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IL-9 Governs Allergen-induced Mast Cell Numbers in the Lung and Chronic Remodeling of the Airways

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Abstract

Rationale—IL-9 is a pleiotropic cytokine that has multiple effects on structural as well as numerous hematopoietic cells, which are central to the pathogenesis of asthma.

Objectives—The contribution of IL-9 to asthma pathogenesis has thus far been unclear, due to conflicting reports in the literature. These earlier studies focused on the role of IL-9 in acute inflammatory models; here we have investigated the effects of IL-9 blockade during chronic allergic inflammation.

Methods—Mice were exposed to either prolonged ovalbumin or house dust mite allergen challenge to induce chronic inflammation and airway remodeling.

Measurements and Main Results—We found that IL-9 governs allergen-induced mast cell (MC) numbers in the lung and has pronounced effects on chronic allergic inflammation. Anti-IL-9

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antibody-treated mice were protected from airway remodeling with a concomitant reduction in mature MC numbers and activation, in addition to decreased expression of the profibrotic mediators transforming growth factor- β 1, vascular endothelial growth factor, and fibroblast growth factor-2 in the lung. Airway remodeling was associated with impaired lung function in the peripheral airways and this was reversed by IL-9 neutralization. In human asthmatic lung tissue, we identified MCs as the main IL-9 receptor expressing population and found them to be sources of vascular endothelial growth factor and fibroblast growth factor-2.

Conclusions—Our data suggest an important role for an IL-9-MC axis in the pathology associated with chronic asthma and demonstrate that an impact on this axis could lead to a reduction in chronic inflammation and improved lung function in patients with asthma.

Keywords

IL-9; mast cells; asthma; airway remodeling; AHR

Asthma is a chronic inflammatory disease of the airways, characterized by airway hyperresponsiveness (AHR) and eosinophilic inflammation and is associated with structural changes termed “airway remodeling” (1). These changes include deposition of extracellular matrix (ECM) proteins, such as fibronectin, collagen, and tenascin; mucus hypersecretion; and proliferation of airway smooth muscle (ASM) cells (1). Airway remodeling is also associated with up-regulation of profibrotic growth factors, in particular transforming growth factor (TGF) β , which has been implicated as a key player in this process (2). Currently, the most effective treatment for asthma is corticosteroids; however, these have only a partial effect on airway remodeling.

Th2 cells are believed to play a central role in the pathogenesis of bronchial asthma by producing a number of key cytokines, including IL-4, IL-5, and IL-13, which, in preclinical animal models, have been shown to contribute to many of the pathophysiological features of the disease (reviewed in Reference 1). IL-9, long described as a Th2-derived cytokine, has also been identified from mouse models as an important mediator of allergic inflammation, and evidence from both human and murine studies suggests IL-9 is associated with susceptibility to develop AHR (3, 4). Overexpression of IL-9 in mice elicits a striking phenotype in the lung, including eosinophilic and lymphocyte inflammation, increased mucus production, AHR, and subepithelial collagen deposition, suggesting that IL-9 may play a role in chronic allergic inflammation (5, 6). However, conflicting data have been published regarding the efficacy of IL-9 blockade in mouse models of asthma. Two independent studies using neutralizing IL-9 antibodies have shown some reduction in inflammation and AHR (7, 8), whereas a study using IL-9-deficient mice described a redundant role for this cytokine in a similar model of asthma (9). Importantly, all of these previous studies were limited in that they examined the effects of IL-9 blockade in acute inflammatory models.

Among the multiple roles for this cytokine, IL-9 has been shown to be a potent mast cell (MC) growth and differentiation factor *in vitro* and in mouse cell lines; IL-9 promotes expression of MC proteases, up-regulates the high-affinity IgE receptor (Fc ϵ R1 α), and induces IL-6 production (10-12). Similarly *in vivo*, IL-9 appears to modulate MC numbers in mouse lung (13, 14). There is compelling evidence that MCs contribute to the acute symptoms of asthma, including bronchoconstriction, mucus secretion, and mucosal edema; however, more recently MCs have been implicated in chronic inflammation and shown to promote angiogenesis and tissue remodeling in a mouse model of asthma (15).

In this study, we present data that point to a central role for IL-9 in regulating resident MC numbers in the lung. Blockade of this pathway during prolonged allergen challenge

significantly attenuates MC numbers in the lung, prevents airway remodeling, and is associated with decreased expression of the growth factors vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2, and TGF β . Furthermore, we report that inhibition of airway remodeling is associated with improved lung function. In human asthmatic lung tissue, we demonstrate that MCs express the IL-9 receptor (IL-9R) and colocalize with these profibrogenic mediators. Taken together, our results identify a unique role for IL-9 in the regulation of MC numbers *in vivo* and airway remodeling, and suggest an important link between IL-9, MCs, and fibrosis of the airways. Some of the results of these studies have been previously reported in the form of an abstract (16, 17).

METHODS

Mice and Antibodies

Female BALB/c mice (6–8 wk) were purchased from Harlan (Indianapolis, IN) and housed at the MedImmune animal facility. To block IL-9, we used a specific mouse monoclonal antibody against mouse IL-9 (MM9C1), which has previously been shown to neutralize IL-9 activity *in vivo* (18, 19).

Induction of Airway Inflammation

Ovalbumin acute model—Airway inflammation was induced in BALB/c mice as previously described (20, 21). Further detail is provided in the online supplement. Anti-IL-9 (MM9C1) or isotype control IgG antibodies were administered 30 minutes (100 μ g/mouse intraperitoneally) before each ovalbumin (OVA) challenge. A schematic of this protocol is shown in Figure E1A in the online supplement.

OVA chronic model—Chronic inflammation and airway remodeling was induced as previously described (22). Additional details are available in the online supplement. Anti-IL-9 (MM9C1) or control isotype IgG antibodies were administered 30 minutes (250 μ g/mouse, intraperitoneally) twice during the first week (Days 19 and 23) and once weekly thereafter. A schematic of this protocol is shown in Figure E1B.

House dust mite chronic model—Chronic airway inflammation and airway remodeling were induced by intranasal administration of house dust mite (HDM) extract (Greer, Lenoir, NC) for 5 weeks as previously described (23). Additional detail is available in the online supplement. Anti-IL-9 or control isotype IgG antibodies were administered (100 μ g/mouse, intraperitoneally) once weekly throughout the HDM challenge. A schematic of this protocol is shown in Figure E1C.

Determination of Cell Numbers in Bronchoalveolar Lavage

Airways were washed with Hanks' balanced salt solution (Sigma, St. Louis, MO) containing 10 mM ethylenediaminetetraacetic acid and *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid, bronchoalveolar lavage (BAL) fluid was centrifuged, and the cells were counted using a Coulter Z1 particle counter (Beckman Coulter, Fullerton, CA). Differential cell counts (of at least 500 cells per slide) were performed on cytospin preparations stained with Diff-Quik (Fisher Scientific, Pittsburgh, PA).

Assessment of AHR

AHR was determined in response to increasing doses of aerosolized methacholine (Sigma) using a modified version of previously described methods, and the flexiVent lung function system (SCIREQ Inc., Montreal, PQ, Canada) (24). Further detail is provided in the online supplement.

mRNA Expression

mRNA was purified with an RNeasy Plus mini kit (Qiagen, Valencia, CA) and cDNA was synthesized using Sprint Power Script Double Preprimed 96 kit (Clontech, Mountain View, CA). Gene expression was measured by TaqMan real-time polymerase chain reaction (Applied Biosystems, Carlsbad, CA) following the manufacturer's protocols. The probe sets were obtained from Applied Biosystems as TaqMan Gene Expression Assays. TaqMan reactions contained either the reference gene GAPDH or the genes of interest, Muc5ac or Gob-5.

Quantification of Cytokines, Growth Factors, and Mouse MC Protease 1

Lung was homogenized in Hanks' balanced salt solution containing protease inhibitor tablets (Roche, Basel, Switzerland). Protein levels of IL-4, IL-13, VEGF, and FGF-2 (R&D Systems, Minneapolis, MN) were measured in homogenate supernatant, and mMCP-1 (Moredun, Penicuik, Scotland) was measured in serum according to the manufacturer's protocol. All samples were assayed in triplicate.

Secreted Alkaline Phosphatase Assay for TGF β

TGF β activity was measured in BAL supernatant as previously described (25). Further details are given in the online supplement.

Histology

Lung inflammation (hematoxylin and eosin-stained sections), goblet cell numbers (periodic acid Schiff-stained sections), and total and proliferating airway smooth muscle cells (proliferating cell nuclear antigen-stained sections) were assessed as previously described (26). Peribronchial collagen deposition was determined in Sirius red-stained lung sections (further details are provided in the online supplement).

Quantification of airway MCs—MCs were counted on toluidine blue-stained tracheal sections, scanned with a Scanscope XT Digital Slide Scanner (Aperio, Vista, CA), and normalized per unit area (μm^2) of tissue examined.

Total Lung Collagen Analysis

Collagen content was measured in lung tissue homogenate supernatant by a biochemical assay according to the manufacturer's instructions (Sircol Assay; Biocolor, Westbury, NY).

Collection of Lung Biopsies from Patients with Asthma

Patients with mild asthma and definite bronchial hyperresponsiveness were included in the study. Healthy volunteers were used as control subjects. Further details of biopsies and patient information are provided in the online supplement. All human work was approved by the Swedish Research Ethical Committee.

Immunohistochemistry of Human Biopsies

Paraffin sections were blocked with 5% dry milk mixed with 20% normal horse/goat/donkey serum (Vector, Burlingame, CA), followed by an avidin/biotin blocking kit (Vector). Incubation was performed with the following primary antibodies: anti-IL-9R (BioLegend, San Diego, CA), anti-IL-9 (BioLegend), anti-VEGF (Santa Cruz, Santa Cruz, CA), anti-FGF-2 (Santa Cruz).

After the primary incubation, sections were rinsed and stained with a biotinylated secondary antibody, followed by Alexa Fluor 555-conjugated streptavidin (Invitrogen, Carlsbad, CA).

To double stain the sections for MCs, slides were incubated with mouse anti-MC tryptase, (Millipore, Billerica, MA) directly labeled with Alexa Fluor 488 using the Zenon Mouse IgG labeling kit (Invitrogen). All sections were mounted in Vectashield mounting medium (Vector). Staining specificity was indicated by lack of staining when replacing the primary antibodies with irrelevant control rabbit antisera or isotype-specific mouse control antibodies. Using an immunofluorescence microscope, the total numbers of tryptase⁺ MCs in each biopsy were quantified manually on blinded sections. The MC numbers were then related to the total tissue area through computerized image analysis (Image-Pro Plus; MediaCybernetics Inc., Silver Spring, MD; and NIS-Elements; Nikon, Kanagawa, Japan). Similarly, using the double-stained sections, the numbers of tryptase⁺ MCs positive for IL-9R, IL-9, VEGF, and FGF-2 were quantified with dual-band ultraviolet filter settings and normalized to the analyzed tissue area.

Statistical Analysis

Results are expressed as mean $\pm$ SEM, analyzed by Mann-Whitney *U* test or two-way analysis of variance, as indicated in the figure legends, and statistical significance accepted when $P < 0.05$. Graph generation and statistic analyses were performed using Prism v4 (GraphPad, La Jolla, CA).

RESULTS

IL-9 Regulates MC Numbers in the Lung during Prolonged Exposure to Allergen

Given the role of IL-9 in MC maturation and proliferation *in vitro*, we examined the effects of IL-9 blockade on MC activation and numbers in the lung after allergen exposure. Acute allergen challenge induced a dramatic increase in MC activation, as assessed by increased serum levels of the mucosal MC biomarker, mouse mast cell protease 1 (mMCP-1), and these levels were significantly reduced by approximately 50% in animals pretreated with anti-IL-9 antibody (Figure 1A). However, short-term IL-9 blockade had no effect on the resident MC population in the lung as we found similar numbers of toluidine blue–positive MCs between all groups (Figure 1B). Interestingly, although IL-9 neutralization resulted in significant reductions in mucus and Th2 cytokines (i.e., IL-4 and IL-13), there was no impact on cellular inflammation or AHR in this acute model (Figures E1D and E1E).

Although the role of IL-9 has been extensively studied in acute models of allergic inflammation, its function in chronic disease and remodeling is less established. We questioned whether prolonged IL-9 blockade would have a greater impact on MC numbers and chronic inflammation. OVA-sensitized BALB/c mice were treated with anti-IL-9 antibody or control IgG, subjected to prolonged exposure to OVA to induce chronic inflammation and airway remodeling, and examined 5 weeks later (22, Figure E1B). We first investigated whether prolonged allergen challenge and/or anti-IL-9 neutralization had any impact on MC numbers in lung. Indeed, chronic allergen exposure induced significant increases in mMCP-1 levels but in contrast to acute allergen provocation, prolonged IL-9 blockade completely attenuated these levels in serum (Figure 1C). Background mMCP-1 levels in sham IL-9 antibody–treated mice were also reduced, suggesting perhaps an impact on resident MC numbers in the absence of allergen challenge. Enumeration of toluidine blue–stained MCs showed that prolonged allergen challenge increased the numbers of MCs in the lungs of control IgG–treated mice. However, in marked contrast to the acute challenge, these cells were markedly reduced to baseline levels (i.e., sham mice) after continued IL-9 neutralization (Figure 1D). Importantly, we observed no impact of IL-9 blockade on cellular inflammation in the lung at this time point; numbers of macrophages, eosinophils, neutrophils, and lymphocytes were comparable to that of control IgG-treated, allergen-challenged mice (Figure E3).

Prolonged IL-9 Blockade during Chronic Allergen Exposure Prevents Airway Remodeling

We next examined the effects of prolonged IL-9 blockade on multiple parameters of airway remodeling during chronic OVA exposure. Neutralization of IL-9 profoundly attenuated all of the pathophysiological features of airway remodeling (Figure 2), namely a significant reduction in mucus production from hyperplastic goblet cells (Figures 2A and 2B), in addition to markedly diminished subepithelial collagen deposition, as assessed by quantitative biochemical measurement of total lung collagen (Figure 2C) and specifically via image analysis of peribronchiolar-associated collagen in Sirius red–stained lung sections (Figures 2D and 2E; reductions in total [i.e., vascular and airway] versus airway-specific collagen were 64 vs. 91%, respectively). Furthermore, there were significant changes in airway smooth muscle mass, as shown by reductions in both total and proliferating (proliferating cell nuclear antigen–positive) airway smooth muscle cells (Figure 2F and 2G).

Similar results were also obtained with IL-9 blockade using the more relevant aeroallergen, HDM. Repeated mucosal exposure to HDM extract has been previously shown to elicit robust Th2 polarized pulmonary inflammation and remodeling that is associated with increased AHR (27). Prophylactic dosing of anti-IL-9 antibody during chronic HDM exposure led to a significant reduction in both mucus hyperplasia (Figure 3A) and subepithelial collagen deposition compared with HDM plus control IgG-treated mice (Figures 3B and 3C). Similarly, we observed a concomitant decrease in lung MC numbers (Figures 3D and 3E).

A Reduction in MCs and Airway Remodeling Correlates with Decreased Expression of Profibrotic Factors

MCs are a robust source of profibrotic factors that contribute to ECM formation. Therefore, we next investigated whether the effects of IL-9 inhibition on airway remodeling were associated with the regulation of various MC-derived growth factors. We found that activated TGF β , VEGF, and FGF-2 levels were significantly elevated in lung tissue after prolonged OVA exposure, and that IL-9 neutralization markedly reduced expression of all three cytokines (Figures 4A–4C), suggesting a potential molecular mechanism for the antifibrotic effects of anti-IL-9 pretreatment.

IL-9 Regulates Airway Remodeling and Changes in Lung Function

The physiological consequences of airway remodeling have been difficult to correlate with changes in lung function. Using the flexiVent lung function system, we were able to study a range of lung function parameters and attempted to correlate structural changes with progression of airway dysfunction. Mice exposed to chronic OVA allergen exhibited significant and sustained increases in total airways lung resistance and elastance (Figures 5A and 5B). Importantly, these changes were also associated with marked elevations in G and H (Figures 5C and 5D), the parameters that are believed to be more closely related to resistance and elastance of the smaller airways and lung parenchyma (28). Interestingly, chronic challenge did not alter the resistance of the central conducting airways (data not shown). When we examined the effects of IL-9 neutralization, we observed a profound and selective effect on airway function. Mice pretreated with anti-IL-9 antibody were completely protected from increases in total airway resistance (Figure 5A) and significantly protected from increases in tissue (Figure 5C) resistance. Despite this, in the anti-IL-9–treated mice, the elastance parameters were profoundly elevated above baseline (i.e., total [Figure 5B] or smaller airways [Figure 5D]). An improvement in tissue resistance after IL-9 neutralization may well reflect a reduced parenchymal distortion in the lung due to decreased remodeling. Because IL-9 blockade prevented airway remodeling and had no

effect on cellular inflammation, these data suggest that structural changes in the smaller airways do contribute to persistent airway dysfunction.

We have previously shown that structural changes in the airways persist long after cessation of allergen challenge, despite an almost complete/partial resolution of inflammatory infiltrates and goblet cell hyperplasia (26). We questioned whether the observed decline in lung function persisted in the absence of airway challenge. Four weeks after the last allergen challenge (Day 80), OVA-sensitized and -challenged wild-type mice had little or no change in BAL inflammatory infiltrates when compared with sham mice (Figure 6A). Most of the goblet cell hyperplasia seen at the earlier time points had resolved by Day 80; however, when we examined collagen deposition at this time point, we found that total lung and Sirius red-stained peribronchiolar collagen deposition (data not shown, *see* Reference 26) were still elevated above baseline. Moreover, when we examined the lung function of these mice, we found that the enhanced changes in total airway resistance (R, Figure 6B) and smaller airway/tissue resistance (G, Figure 6C) were still present at Day 80. As expected, the anti-IL-9 antibody-treated, challenged mice remained completely protected from changes in collagen deposition and airway resistance (Figures 6B and 6C). These results suggest that collagen deposition may contribute to the altered lung dysfunction observed after chronic allergen provocation and, importantly, highlight a potential for antifibrotic treatments in asthma.

MCs Are the Major Cell Population Expressing the IL-9 Receptor in Human Asthmatic Airways: Colocalization with the Profibrotic Mediators VEGF and FGF-2

To substantiate the effects of anti-IL-9 treatment in a mouse model of asthma, we attempted to corroborate some of our described observations in human subjects with asthma. We determined the numbers of MCs in bronchial biopsies from subjects with mild asthma and normal healthy control subjects. The total density (median [range]) of MCs in bronchial biopsies from subjects with mild asthma was 151 (53–350) MCs/mm² and similar to that observed in biopsies from control subjects (135 [45–244] MCs/mm²) (Figure 7A). We then examined the expression of IL-9R in subjects with mild asthma and normal airways. Although some staining was detected in scattered epithelial cells (data not shown), we found that MCs were the major IL-9R-expressing cell in the lungs of both subjects with asthma and control subjects (Figures 7D–7F, IL-9R expression is shown for subjects with asthma only). There were no differences in the number of IL-9R⁺ MCs when subjects with mild asthma were compared with healthy control subjects (total density of IL-9R⁺ MCs 151 [44–350] vs. 132 [45–244] MCs/mm², $P = 0.82$, respectively; Figure 7B).

We next looked at mediators that may be produced by MCs. Interestingly, we identified a subpopulation of MCs that stained positive for tryptase and IL-9 (Figures 7C and 7G), suggesting a potential autocrine IL-9/IL-9R system in bronchial MCs as previously shown in mice (29). However, this needs to be further examined, as positive staining could also reflect receptor-bound IL-9 at the cell surface. Numbers of IL-9⁺ MCs were also unchanged between subjects with mild asthma and healthy subjects (total density of IL-9⁺ MCs, 7 [0–85] vs. 6 [0–19] MCs/mm², $P = 0.78$, respectively; Figure 7C).

As the levels of VEGF and FGF-2 were reduced after IL-9 neutralization *in vivo*, the expression of these growth factors was also explored in human asthmatic airways. The expression of VEGF, in both subjects with asthma and normal subjects, was mainly localized to the airway epithelium and vascular endothelial cells (Figure 7H). However, MCs did constitute a major proportion of the VEGF⁺ nonstructural cells (Figure 7H, *white arrows*). Notably, the total numbers of VEGF⁺ MCs were increased in subjects with mild asthma (65 [21–158] MCs/tissue area) compared with healthy control subjects (35 [23–105] MCs/tissue area). In addition, when we examined the expression of FGF-2, we found

positive immunoreactivity in epithelial and endothelial cells as well as in subepithelial nonstructural cells (Figure 7I). Among these nonstructural cells were occasional MCs (*inset* in Figure 7I), although the majority of the FGF-2⁺ cells were of a non-MC phenotype.

DISCUSSION

Although originally cloned and characterized as a T-cell growth factor (30), the precise role of IL-9 in inflammation and autoimmune diseases remained unclear. Although described as a pleiotropic cytokine, one of the most important functions for IL-9 appears to be a major role in mediating MC survival and function. IL-9R is strongly expressed on pluripotential precursors in bone marrow, committed immature, and mature MCs (30) (Figure 7). Furthermore, IL-9 has been shown to induce proliferation and survival of MCs (10). Likewise, transgenic mouse studies have implicated a role for this cytokine in pulmonary mastocytosis (13, 14), and in an acute mouse model of asthma, IL-9 has been shown to regulate allergen-induced MC progenitor recruitment to the lung (21). Notably, the airways of human subjects with chronic asthma exhibit features of ongoing mucosal MC activation, and in some studies this correlates with disease severity (31, 32). Furthermore, MC localization within ASM bundles is believed to contribute to the disordered airway physiology associated with this disease (33). Thus, an impact on MC function may have therapeutic potential in all aspects of chronic asthma. In this study, we provide direct evidence for a pathogenic role of IL-9 in mechanisms that govern pulmonary MC numbers after allergen challenge and chronic remodeling of the airways that is associated with a decline in lung function.

We found that short-term IL-9 neutralization, during an acute allergen provocation, had no effect on resident lung MC numbers, albeit it did significantly reduce mucosal MC activation. Similarly, we observed no effect of IL-9 blockade on lung function during the acute phase, despite significant reductions in mMCP-1, IL-13 levels, and mucus production. However, the presence of functional and mature lung MCs in these mice would explain the lack of effect of short-term IL-9 neutralization on AHR and is consistent with the observation that IL-9 deficiency did not confer protection in a similar acute model (9). In contrast, extended inhibition of IL-9 during prolonged allergen exposure markedly diminished mucosal MC activation and dramatically reduced the number of MCs in the lung. These data suggest that IL-9 regulates the recruitment of MC progenitors to the lung during chronic inflammation and support a role for IL-9 in MC hyperplasia (13, 14, 21). Interestingly, we observed lower mMCP-1 levels in chronically, versus acutely, challenged mice even though MC numbers were significantly higher than animals undergoing acute exposure. The reason for this discrepancy is unclear given that mMCP-1 levels have previously been shown to correlate with MC numbers in tissue (34, 14). The acute and chronic models used in our study markedly differ in the allergen challenge regime and are thus difficult to compare. However, we have previously observed that consecutive, short-term MC activation (i.e., 3–4 d) results in a cumulative and significant enhancement of mMCP-1 levels in the absence of MC hyperplasia (data not shown). Importantly, the dramatic reduction in MC numbers correlated with a profound effect on the development of airway remodeling and subsequent changes in lung function after chronic allergen exposure. These changes included a reduction in mucus hyperplasia and subepithelial fibrosis as well as attenuation in airway smooth muscle mass and an overall improvement in lung function. Conclusively, these data predict that prolonged IL-9 inhibition is required to diminish MC activity and mediate significant beneficial effects in the lung.

The correlation between a reduction in MC numbers and decreased airway remodeling, after IL-9 inhibition, is consistent with reports that MC-deficient mice demonstrate significantly attenuated fibrosis and inflammation after silica (35), ozone (36), or bleomycin injury (37).

Additional evidence stems from reports indicating the abundance of MCs in diseases associated with fibrosis (38). Of relevance was the recent report that MC-deficient mice have reduced structural changes after chronic allergen exposure (15). We and others have previously identified a critical role for eosinophils in this process (22, 39). Moreover, we have observed that eosinophil-deficient and CCR3^{-/-} mice, the latter in which eosinophil trafficking is severely impaired to the lung, have significantly attenuated levels of IL-9 in the lung as well as reduced fibrosis after prolonged allergen exposure (A. A. Humbles and coworkers, unpublished observations). It is tempting to speculate that, in addition to lymphocytes, eosinophils are a major source of IL-9 in the lung and are indirectly contributing to fibrosis via an IL-9–MC dependent manner.

Of significance was our finding that anti-IL-9 pretreatment also protected mice from chronic HDM-induced remodeling. HDM is a clinically relevant aeroallergen and is, in contrast to OVA, a complex antigen with proteolytic activity. These allergens are believed to elicit very different immune responses, which is supported by recent data identifying opposing roles for TGFβ blockade in both of these models (2, 23). Remarkably, we found a protective and similar phenotype with anti-IL-9 therapy in both OVA- and HDM-driven responses, suggesting that an IL-9–MC axis is central to allergen-induced chronic inflammation.

Importantly, attenuated airway remodeling by anti-IL-9 antibody was also associated with a decrease in lung expression of the growth factors TGFβ, VEGF, and FGF-2. TGFβ has been reported to play a critical role in airway remodeling and is a key player in the tissue repair response (2, 40). Similarly, overexpression of VEGF in mouse lung provokes not only a vascular phenotype but also marked subepithelial fibrosis, mucus hyperplasia, and AHR (41), and FGF-2 is up-regulated in the airways of subjects with asthma undergoing segmental challenge (42) as well as in a nonhuman primate model of asthma wherein expression was associated with remodeling (43). Moreover, MC activation/proteases have been linked to the release of many of these growth factors, including FGF-2 release after allergen challenge (44–47). Our analysis of VEGF and FGF-2 expression in lung tissue from subjects with mild asthma clearly implicates MC as a potential and relevant source of these growth factors *in vivo*. Moreover, we found increased numbers of VEGF-expressing MCs in subjects with asthma compared with control subjects. These data are consistent with a recent study demonstrating that tryptase⁺ chymase⁺ MCs (MC_{TC}) are increased in asthma and correlate with the number of VEGF⁺ cells within the vascular area of the bronchial mucosa (48). Thus, the combined reduction of TGFβ, VEGF, and FGF-2 during chronic inflammation provides a possible mechanism whereby IL-9 blockade could prevent fibrosis and potentially alleviate asthma symptoms.

Airway remodeling is believed to be a determinant of AHR and associated with the accelerated loss of lung function that is observed over time in chronic asthma. Structural changes, including subepithelial fibrosis, are believed to contribute to the thickened airway walls and progression of airway dysfunction in subjects with asthma by potentiating airway narrowing (49). Furthermore, a recent study in subjects with asthma has shown that the persistence of AHR is associated with airway remodeling and not dependent on sustained inflammatory cell recruitment (50). Notably, this is consistent with the observation that AHR persists in more severe disease despite prolonged treatment with inhaled corticosteroids (51). Interestingly, we found that the effects of anti-IL-9 therapy on changes in lung physiology were very specific. The observed increases in total lung and smaller airways elastance were not affected by IL-9 blockade. Changes in these specific airway parameters are believed to be associated with inflammation (28) and therefore concur with anti-IL-9 treatment having no effect on leukocyte accumulation in the lung. In contrast, increases in total airway resistance (R) and resistance of the smaller airways (G) were

completely attenuated by IL-9 blockade, implying that airway remodeling significantly contributes to changes in the resistance of the peripheral airways. Similar findings describing a correlation between subepithelial fibrosis and progression of AHR have also been reported in mice after chronic HDM exposure (52). Notably, and in agreement with our observations, a recent study in allergic cynomolgus monkeys suggests that treatments aimed at improving smaller airways dysfunction might be more effective in asthma (53).

In conclusion, our studies demonstrate a critical role for IL-9 in regulating MC numbers in the airways after allergen challenge. Prolonged IL-9 neutralization results in a profound inhibition of MC numbers, airway remodeling, and AHR after chronic allergen exposure. Furthermore, we correlate a reduction in MCs with decreased expression of TGF β , VEGF, and FGF-2 in the lung. Our data suggest a novel IL-9–MC axis that regulates airway fibrosis and is further supported by studies in human asthmatic airways, demonstrating that lung MCs express the IL-9 receptor and can exhibit colocalization with VEGF and FGF-2 expression. These data suggest that IL-9 may play a critical role in allergic inflammation as well as other respiratory fibrotic-related diseases, such as idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease. Furthermore, these studies highlight the MC-enhancing activity of IL-9, which is unique among the Th2 cytokines, and predict that targeting IL-9 could provide a novel approach for the treatment of asthma.

Supplementary Material

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AT A GLANCE COMMENTARY**Scientific Knowledge on the Subject**

The contribution of the Th2 cytokine IL-9 to asthma pathogenesis has thus far been unclear, with several groups reporting conflicting data as to whether this cytokine is a critical mediator in disease.

What This Study Adds to the Field

Here we provide evidence on a role for IL-9 in controlling lung mast cell numbers and regulating airway remodeling in response to chronic allergen challenge. Furthermore, we highlight a link between IL-9, mast cells, and fibrosis of the airways and suggest that modulating this pathway could lead to a reduction in chronic inflammation and improved lung function in patients with asthma.

Figure 1.

Prolonged IL-9 neutralization throughout chronic allergen challenge is required to reduce resident mast cell (MC) numbers in the lung. Mice were sensitized and challenged according to the acute and chronic challenge protocol (Figures E1A and E1B). (A, C) MC activation was determined by levels of mMCP-1 in serum measured by ELISA. (B, D) Tracheal sections were stained with toluidine blue, and MC numbers were determined and normalized per unit area (μm^2) of tissue counted. Data are expressed as mean $\pm$ SEM, $n = 8$ –27 mice/group from one to three independent experiments, * $P < 0.05$ or *** $P < 0.001$, comparing groups as indicated by Mann-Whitney U test. OVA = ovalbumin.

Figure 2.

IL-9 blockade significantly attenuates airway remodeling after chronic ovalbumin (OVA) challenge. Mice were sensitized and chronically challenged with allergen (Figure E1B). (A, B) Mucus production was determined from periodic acid-Schiff–stained lung sections according to criteria in METHODS. (C) Total lung collagen was assessed biochemically in lung tissue homogenates, and (D) peribronchial collagen deposition was quantified by image analysis of Sirius red–stained lung sections for which representative sections of each group are shown in (E). (F) Numbers of airway smooth muscle (ASM) cells beneath the basement membrane were determined morphologically, and (G) proliferating ASM cell numbers were quantified from proliferating cell nuclear antigen–stained lung sections. Data are expressed as mean $\pm$ SEM, $n = 10$ –27 mice/group from two to three independent experiments, * $P < 0.05$, *** $P < 0.0001$ comparing groups as indicated by Mann-Whitney U test.

Figure 3.

IL-9 blockade is effective at preventing airway remodeling due to house dust mite (HDM) exposure. Mice were chronically challenged with intranasal HDM (Figure E1C). (A, B) Mucus production was quantified from periodic acid-Schiff–stained lung sections, according to criteria in *METHODS*. Peribronchial collagen deposition was examined from Sirius red–stained lung sections for which representative sections of each group are shown in (C) and quantified by image analysis (D). (E, F) Tracheal sections were stained with toluidine blue and mast cells counted and normalized according to area. Data shown in A, C, and D are expressed as mean $\pm$ SEM, $n = 4$ –6 mice/group. * $P < 0.05$, ** $P < 0.01$ comparing groups as indicated by Mann-Whitney *U* test. PBS = phosphate-buffered saline.

Figure 4.

Anti-IL-9 treatment reduced expression of profibrotic growth factors. (A) Transforming growth factor (TGF) β activity was measured in bronchoalveolar lavage supernatants using the secreted alkaline phosphatase bioassay as described in *METHODS*. Levels of (B) vascular endothelial growth factor (VEGF) and (C) fibroblast growth factor (FGF)-2 were quantified in lung tissue homogenate by ELISA. Data are expressed as mean $\pm$ SEM, $n = 6$ –18 mice/group from one to two independent experiments, * $P < 0.05$, *** $P < 0.001$ comparing groups as indicated by Mann-Whitney U test. OVA = ovalbumin.

Figure 5.

Chronic allergen challenge is associated with impaired lung function, which is reversed by anti-IL-9 blockade. AHR was induced by chronic allergen challenge (Figure E1B) and assessed using the flexiVent system in response to increasing concentrations of methacholine. The snapshot parameters (*A*) resistance and (*B*) elastance, and the prime wave parameters (*C*) G (tissue resistance), and (*D*) H (tissue elastance) were measured. Data are expressed as mean $\pm$ SEM, $n = 10$ – 27 mice/group from three independent experiments, ** $P < 0.01$, *** $P < 0.001$, comparing ovalbumin (OVA)-sensitized IgG-treated mice with sham-sensitized IgG-treated mice and # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ comparing OVA-sensitized anti-IL-9 treated mice with sham-sensitized anti-IL-9 treated mice by two-way analysis of variance.

Figure 6.

Airway hyperresponsiveness (AHR) persists after cessation of allergen exposure. Mice were sensitized and subjected to chronic allergen challenge (Figure E1B). One month after the cessation of allergen challenge (Day 80), cellular recruitment to the airway lumen was determined by bronchoalveolar lavage, followed by (A) differential counting. AHR was measured using the flexiVent system in response to increasing concentrations of methacholine. (B) Total airways resistance (R) and (C) small airways resistance (G) were measured. Data are expressed as mean $\pm$ SEM, $n = 6-8$ mice/group, *** $P < 0.001$ comparing OVA-sensitized mice with sham-sensitized mice by (A) Mann-Whitney U test or (B, C) two-way analysis of variance.

Figure 7.

Human lung mast cells (MCs) express IL-9 receptor (IL-9R), and are a source of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-2. (A) Tryptase⁺ MC numbers were calculated in bronchial biopsies from healthy control subjects and subjects with mild asthma. Double immunofluorescence staining of MCs and IL-9R or IL-9 was performed on paraffin sections using direct-labeled Alexa-488 anti-MC tryptase (*green*) and Alexa-555 detection system (*red*) to detect MCs and IL-9 or IL-9R, respectively. (B) Numbers of IL-9⁺ and (C) IL-9R⁺ MCs were determined in bronchial biopsies from subjects with asthma and healthy control subjects. (D) IL-9R⁺ subepithelial cells in the human airway mucosa of subjects with mild asthma, as visualized by AP/new Fuchsin detection (*red*) and HTX counter stain. Examples of tryptase and IL-9R colocalization within same sections are shown by (E) separate filter settings and (F) as fused images. (G–I) Examples of colocalization of MC tryptase (*red*) and (G) IL-9, (H) VEGF, and (I) FGF-2. Scale bars: D, E, G, I = 65 μ m; F, H = 40 μ m.