Il1rn−/− (right images) mice. Original magnification 20x. Bar = 100 μm. (D) Compliance, (E) tissue damping and (F) tissue elastance was analyzed via mechanical ventilation using the flexiVent pulmonary function system. The Figures illustrate cumulative data from two independent studies (n = 4-5 mice per group per study). Data expressed as mean ± SEM. *** represents a p value of < 0.001 (2-way analysis of variance). (G) Twenty-four hours after the last challenge, the left lungs were collected and Muc5ac and Gob5 gene expression was quantified by real-time PCR and normalized to HPRT. Data are expressed as 2−ΔΔCt. The Figure illustrates cumulative data from two independent studies (n = 4 mice per group per study). Quantitative data is represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, line representing the median, whiskers ranging from minimum to maximum values and “+” indicating the mean. For (H), * and *** represent P values of < 0.05 and < 0.001, respectively (two-tailed Student’s t test).
A. Neutrophils and their associated inflammatory mediators are differentially regulated by IL-1R1 and IL-1RA during experimental fungal-associated allergic airway inflammation. C57BL/6 wild-type (WT), IL-1 receptor deficient (Il1r1−/−) and IL-1 receptor antagonist deficient (Il1rn−/−) mice were chronically exposed to A. fumigatus as described in the Methods. (A, B) Twenty four hours after last challenge, lung cells from (A) WT and Il1r1−/− mice and (B) WT and Il1rn−/− mice were isolated by bronchoalveolar lavage, enumerated, Fc-blocked, stained with a live/dead staining kit and stained for neutrophils (CD45+, CD11b+, Ly-6G+, Siglec F−). The Figures illustrate cumulative data from two to three independent studies (n = 4-5 mice per group per study). (C, D) 24 hours after last challenge, right lungs were collected, enzymatically digested and unfractionated lung cells co-cultured with A. fumigatus conidia for 24 h at a cell to organism ratio of 1:1. IL-17A and IL-22 levels in lung digest cell culture supernatants from (C) WT and Il1r1−/− mice and (D) WT and Il1rn−/− mice were quantified by MilliPlex or ELISA. The Figures illustrate cumulative data from two independent studies (n = 3 mice per group per study). (E, F) Samples obtained as described for C and D. Granulocyte-colony stimulating factor (G-CSF), CCL3 and CCL4 levels in lung digest cell culture supernatants from (E) WT and Il1r1−/− mice and (F) WT and Il1rn−/− mice were quantified by MilliPlex. The Figures illustrate cumulative data from three independent studies (n = 3-5 mice per group per study). For all graphs: quantitative data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, line representing the median, whiskers ranging from minimum to maximum values and “+” indicating the mean. *, ** and *** represent P values of < 0.05, < 0.01 and < 0.001, respectively (two-tailed Student’s t test).
The absence of IL-1R1 and IL-1RA results in varied type 2 responses during experimental fungal-associated allergic airway inflammation. C57BL/6 wild-type (WT), IL-1 receptor deficient (Il1r1−/−) and IL-1 receptor antagonist deficient (Il1rn−/−) mice were chronically exposed to A. fumigatus as in the Methods. (A, B) Twenty four hours after last challenge, lung cells from (A) WT and Il1r1−/− mice and (B) WT and Il1rn−/− mice were isolated by bronchoalveolar lavage, enumerated, Fc-blocked, stained with a live/dead staining kit and stained for eosinophils (CD45+, CD11b+, Siglec F+, Ly-6G−). The Figures illustrate cumulative data from two to four independent studies (n = 4–5 mice per group per study). (C, D, E, F) 24 h after last challenge, right lungs were collected, enzymatically digested and unfractionated lung cells co-cultured with A. fumigatus conidia for 24 h at a cell to organism ratio of 1:1. IL-4, IL-5 and CCL11 levels in lung digest cell culture supernatants from WT and Il1r1−/− mice (C, E) and WT and Il1rn−/− mice (D, F) were quantified by MilliPlex. The Figures illustrate cumulative data from three to four independent studies (n = 3–5 mice per group per study). (G, H, I, J) Twenty four hours after last challenge, left lungs were collected, homogenized and CCL17, CCL22 and IL-33 levels were quantified by ELISA in clarified lung homogenates from WT and Il1r1−/− mice (G, I) and WT and Il1rn−/− mice (H, J). The Figures illustrate cumulative data from two to three independent studies (n = 3–5 mice per group per study). For all graphs: quantitative data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, line representing the median, whiskers ranging from minimum to maximum values and “+” indicating the mean. *, ** and *** represent p values of < 0.05, < 0.01 and < 0.001, respectively (two-tailed Student's t test).
Figure 6. Cellular changes in whole lung during experimental fungal-associated allergic airway inflammation. C57BL/6 wild-type (WT), IL-1 receptor deficient (Il1r1−/−) and IL-1 receptor antagonist deficient (Il1rn−/−) mice were chronically exposed to A. fumigatus as in the Methods. Twenty-four h after last challenge, right lungs were collected, enzymatically digested, Fc-blocked, stained with a live/dead staining kit and stained for eosinophils (CD45+, CD11b+, Siglec F+, Ly-6G+), neutrophils (CD45+, CD11b+, Ly-6G+), CD4 T cells (CD45+, CD4+, CD3+), γδ T cells (CD45+, γδ TCR+, CD3+) and iNKT cells (TCRβ+, PBS-57 CD1d tetramer+) in (A) WT and Il1r1−/− mice and (B) WT and Il1rn−/− mice. The Figures illustrate cumulative data from two independent studies (n = 3-4 mice per group per study). For all graphs: quantitative data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, line representing the median, whiskers ranging from minimum to maximum values and “+” indicating the mean. *, ** and *** represent p values of < 0.05, < 0.01 and < 0.001, respectively (two-tailed Student’s t test).
Figure 7. Type 1 responses are differentially regulated by IL-1R1 and IL-1RA during experimental fungal-associated allergic airway inflammation. C57BL/6 wild-type (WT), IL-1 receptor deficient (Il1r1-/-) and IL-1 receptor antagonist deficient (Il1rn-/-) mice were chronically exposed to A. fumigatus as in the Methods. (A, B) Twenty four hours after last challenge, right lungs were collected, enzymatically digested and unfractionated lung cells co-cultured with A. fumigatus conidia for 24 h at a cell to organism ratio of 1:1. IFN-γ, CXCL9 and CXCL10 levels in lung digest cell culture supernatants from (A) WT and Il1r1-/- mice and (B) WT and Il1rn-/- mice were quantified by MilliPlex. The Figures illustrate cumulative data from three independent studies (3-5 mice per group per time point). For all graphs: quantitative data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, line representing the median, whiskers ranging from minimum to maximum values and “+” indicating the mean. *, ** and *** represent p values of < 0.05, < 0.01 and < 0.001, respectively (two-tailed Student's t test).
**Figure 8. In vivo administration of human IL-1RA improves lung function during experimental fungal-associated allergic airway inflammation.** C57BL/6 wild-type (WT) mice were chronically exposed to *A. fumigatus* as described in the Methods and treated daily from day 0 to day 16 with 10 mg/kg or 50 mg/kg human recombinant IL-1RA (Kinere®/Anakinra) or vehicle i.p. Twenty-four hours after the last organism challenge, (A, C) Airway (Newtonian) resistance and (B, D) total lung resistance was analyzed via mechanical ventilation using the flexiVent pulmonary function system. The Figures illustrate cumulative data from two independent studies (n = 4-5 mice per group per study). Data expressed as mean ± SEM. * and *** represent P values of < 0.05 and < 0.001, respectively (2-way analysis of variance). (E, F, G) 24 h after the last organism challenge, the right lungs were collected, enzymatically digested and unfractionated lung cells cultured for 24 h in the presence of *A. fumigatus* conidia at a cell to organism ratio of 1:1. (C) IFN-γ, CXCL9 and CXCL10 levels, (D) IL-17A and IL-22 levels and (E) IL-4 and IL-5 levels in lung digest cell culture supernatants from vehicle treated vs. Kinere®/Anakinra were quantified by MilliPlex or ELISA. The Figures illustrate cumulative data from two independent studies (n = 4-5 mice per group per study). Data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, line representing the median, whiskers ranging from minimum to maximum values and “+” indicating the mean. *, ** and *** represent p values of < 0.05, < 0.01 and < 0.001, respectively (two-tailed Student’s t test). (H) Twenty-four hours after the last challenge, the left lungs were collected and *Muc5ac* and *Gob5* gene expression was quantified by real-time PCR and normalized to HPRT. Data expressed as $2^{-\Delta\Delta Ct}$. The Figure illustrates cumulative data from two independent studies (n = 4 mice per group per study). Data are represented as a box-and-whisker plot, with bounds from 25th to 75th percentile, line representing the median, whiskers ranging from minimum to maximum values and “+” indicating the mean. *, ** and *** represent p values of < 0.05, < 0.01 and < 0.001, respectively (two-tailed Student’s t test).
Figure 9. Impact of IL-1R1 signaling during experimental fungal-associated allergic airway inflammation. Chronic exposure to live airborne *A. fumigatus* conidia induces IL-1α and IL-1β production and subsequent signaling via IL-1R1. IL-1R1 signaling induces the production of type 1 and type 17 responses which augments neutrophil recruitment to the lung. Enhanced neutrophil recruitment negatively impacts both central and peripheral airway function. The IL-1R1 antagonist IL-1RA is essential for regulating the magnitude of IL-1R1 signaling and augmenting IL-1RA levels result in lower type 1 and type 17 responses and improved lung function.
Table I. Demographics and clinical characteristics of fungal (-) and fungal (+) asthmatics.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fungal-positive (n = 51)</th>
<th>Fungal negative (n = 77)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>33 (17-29)</td>
<td>39 (11-32)</td>
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<tr>
<td>Asthma duration</td>
<td>21 (17-29)</td>
<td>22 (11-32)</td>
<td>0.511</td>
</tr>
<tr>
<td>BMI</td>
<td>29.7 (25-37)</td>
<td>30 (25-35)</td>
<td>0.992</td>
</tr>
<tr>
<td>Age onset</td>
<td>5 (2-16)</td>
<td>14 (5-24)</td>
<td>&lt;0.001</td>
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<tr>
<td>Basal preBD FEV1%pred</td>
<td>75.8 (17.1)</td>
<td>76.4 (18.1)</td>
<td>0.859</td>
</tr>
<tr>
<td>Basal preBD FVC%pred</td>
<td>91.2 (15.5)</td>
<td>87 (15.6)</td>
<td>0.165</td>
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<tr>
<td>FEV1/FVC</td>
<td>0.716 (0.62-0.77)</td>
<td>0.751 (0.66-0.80)</td>
<td>0.078</td>
</tr>
<tr>
<td>Max FEV1%pred</td>
<td>88.9 (17.3)</td>
<td>89.2 (15.8)</td>
<td>0.928</td>
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<tr>
<td>Max FVC%pred</td>
<td>100 (14.4)</td>
<td>96 (15.1)</td>
<td>0.124</td>
</tr>
<tr>
<td>Max Reversal</td>
<td>14.1 (9.2)</td>
<td>11.1 (7.2-19.4)</td>
<td>0.131</td>
</tr>
<tr>
<td>PC20</td>
<td>1 (0.24-1.85)</td>
<td>1 (0.28-4.8)</td>
<td>0.342</td>
</tr>
<tr>
<td>Total IgE</td>
<td>252 (134-584)</td>
<td>121 (44-273)</td>
<td>&lt;0.001</td>
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<tr>
<td>Pos skin prick tests (#)</td>
<td>8 (5-9)</td>
<td>3 (2-6.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FeNO</td>
<td>26.7 (17-60)</td>
<td>27.3 (18-43)</td>
<td>0.761</td>
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<tr>
<td>Blood eos (#)</td>
<td>300 (200-400)</td>
<td>200 (100-300)</td>
<td>0.005</td>
</tr>
<tr>
<td>Blood eos (%)</td>
<td>5 (3-7)</td>
<td>3 (1.5-4.5)</td>
<td>&lt;0.001</td>
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<tr>
<td>ER visits for breathing (ever)</td>
<td>72.50%</td>
<td>67.50%</td>
<td>0.684</td>
</tr>
<tr>
<td>ER visits for breathing (last 12 mos)</td>
<td>25.50%</td>
<td>18.40%</td>
<td>0.548</td>
</tr>
<tr>
<td>Intubation for breathing</td>
<td>23.90%</td>
<td>9.40%</td>
<td>0.07</td>
</tr>
<tr>
<td>Hospitalization for breathing</td>
<td>42%</td>
<td>32.50%</td>
<td>0.367</td>
</tr>
<tr>
<td>Inhaled corticosteroid</td>
<td>72.50%</td>
<td>53.20%</td>
<td>0.045</td>
</tr>
<tr>
<td>Long-acting beta-agonist</td>
<td>60.90%</td>
<td>45.30%</td>
<td>0.156</td>
</tr>
</tbody>
</table>