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Modeling T_H2 responses and airway inflammation to understand fundamental mechanisms regulating the pathogenesis of asthma

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Summary

In this review, we highlight experiments conducted in our laboratories that have elucidated functional roles for CD4⁺ T-helper type-2 lymphocytes (T_H2 cells), their associated cytokines and eosinophils in the regulation of hallmark features of allergic asthma. Notably, we consider the complexity of Type-2 responses and studies that have explored integrated signaling among classical T_H2 cytokines (IL-4, IL-5 and IL-13), which together with CCL11 (eotaxin-1) regulate critical aspects of eosinophil recruitment, allergic inflammation and airways hyperresponsiveness (AHR). Among our most important findings, we have provided evidence that the initiation of T_H2 responses is regulated by airway epithelial cell-derived factors, including TRAIL and MID1, which promote T_H2 cell development via STAT6-dependent pathways. Further, we highlight studies demonstrating that microRNA are key regulators of allergic inflammation and potential targets for anti-inflammatory therapy. On the background of T_H2 inflammation, we have demonstrated that innate immune cells (notably, airway macrophages) play essential roles in the generation of steroid-resistant inflammation and AHR secondary to allergen and pathogen-induced exacerbations. Our work clearly indicates that understanding the diversity and spatiotemporal role of the inflammatory response and its interactions with resident airway cells is critical to advancing knowledge on asthma pathogenesis and the development of new therapeutic approaches.

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Keywords

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Background

Asthma as an inflammatory disease

Asthma is a disease of the airways, which affects approximately 300 million people worldwide. Further, asthma prevalence has been increasing over the last 50 years. The disorder is clinically characterized by chronic airway inflammation, remodeling of the airway wall and airways hyperresponsiveness (AHR) to non-specific spasmogenic stimuli. While previous studies focused on asthma as a uniform entity, asthma is now recognised as a complex heterogeneous inflammatory disease. Asthma encompasses distinct clinical phenotypes that can be further refined by the assessment of pathophysiological mechanisms (endotypes), characterised by differing inflammatory cell infiltrate profiles and immune cell activation patterns. This has led to the description of four sub-types of asthma, including allergic/eosinophilic, neutrophilic, mixed eosinophilic/neutrophilic and paucigranulocytic. Furthermore, individual disease severity can range from mild through to severe. While steroids (primarily inhaled glucocorticoids) and bronchodilators (β -adrenergic receptor-agonists) remain as mainstay therapy for most asthma patients, responsiveness to these agents often depends on the phenotype and severity of disease. Thus, while the majority of individuals with mild-to-moderate allergic/eosinophilic asthma achieve control of their symptoms through standard treatment with steroids and bronchodilators, patients with other subtypes or severe disease can be resistant to these therapies. These (above) subjects are covered in substantial detail in several excellent recent reviews (1–5).

Controlling exacerbations of asthma remains difficult. Exacerbations are commonly induced by infection (particularly secondary to respiratory virus infection) as well as allergens and are a major cause of hospitalization, especially in children. Further, exacerbations account for a large proportion of the healthcare costs associated with asthma management (reviewed in (6)). Those at greatest risk have more severe airways disease and poor symptom control (termed steroid-resistant or treatment-refractory asthma). However, exacerbations can also afflict patients whose disease is typically well-controlled by steroid therapy. In addition, exacerbations predispose to disease progression characterized by an accelerated decline in lung function. The mechanisms underlying asthma exacerbation have not yet been fully elucidated. However, activation of the innate immune system on the background of chronic allergic inflammation appears to play a critical role in this process. Crosstalk between innate and allergic inflammatory pathways may result in the induction of an exaggerated steroid-resistant inflammatory response in asthma.

It is important to recognize that current therapies only treat asthma symptoms and not the underlying cause of disease. Furthermore, it is equally crucial to recognize that a significant proportion of individuals suffer from severe disease, and remain symptomatic despite medication use (7). The challenge for the future is to develop tools that can easily classify patients into specific subtypes, to facilitate treatments with appropriate disease-modifying

therapeutics. However, the development of targeted, rational treatment approaches for asthma requires a thorough understanding of disease mechanisms. The development of these approaches requires coordination between clinical, translational and basic research studies to identify important disease targets in appropriate models of disease pathogenesis.

An important example of this approach is the examination of the T_H2 paradigm; specifically, recognition of the role of dysregulated T_H2 immune responses in mediating disease. This recognition encompasses the initial discovery in mice of distinct T_H1 and T_H2 subsets; the association of T_H2 cells, the cytokines they secrete (e.g. interleukin-(IL)-5) and pathways they operate (e.g. IgE production and eosinophil development) with asthma pathogenesis; modeling the role of T_H2 cell-regulated pathways in animal models; and the implementation of clinical trials targeting candidate cytokines and molecules.

In this review, we were invited to highlight experiments conducted primarily in our laboratories (in conjunction with many collaborators) that have focused on understanding the fundamental pathways underpinning the pathogenesis of asthma by employing a range of mouse models (see (8–14) for excellent reviews with broader field focus). In the development of these models, we have utilized the clinical data of the ‘time’ to shape the development of appropriate models to gain insights into pathogenic mechanisms. These include models of acute T_H2 -driven allergic airways disease, eosinophil expansion, migration and homing, chronic disease, steroid-resistant inflammation and disease exacerbations induced by a range of triggers.

In particular, we will focus on early investigations that dissected the role of allergen-specific T_H2 cells and associated cytokines/chemokines, together with the role of eosinophils in driving hallmark clinical features of allergic asthma. We also describe our investigations into the molecular and cellular processes regulating the development of T_H2 cells, chronic asthma, steroid-resistant inflammation and asthmatic exacerbations. Although the T_H2 paradigm may have temporarily polarized experimentation in the field toward a single hypothesis, collectively, its examination by many investigators led to the realization that other inflammatory phenotypes existed in asthma and the recognition of more multi-faceted triggers of disease (e.g. infection). We propose that understanding the diversity and spatiotemporal role of inflammation and its interactions with resident airway cells will be crucial to advancing knowledge on asthma pathogenesis and the development of new therapeutic approaches.

Modeling the role of T_H2 cells, associated cytokines and CCL11 in the regulation of allergic airways disease

Role of T_H2 cells and IL-4, IL-5 and IL-13

At the beginning of our investigations into the pathophysiology of asthma (in early 1992), asthma was understood as a chronic disease of the airways, characterized clinically by reversible airways obstruction, enhanced bronchial responsiveness to spasmogens (also known as airways hyperresponsiveness, or AHR) and airways inflammation. The underlying inflammatory response was thought to play a critical role in the development and

progression of disease and the induction of AHR. This included a role in altering the structural integrity of the epithelium and in remodeling of the airway wall. Notably, at this time the cellular composition of inflammatory infiltrates into the lung was recognized to be diverse; lung infiltrates included activated neutrophils, mast cells, eosinophils and monocytes/macrophages. The recruitment and/or activation of these cells was thought to be controlled by the secretion of cytokines and chemotactic factors released from antigen-stimulated T lymphocytes and other structural (e.g. epithelial) and inflammatory cells (e.g. dendritic cells) (15–20).

At this time, the concept of distinct inflammatory phenotypes of asthma had not yet emerged and there was increasing interest in the involvement of eosinophils and factors secreted from T_H2 cells in disease pathogenesis (16, 17, 19–23). This largely stemmed from a rapidly emerging body of clinical studies where eosinophil accumulation in the airways was a predominant feature of the inflammatory infiltrate, which often correlated with disease severity (15, 18, 24, 25). Furthermore, increased numbers of activated T_H2 cells and levels of the eosinophil-specific regulatory cytokine, interleukin-5 (IL-5), had been observed in the lungs of asthmatic patients and after experimental allergen-induced late-phase reactions (16, 17, 19, 20, 26). Airway narrowing and disease progression through tissue damage was directly attributed to eosinophil activation and the release of cationic granule proteins into the airways (21, 27, 28). Roles for other T_H2 cytokines remained largely unknown; while IL-4 was linked to the regulation of Type-2 immune responses and IgE production, the role of IL-13 had not been explored.

Role of IL-5 and eosinophils

To gain some insight into the role of IL-5 and eosinophils in regulating asthmatic responses, we employed IL-5 gene-deleted mice (*IL5*^{−/−} mice) (29, 30) in conjunction with models of acute T_H2-mediated allergic inflammation. For our studies, we employed a classic model of allergic airways disease induced by sensitization and aeroallergen challenge with the archetypal Type-2 antigen ovalbumin (OVA) (30). This model allowed the direct dissection of T_H2 cells and secreted cytokines in the mechanism leading to pathogenic features of allergic eosinophilic asthma and significantly advanced our understanding of the role of this pathway in disease. Furthermore, they built on earlier studies highlighting the potential role of IL-5 and eosinophils in the pathogenesis of asthma (26, 29, 31–35).

Sensitization and aeroallergen challenge of wildtype C57Bl/6 mice with OVA induced allergic airways disease (AAD) and hallmark features of asthma, including the development of antigen-specific T_H2 cells, eosinophilia, eosinophil accumulation in the lung, mucus hypersecretion, epithelial cell shedding and occlusion of the airways (30). These inflammatory and pathophysiological changes correlated with the development of AHR in response to administered methacholine, a spasmogen that is also used as a clinical tool to assess airway responses in asthmatic patients (30). Notably, *IL5*^{−/−} C57Bl/6 mice, in the absence of eosinophils, were protected from all features of allergic airways disease (30). Interestingly, allergic sensitization was unaffected, with no difference in circulating levels of allergen-specific IgE (30). Further, reconstitution of IL-5 production (by delivery of vaccinia virus expressing IL-5 to the lung) completely restored all features of disease (30). Of note,

earlier studies that featured monoclonal antibodies (mAbs) targeting IL-5 had been shown to suppress, but not abolish, pulmonary eosinophil accumulation in response to antigen challenge and the correlation of this effect with changes in lung morphology and AHR were controversial (32, 34). Our study, utilizing mice fully devoid of IL-5, clearly demonstrated the central importance of this cytokine in inducing eosinophilia and subsequent lung damage and inflammation in response to T_H2-inducing antigens.

In a subsequent study, we extended these findings and demonstrated that treatment with an anti-IL-5 mAb before allergen challenge could profoundly suppress eosinophil recruitment to the peripheral blood and airways, lung damage, and reduce the magnitude of AHR (36). The central importance of IL-5 derived from T_H2 cells for the development of eosinophilia and allergic inflammation was also demonstrated (37). The transfer of primed OVA-specific CD4⁺ T_H2 cells from OVA-sensitized wild-type mice into OVA-sensitized *IL5*^{-/-} mice was sufficient to restore blood and airway eosinophils, lung damage and AHR after antigen challenge (37). These CD4⁺ T_H2 cells produced IL-4 and IL-5, but not IFN γ , when stimulated with OVA *in vitro* (37). By contrast, OVA-specific CD4⁺ T cells that produced IFN γ (but not IL-4 or IL-5) *in vitro* failed to restore features of AAD (37). OVA sensitization of the recipient mouse was not required, but aeroallergen OVA challenge was required to induce disease (37). We also demonstrated that delivery of antigen to the airways directly promoted activation of transferred T_H2 cells via antigen presenting cells (37). Collectively, the aforementioned studies demonstrated that antigen-specific IL-5-secreting T_H2 cells played a central role in eosinophil recruitment and activation. Furthermore, they demonstrated that T_H2 cells can provide all the signals essential for the induction of allergen-induced AHR and lung damage, and supported the concept that accumulation and activation of eosinophils in the airways during asthmatic responses may be initiated and sustained by cytokines released from this lymphocyte subset.

Role of IL-4 and IgE

We next investigated the roles of IL-4 and IgE in the regulation of AAD (38–41). Both of these molecules have been implicated as central mediators of asthma. At the time, IL-4 was thought to be a critical factor in the regulation of T cell commitment to the T_H2 phenotype, based on studies of T cell differentiation carried out *in vitro*, and considered to play an essential role in IgE isotype-switching in B cells (38, 42–44). IL-4 also had potential roles in the maintenance of the inflammatory response to recall antigens and regulating production of the eosinophil-specific chemoattractant CCL11 (38, 39, 45). IgE had been identified as a key factor for the activation of mast cells and there was emerging evidence that it played a role in the regulation of eosinophil-mediated AHR by modulation of T_H2 cell function via B-cells (35).

To ascertain the roles of IL-4 and IgE in disease processes, we employed our model of acute AAD and evaluated mice deficient in IL-4 and CD40 (on a mixed 129Sv X C57BL/6 background). In *IL4*^{-/-} mice following induction of allergic inflammation, we observed a reduction in the numbers of eosinophils recruited into the airways and decreased levels of antigen-specific IgE and IgG1 (46). However, tissue damage and AHR were not affected and antigen-specific IgG2a and IgG3 were detected (46). Further, treatment of *IL4*^{-/-} mice with

anti-IL-5 reduced blood and airway eosinophil recruitment and AHR (46). Next we used *CD40*^{-/-} mice in the aeroallergen-induced allergic airways disease model. *CD40*^{-/-} mice exhibit impaired T cell-dependent immunoglobulin class switching and germinal center formation (47). Aeroallergen challenge of sensitized *CD40*^{-/-} mice resulted in a severe inflammatory response, characterized by elevated numbers of blood and BALF eosinophils, mucous hypersecretion and AHR (46).

Notably, our studies on the role of IL-4 were in disagreement with those of other investigators. At the time, a key role for IL-4 had been demonstrated in the induction of AHR in BALB/c mice, when IL-4 was targeted during the initial sensitization phase (but not effector phase) (48). Pre-treatment with anti-IL-5 mAb during the sensitization phase had also been shown to inhibit airway eosinophilia, but not AHR (48). Our work demonstrated that IL-5 was also required for AHR development and played a functional role in the allergic challenge phase of disease (36). Moreover, it demonstrated that IL-4 (as well as IgE) was not required to induce hallmark features of AAD induced by antigen-specific T_H2 cells. It did not, however, exclude a role for these factors in regulating other pathways that may drive disease either independently or in parallel with the T_H2/IL-5/eosinophil effector arm.

Antigen-specific T_H2 cells can develop independently of IL-4 in vivo

Notably, while other investigators had demonstrated that *Il4*^{-/-} mice had significantly impaired T_H2 cell responses and did not develop serum IgE responses to nematode infestation (44), they could produce IL-5 and mount an eosinophilic response (albeit at lower levels) in response to infestation or after sensitization and subsequent challenge with OVA (39, 44). Eosinophilic inflammation has also been observed in models of parasite infestation and malaria infection, when IL-4 was absent during the initiation phase of the immune response (49, 50). In these contexts, the source of IL-5 was thought to be CD4⁺ T cells (either non-classical T_H2 cells or T_H0) or non-CD4⁺ cells (which may have been cells now known as type 2 innate lymphoid cells (ILC2s)) (44, 51, 52). ILC2s are now known to play a key role in eosinophil homeostasis and the regulation of allergic inflammation (53, 54). In our view, by using genetically-deficient mice and a strong T_H2-polarizing antigen, we were able to unmask the existence of two pathways for the development of antigen-specific T_H2 cells; one pathway, which was known to be dependent on IL-4 and another, which was independent of this factor. At the time this concept created controversy, but was further substantiated by Cohn *et al.* and subsequently Le Gros and colleagues (55, 56). This observation also highlighted that critical factors identified in T cell polarization assays *in vitro* may not be essential in the dynamic living system.

Variations in mouse strains can influence the role of T_H2 factors in the development of allergic inflammation

One difference between the elegant work of Corry *et al.* (which demonstrated a role for IL-4 but not IL-5) and our studies was the use of different strains of mice. Corry *et al.* (48) used BALB/c mice, whereas we primarily employed C57Bl/6 mice. Although pronounced and reproducible T_H2 responses can be induced by sensitization and challenge of the C57Bl/6 strain, BALB/c mice were thought to be innately more “T_H2-like”/prone. Therefore, the

relative contribution of the IL-4/IgE and IL-5/eosinophil pathways to the induction of AAD in different strains of mice may account for the differences observed in these studies (57).

In order to explore this possibility, we utilized *Il4*^{-/-} and *Il5*^{-/-} mice backcrossed onto the BALB/c strain in combination with inhibitory mAbs to these cytokines, to identify the contribution of these factors to the induction of allergic inflammation and AHR (58). Inhibition of the actions of IL-4 and/or IL-5 in mice on a BALB/c genetic background did not abolish AHR (58). Further, in the case of *Il4*^{-/-} mice pre-treated with anti-IL-5 mAb, AHR persisted in the absence of pronounced airways inflammation (58). AHR was abolished only following anti-CD4 mAb treatment, to deplete CD4⁺ T cell populations (58). However, aeroallergen challenge of *Il5*^{-/-} mice showed that morphologic changes to the airways were critically linked to IL-5 and eosinophil recruitment (58). Thus, in BALB/c mice, a novel CD4⁺ T cell pathway that modulated AHR was present, which did not require IL-4 or IL-5. Further, this study indicated that CD4⁺ T cells operate at least two pathways that can act independently to induce AHR in BALB/c mice. These findings suggested a novel role for CD4⁺ T cells, beyond the production of IL-4 and IL-5 in the regulation of AHR. Later, we demonstrated that polymorphisms in the IL-4R α chain in various strains of mice can profoundly regulate AHR, spatial aspects of tissue accumulation of eosinophils and generation of T_H2-biased T cell responses (59).

Role of IL-13

The coexistence of parallel pathways may account for the dissociation of airway eosinophils from the development of AHR observed in BALB/c mice. Further, this observation may have important implications for the treatment of asthma where eosinophils had been dissociated from AHR (60–62). In subsequent elegant studies published back-to-back in *Science*, Corry and colleagues (63) and Wills-Karp and colleagues (64) demonstrated that IL-13 was a novel T_H2 cell factor that induced AHR. Further, IL-13 could promote eosinophil accumulation in the lung and induce mucus hypersecretion (63, 64). Collectively, these papers suggested that IL-13 induced AHR independently of IL-5 and eosinophils and may represent a parallel pathway whereby T_H2 cells modulate AHR and airway inflammation. Of note, in later experiments using mice devoid of eosinophils, Lee and colleagues (65) identified eosinophils as critical for promoting AHR, while Humbles and colleagues (66) found that eosinophils were primary modulators of airway remodeling, demonstrating that eosinophils can play an important role. The importance of IL-13 in the induction of hallmark features of allergic airways inflammation such as AHR, mucous hypersecretion, eosinophil recruitment into tissues and tissue remodeling is now well recognized (67–71).

Integrated signaling between T_H2 cytokines regulates allergic inflammation

In the next series of experiments, we demonstrated the complexity of the T_H2 response in the regulation of allergic inflammation and AHR. In the first paper, by employing *Il13*^{-/-} mice in combination with mAbs targeting specific cytokines, we demonstrated that integrated signaling events between IL-4, IL-5 and IL-13 regulated allergic inflammation and AHR (72). Notably, these cytokines could operate in parallel, but their signals were also integrated through crosstalk with cellular infiltrates such as eosinophils. These cytokines

also had distinct temporal roles in the development of inflammation and AHR (e.g. IL-4 promoted the early phase of inflammation and IL-13 and IL-5 acted as late-stage effector molecules) (72). Thus, T_H2 cytokines did not operate on separate linear pathways in isolation, but rather their signals were integrated into a network that modulated the type and severity of lung inflammation.

At this time, Joerg Mattes and I started to think about how T_H2 pathways operated by cytokines such as IL-13 and IL-5 could be linked. In *IL5*^{-/-} BALB/c mice that were sensitized and aeroallergen challenged, we observed that while eosinophilia was absent, tissue eosinophils still accumulated, albeit at much lower levels than observed in wildtype mice (58, 73, 74). This was in marked contrast to *IL5*^{-/-} C57Bl/6 mice, where eosinophilia and tissue eosinophils were absent after the identical aeroallergen challenge (30). This suggested to us that a pool of eosinophils could develop independently of IL-5, which could accumulate in inflamed tissues if a sufficiently strong chemotactic stimulus was present. Thus, in the absence of IL-5, circulating and airway lumen eosinophils were absent, but tissue accumulation could still occur at a reduced level. Interestingly, in the first proof-of-principle clinical trials of anti-IL-5 mAb for the treatment of asthma, similar effects were observed (75–77). While blood and airway eosinophil levels were reduced by anti-IL-5 treatment after allergen provocation and induction of the late-phase response (75, 78), tissue-dwelling eosinophils persisted (79).

We set out to define the role of this tissue-resident pool of eosinophils in regulating AHR and to further investigate the interplay between IL-5, CCL11 and IL-13 for its regulation. Although IL-5 and IL-13 were thought to utilize different pathways to induce inflammation and AHR, we and others had observed that both molecules employed the eosinophil-specific chemokine CCL11 to promote tissue accumulation (45, 63, 64, 80–85). For example, delivery of recombinant IL-13 to the lungs of mice induced a pronounced inflammatory response characterized by eosinophil accumulation, mucous hypersecretion and increased expression of a range of inflammatory mediators (63, 64, 69). This eosinophilic response was dependent on CCL11 and STAT6 and suggested that while IL-5 can operate as a cofactor with CCL11 to induce eosinophil recruitment to tissues, it can also operate independently of this cytokine (69, 86). These investigations also highlighted the broad pro-inflammatory activity of IL-13, which could critically activate a range of immune pathways that linked innate and adaptive immunity. These observations are currently very relevant to how IL-13- or IL-5- producing ILC2s may exaggerate innate immune responses during infection-induced exacerbation of asthma. Furthermore, we had demonstrated unique cooperative interactions between IL-5 and CCL11 for the regulation of blood and tissue eosinophils (see section below for a detailed description of this relationship). Subsequent investigations by others identified two more eotaxin family members CCL-24 (eotaxin-2) and -26 (eotaxin-3, which is exclusively human), which also acted to specifically recruit eosinophils into tissue. However, these molecules had different temporal contributions to the phases of recruitment (87–89).

To further explore the unique nature of the IL-5/IL-13/CCL11 axis and its role in the regulation of eosinophil migration and the development of allergic inflammation and AHR, we generated BALB/c mice deficient in both IL-5 and CCL11 (*IL-5*^{-/-}*CCL11*^{-/-}) (74). In

Il-5^{-/-}CCL11^{-/-} mice, eosinophil accumulation was totally abolished in the lung after aeroallergen challenge, providing evidence that the accumulation of tissue eosinophils observed in *Il5^{-/-}* mice was regulated by CCL11 (74). The absence of eosinophils correlated with inhibition of AHR (74). Further, we determined that eosinophils may be contributing to the development of AHR by regulating the production of T_H2-derived IL-13 (74).

Bidirectional signaling between eosinophils and T_H2 cells regulates T cell cytokine production

As signals elicited by T_H2 cells were shown to play an obligatory role in the induction of AHR, the impact of IL-5/CCL11-deficiency on T_H2 cell production of IL-13, IL-4, and IL-5 was examined. T_H2 cells were isolated from allergic wildtype, *Il5^{-/-}*, *CCL11^{-/-}*, and *Il5^{-/-}CCL11^{-/-}* mice for evaluation (74). The level of IL-13 produced by activated CD4⁺ T_H2 cells from gene-deficient animals was significantly reduced when compared with those derived from wildtype mice (74). Notably, the levels of IL-13 in *Il5^{-/-}CCL11^{-/-}* CD4⁺ T cell cultures were 10-fold lower than wildtype cell cultures and were further reduced when compared to the levels observed with cells from *Il5^{-/-}* or *CCL11^{-/-}* mice (74). The reduced levels of IL-13 did not correlate with an overall suppression in T_H2-type cytokine production, as IL-5 levels produced by *CCL11^{-/-}* CD4⁺ T cells and IL-4 levels in all cultures were similar to those observed in wildtype CD4⁺ T cell cultures (74). Furthermore, IL-13 levels in the lung were significantly diminished in the absence of eosinophils, with the lowest levels found in aeroallergen-challenged *Il5^{-/-}CCL11^{-/-}* mice, where eosinophils and AHR were absent (74). Notably, transfer of wildtype antigen-specific T_H2 cells into *Il5^{-/-}CCL11^{-/-}* mice promoted eosinophil development and recruitment to the lung and induced AHR after aeroallergen challenge (74). Furthermore, transfer of wildtype eosinophils to sensitized and aeroallergen-challenged *Il5^{-/-}CCL11^{-/-}* mice restored the ability of their T_H2 cells to produce IL-13 (74). Thus, in the absence of eosinophils, T_H2 cells had an intrinsic defect in their ability to produce IL-13 and this was directly correlated with the number of eosinophils in the microenvironment. In further experiments, we showed that IL-13 production from CD4⁺ T cells taken from IL-5 overexpressing transgenic mice was significantly greater than from wildtype T-cells (74). Further, the level of T_H2-derived IL-13 and IL-13 in peribronchial lymph nodes directly correlated with the development and degree of AHR (74). We also indirectly demonstrated a potential role for IL-18 derived from eosinophils as the factor regulating IL-13 production (74). This study demonstrated bidirectional signaling between eosinophils and T_H2 cells in the regulation of allergic disease and highlighted the integrated and complex nature of T_H2 cytokine regulated responses. Thus, the IL-5/eosinophil and IL-13 pathways were not separate effector pathways but were integrated to regulate allergic inflammation. In later investigations, Jacobsen *et al.* demonstrated that the induction of allergic inflammation in mice was dependent on eosinophil regulated recruitment and function of DCs and T_H2 cells in the lung, further highlighting the intimate relationship between these cells (90). Collectively, these observations also had significant implications for therapies targeting IL-5 in isolation, with a view to abolishing tissue accumulation of eosinophils.

We and others went on to demonstrate that eosinophils could act as antigen presenting cells and directly promote allergic inflammation by activating T_H2 cells in an antigen-specific

manner (91–94). After aeroallergen challenge of sensitized mice, eosinophils rapidly trafficked to the lung and then to sites of antigen presentation, including the lung-draining lymph nodes (94). Eosinophils expressed MHCII and the costimulatory molecules CD80/86 and could internalize and present antigen (94). Further, antigen-loaded eosinophils could promote the production of IL-4/-5/-13 from antigen-specific T_H2 cells and could prime for allergic inflammation upon transfer to naïve animals (94). Thus, eosinophils have the potential to not only activate T_H2 cells to release disease-modulating cytokines, but also to assist in priming the immune system for allergic responses. Collectively, these investigations highlighted the potential of eosinophils to not only act as terminal effector cells, but also to actively modulate allergic inflammation by amplifying T_H2 cell responses. The role of eosinophils and other atypical antigen presenting cells in immune responses has recently been reviewed (95).

Defining the relationship between IL-5 and CCL11 for the regulation of eosinophil migration

Although we had demonstrated a critical role for IL-5 in the expansion of eosinophils in the bone marrow and induction of eosinophilia during allergic inflammation (supporting previous studies of parasitic infestation (49)), the critical signal for eosinophil-specific homing to tissue remained unknown. At the time of our initial investigations into the role of IL-5 in the regulation of allergic inflammation, the eosinophil-specific chemoattractant CCL11 was discovered and cloned (45, 80, 96–98). This was exciting, as this molecule appeared to be the critical homing signal for eosinophils. We designed fundamental experiments to determine the relationship between IL-5 and CCL11 for the regulation of eosinophil migration.

Systemic intravenous (i.v.) administration of recombinant IL-5 or CCL11 into wildtype mice induced a rapid eosinophilia, while CCL11 administration could also induce eosinophilia in *Il5*^{-/-} mice (86). These studies highlighted a role for IL-5 in the mobilization of bone marrow eosinophils and CCL11 in the recruitment of eosinophils from tissues into the blood (86). Further, pre-treatment of *Il5*^{-/-} mice with IL-5 was necessary to promote eosinophil homing and tissue accumulation in response to CCL11, highlighting cooperative roles shared between IL-5 and CCL11 in eosinophil activation and migration (86). Interestingly, although intravenous CCL11 induced eosinophilia in wildtype mice, this did not correlate with a significant reduction in the level of bone marrow eosinophils (86). By contrast, i.v. IL-5 administration resulted in depletion of the bone marrow store of eosinophils in wildtype mice (86). These results suggest the existence of two mechanisms by which eosinophilia can be rapidly induced. One mechanism occurs through expansion and mobilization of an IL-5-dependent bone marrow pool, while the second involves the mobilization of eosinophils from tissues into the circulation by eosinophil-specific chemoattractants, such as CCL11. Interestingly, the fact that CCL11 could direct eosinophils into systemic circulation suggests the existence of a pool of eosinophils located in tissues other than the bone marrow, which could be rapidly mobilized independently of IL-5 stimulation (this population would be mature effector cells). Furthermore, this pool of eosinophils, which is recruited to the lung in the absence of IL-5, then contributed to AAD. Tissue-resident eosinophils are found at many

sites in the body (e.g. gastrointestinal tract, adipose tissue, thymus, uterus, lung and mammary glands) where they have been proposed to play important biological roles (85). Recently, Mesnil *et al.* have also proposed that resident pulmonary eosinophils may contribute to homeostatic immune processes (99). They report resident pulmonary parenchymal eosinophils (rEOS) at steady-state, which are described as IL-5-independent Siglec-F^{int}CD62L⁺CD101^{lo} cells (99). During induction of AAD, the phenotype of rEOS does not change, and mice deficient in these cells showed enhanced T_H2 cell responses to inhaled allergens (99). The regulatory role of rEOS was linked to their ability to inhibit DC maturation and attenuate T_H2 responses (99). We would speculate that the IL-5 independent pool of eosinophils observed in our studies during allergic inflammation are recruited to the lung from other tissues, and do not represent steady-state rEOS (99).

To model tissue recruitment, we next injected recombinant CCL11 and/or IL-5 subcutaneously into the skin (86). When injected into this tissue compartment, either molecule promoted the recruitment of eosinophils to this locale. Recruitment was significantly enhanced if the number of circulating eosinophils was increased (either by i.v. administration of recombinant IL-5 or adoptive transfer of eosinophils) (86). Furthermore, eosinophil accumulation in the skin was amplified by co-administration of both molecules (86). This data further shows the cooperative nature of the interaction between CCL11 and IL-5 for eosinophil homing. This study suggested that CCL11 secreted from inflamed tissue may play an important role in initiating both blood and tissue eosinophil recruitment in the early phases of allergic inflammation. Furthermore, IL-5 is not only essential for expanding and mobilizing eosinophils from the bone marrow during allergic inflammation, but also plays a critical role in regulating eosinophil homing and migration into tissues in response to CCL11 and possibly other eosinophil chemotactic stimuli.

To further investigate the relationship between IL-5 and CCL11 for the selective recruitment of eosinophils into the airways, we delivered vaccinia virus expressing either CCL11 or IL-5 to the lungs of naïve WT mice (100). Again, we demonstrated that IL-5 and CCL11 cooperated to specifically recruit eosinophils into the airways. Expression of IL-5 and/or CCL11 in the lungs induced a pronounced and selective recruitment of eosinophils to the airways, but did not result in eosinophil degranulation or AHR (100). This is in contrast to later studies by Ochkur *et al.* that demonstrated that sustained overexpression of IL-5 and CCL24 (eotaxin-2) in the lung of transgenic mice induced pronounced eosinophil accumulation and degranulation and hallmark features of allergic inflammation (101). This may reflect a differential role for CCL11 and CCL24 in regulating degranulation or their levels of expression overtime (transient vs. sustained). In our study, airways accumulation of eosinophils could be induced independently of the induction of eosinophilia, but was markedly augmented by the co-expression of both molecules and/or by the transient mobilization of eosinophils from the bone marrow by i.v. administration of recombinant IL-5 (100). These experiments again implied that circulating and tissue eosinophils can provide important pools for recruitment to sites of inflammation. However, for eosinophil degranulation and AHR to occur, in our studies the inhalation of antigen and activation of resident CD4⁺ T cells by dendritic cells was required in association with IL-5 and CCL11 expression (100). Importantly, these investigations demonstrated that IL-5 regulates eosinophil accumulation within the lung as well as in the circulation, and also amplifies

CCL11-induced chemotaxis in the airway compartment (probably by increasing the number of eosinophils available to be recruited and by priming this cell). Furthermore, it highlights that signals from antigen-activated CD4⁺ T cells are required for eosinophil activation. Antigen-activated T helper cells have also been demonstrated to play important roles in regulating Type-2 innate immune responses and eosinophil degranulation (102). The interaction of T_H2 cytokines and CCL11 for the regulation of bone marrow, blood and tissue eosinophilia is reviewed in (73, 85, 103). We also demonstrated cooperative signaling between CCL24 and IL-5 for the recruitment of eosinophils into the lung (104). Findings in this investigation establish the potential importance of CCL24 in the induction of key phenotypic characteristics of experimental asthma and assisted in clarifying the link between eosinophils and IL-13 for the induction of AHR.

Anti-IgE and T_H2 Cytokine Therapies: Early failures and long-term success

Collective investigations by many groups looking at the role of T_H2 cells and the cytokines IL-4, -5, -13 and IgE in asthma pathogenesis lead to clinical trials. In initial clinical trials, humanized anti-IL-5 (mepolizumab) treatment decreased blood eosinophil numbers and reduced blood and sputum eosinophils after allergen challenge (78). However, treatment had no effect on AHR or late-phase allergic responses (78). A second study of patients with poorly-controlled asthma demonstrated that treatment effectively decreased blood and sputum eosinophil levels, but did not provide significant improvements in symptoms, quality of life or exacerbation frequency (105). These trials recruited patients based on asthma severity, rather than assessing underlying drivers of disease pathogenesis. The poor results that were obtained caused a severe setback to the field. However, the numbers of eosinophils remaining in lung tissue after treatment with mepolizumab may have been a confounding factor limiting therapeutic benefit, based on data from IL-5-deficient mice and anti-IL-5 mAb-treated models of AAD (58, 73, 74, 79).

It was not until follow-up studies were conducted, in which participants were selected based on the level of eosinophils detected in sputum samples (≥ 3% eosinophils in sputum) that clinical benefit was successfully and clearly demonstrated. In these studies, anti-IL-5 treatment was shown to reduce sputum and blood eosinophils and also improve asthma control, lung function and reduce corticosteroid dose (77). In other words, when patients were selected based on the presence of eosinophilic inflammation and recurrent exacerbations, treatment reduced exacerbation frequency (75). Building on this success, mepolizumab was recently approved by the United States Food and Drug Administration (FDA) for the treatment of severe eosinophilic asthma. Further investigations into the mechanisms underpinning non-eosinophilic phenotypes are now required to develop new therapeutics for these patients.

Favorable results have also been demonstrated with anti-IgE/omalizumab for the treatment of severe allergic asthma, which was approved by the FDA in 2003. Safety and efficacy for this treatment have been well established, although it should be noted that treatment is only effective in a subset of patients with IgE-mediated allergic asthma (106). Omalizumab treatment reduces exacerbation frequency from 26% to 16%, hospitalizations from 3% to 0.5% and can allow reduction in inhaled corticosteroid treatment (106). Further, treatment

with omalizumab therapy improved quality of life and symptoms, although no consistent improvement in lung function was seen (106).

A number of mAbs targeting IL-13 have also been assessed for therapeutic potential (e.g. lebrikizumab, tralokinumab, GSK679586 and anrukinzumab). Results from clinical trials have been mixed, with a number showing trends towards disease improvement, which failed to reach statistical significance. However, in a defined cohort of patients that had inadequately controlled asthma despite inhaled glucocorticoid therapy, anti-IL-13 treatment (lebrikizumab) improved lung function (107). These patients had evidence of IL-13-regulated biological activity as measured by increased circulating levels of periostin. Notably, the higher the serum periostin levels the greater therapeutic benefit (107). Clinical trial findings of an antibody targeting IL-4R α (dupilumab) was also recently reported to improve lung function and reduce exacerbations in patients with uncontrolled persistent asthma (108). Further studies on the IL-13/ IL-4R α axis for treatment are required.

Overall, these clinical studies provide confirmation that Type-2 inflammation underlies the pathological changes of allergic asthma, and they highlight the importance of disease heterogeneity even within this disease subtype. However, no treatment options are available that focus on disease onset, and there are limited options for individuals with non-allergic phenotypes, as well as for the treatment of severe, acute exacerbations of disease.

Understanding factors that initiate and regulate T_H2-induced allergic inflammation

The overlapping functions and integrated circuits regulating T_H2 cytokine responses suggested that targeting individual cytokines may have limited therapeutic efficacy. Indeed, redundancy in cytokine signaling pathways has limited the effectiveness of targeting a single molecule for therapeutic intervention in asthma (e.g. IL-4, IL-5, IL-13 and IgE) (109, 110). Thus, after characterizing the role of the T_H2/eosinophil axis in regulating allergic inflammation, our focus turned to understanding the pathways that initiated and regulated the development of T_H2 responses. The goal was to understand new ways to suppress T_H2 responses, with a view to developing new specific anti-inflammatory approaches.

Role of IL-3/GM-CSF/IL-5 beta common chain

IL-3 and GM-CSF, in association with IL-5, are the only known cytokines capable of inducing eosinophil differentiation from bone marrow progenitors and are central to the survival, migration, and activation of this granulocyte (111–113). The receptors for each of the three factors have separate alpha chains, but share a common beta-receptor chain (β c) (note that unlike human cells, mouse cells also possess an additional β -receptor specific for IL-3 (β IL-3), which allows IL-3 signaling independent of the β c (114)). Activated antigen-specific T_H2 cells are an important source of IL-3, GM-CSF and IL-5 and lung epithelial cells also produce large amounts of GM-CSF. We speculated that signaling redundancy between these cytokines may be the critical pathway that promotes the development and recruitment of eosinophils, independently of IL-5 at sites of allergic inflammation. IL-3 and GM-CSF signaling through the β c is also important in the development and function of other

immune cells that have been implicated in the pathogenesis of asthma (e.g. dendritic cells, macrophages and neutrophils). This observation also raises the possibility that targeting the β_c may have therapeutic potential for the treatment of severe asthma and neutrophilic or paucigranulocytic forms of the disease.

By using our mouse model of allergic airways inflammation in conjunction with mice deficient in β_c/β_{IL-3} chains, we demonstrated that aeroallergen-induced expansion and accumulation of eosinophils in the lung was abolished (115). Moreover, deficiency inhibited hallmark features of asthma, including AHR, mucus hypersecretion, and surprisingly, production of antigen-specific IgE (115). T_H2 cell function was also significantly attenuated, as we had previously observed in the absence of eosinophils. However, the effect on disease pathology was much more marked in the absence of β_c signaling, than was observed in the absence of eosinophils alone. T_H2 cells in the lung of allergen-challenged $\beta_c/\beta_{IL-3}^{-/-}$ mice were limited in their ability to proliferate, produce cytokines (IL-4, IL-5 and IL-13), and migrate to effector sites, which we attributed to reduced numbers of myeloid dendritic cells in the lung compartment (115). Thus, this study demonstrated that the β_c plays a novel role in regulating allergic disease by regulating multiple effector arms (T_H2 cell, mast cell, dendritic cell and eosinophil function) and represents a novel anti-inflammatory target. The therapeutic value of targeting the β_c for the treatment of asthma is currently being explored (116).

Role of IL-25 in allergic inflammation

Epithelial cell production of IL-25 plays an important role promoting T_H2 responses, in part by promoting the development of ILC2s (117, 118). In one of the earlier studies that demonstrated a role for IL-25 in allergic inflammation, we showed that administration of IL-25 to the lungs of naïve mice induced AHR, eosinophilic inflammation, mucus hypersecretion and T_H2 cytokine expression (119). STAT6 plays a critical role in the development and maintenance of T_H2 cells and the Type-2 immune response (120, 121). Further, arginase-I and CCL11 expression were increased following IL-25 exposure (119). Induction of AHR and mucus overproduction required IL-13, IL-4R α and STAT6, while AHR alone required IL-4 and IL-5/CCL11 (119). IL-25 is produced from respiratory epithelial cells in response to antigen challenge and has now been implicated as an early innate cytokine responsible for induction of allergic inflammation (reviewed in (122)). IL-25, similar to IL-33 (123) and thymic stromal lymphopoietin (TSLP) (124) plays an important role in promoting T_H2 responses, in part, by promoting the development of ILC2s, which respond by producing IL-13 and/or IL-5 (reviewed in (125)). ILC2s can induce allergic inflammation, mucous hypersecretion and AHR (126). Interestingly, concurrent studies in our laboratory demonstrated that arginase-I expression in the lung is induced by IL-13 and inhibition of its activity by RNA interference (RNAi) treatment of the airways attenuated IL-13-induced AHR (127). Arginase-I had also been identified as a potential critical regulatory factor of disease in several models of experimental asthma (128).

In further studies, we went on to demonstrate that IL-25 played a central role in the development of respiratory syncytial virus (RSV)-specific T_H2 cells and bystander allergen-specific T_H2 cells (129). This occurred in an immune-compromised environment, where NK

cells and IFN γ levels were deficient, as is also observed in patients with severe infection. Airway epithelial cells were the primary source of IL-25 after RSV infection, and IL-25 expression was inversely correlated with the levels of IFN γ (129). Thus, NK cells, via production of IFN γ , negatively regulate the development of viral-specific T_H2 responses, which has implications for how severe RSV infections may exert deleterious effects in promoting the pathogenesis of T_H2 responses in asthma.

The role of the epithelium in regulating T_H2 type responses in asthma

In a series of experiments with Joerg Mattes and colleagues (The University of Newcastle, Australia), we identified new pathways in airway epithelial cells that play central roles in the development of the T_H2 response. In particular, our work has highlighted a role for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and midline 1 (MID1; also known as tripartite motif-containing protein (TRIM) 18) in the development of Type-2 inflammation and AHR (130–134).

TRAIL is a member of the tumor necrosis factor (TNF) superfamily of cytokines (135, 136), which have essential roles in the regulation of immune and inflammatory responses (reviewed in (137)). We were particularly interested in this molecule, as its expression was increased in the airway epithelium of asthmatics and increased further during allergen-induced late-phase response in asthma, correlating with inflammation and a decline in lung function (138). Further, TRAIL was also expressed in immune cells linked to asthma pathogenesis (e.g. human T_H2 cell clones, eosinophils, neutrophils and dendritic cells) (138–140).

In our first study, we demonstrated that TRAIL is abundantly expressed in the airway epithelium of aeroallergen-challenged mice and is increased in sputum from individuals with asthma (130). TRAIL-deficient (*Tnfsf10*^{−/−}) mice or siRNA-mediated suppression of TRAIL attenuated the development of hallmark features of airways inflammation (e.g. eosinophil recruitment) and AHR, through decreased CCL20 production and thus reduced recruitment of myeloid DCs (mDCs) and memory CCR6⁺CD4⁺ T cells into the lung (130). This resulted in inhibition of T_H2 cytokine release, inflammation and AHR in a STAT6-dependent manner. Further, administration of recombination IL-13 to *Tnfsf10*^{−/−} mice could restore AHR and cardinal features of disease (130). Administration of recombinant TRAIL was also sufficient to induce CCL20 production in human bronchial epithelial cells *in vitro* (130). A critical role for airways epithelial-derived CCL20 in regulating HDM-induced allergic inflammation by regulating DC recruitment was also demonstrated by Nathan *et al.* (141). Thus, we identified TRAIL as an early signal released from respiratory epithelial cells in response to allergen exposure, which plays an essential role in promoting T_H2 responses and induction allergic inflammation. TRAIL represents a novel target up-stream of T_H2 cells for therapeutic intervention.

To identify new signaling pathways activated by allergens and linked to the TRAIL pathway, we characterized the transcriptional programs in airway wall tissue taken from allergen challenged wildtype and *Tnfsf10*^{−/−} mice, and identified factors that were differentially expressed in models of OVA and house dust mite (HDM)-induced allergic airways disease (132). We observed that the microtubule-associated E3 ubiquitin ligase MID1 was

upregulated in wildtype mice sensitized and challenged with HDM, but not in similarly treated *Tnfsf10*^{-/-} mice (132).

Increased expression of MID1 in airway epithelium was also observed after exposure to rhinovirus (a major trigger of exacerbations in asthma) and in cultures of primary human bronchial epithelial cells (132). Our findings indicated that MID1 expression was induced downstream of TLR4 (which we and others had demonstrated earlier as an important receptor for HDM-induced allergic inflammation (142, 143) and TRAIL activation (132)). Inhibition of MID1 activity reduced AHR and airways inflammation, including expression of IL-25, IL-33 and CCL20, and the production of IL-5 and IL-13 (132). Further, inhibition of MID1 could suppress rhinovirus-induced exacerbations of disease (132). MID1 appears to function through suppression of protein phosphatase 2A (PP2A) activity (132). This was an important observation, as MID1, like TRAIL, represents a novel target in airway epithelial cells that promotes the up-regulation of a number of critical factors that promote T_H2 responses.

We have also demonstrated key roles for TRAIL in airway remodeling induced by chronic allergic inflammation. Following 12 weeks of chronic allergen (OVA) exposure, TRAIL-deficient mice had reduced AHR, tissue eosinophils and mast cells, and lower levels of IL-4, IL-5, IL-10 and IL-13 in lung tissue (133). Further, *Tnfsf10*^{-/-} mice had decreased airways remodeling, with reduced peribronchial fibrosis, smooth muscle hypertrophy and mucus hypersecretion (133). MID1 levels were increased by chronic allergen exposure, but suppressed in *Tnfsf10*^{-/-} mice (133). Finally, we demonstrated that increasing PP2A activity reduced eosinophilia and peribronchial fibrosis (133). These studies further highlighted the potential therapeutic value of targeting these novel epithelial cell factors for treatment of the T_H2 arm of asthma.

In collaboration with Phil Hansbro and colleagues (see review in this issue), a role for TRAIL in the pathogenesis of neonatal *Chlamydia* infection was also identified. We characterized neonatal *Chlamydia* infection in TRAIL-deficient mice or following antibody-mediated blockade of TRAIL function (134). TRAIL was required for the induction of inflammation, morphological changes to the airways, mucus hypersecretion and reduced lung function (134). Thus, TRAIL played a role that may be relevant to the development of allergic inflammation and exacerbation by infection.

Role of MicroRNA in the regulation of allergic airways disease

MicroRNAs (miRNAs) are small non-coding RNAs that act as endogenous sequence-specific regulators of gene expression and control many cellular pathways. miRNAs are approximately 22 nucleotides in length and can act by directly blocking the translational machinery or by regulating mRNA stability (144). For a detailed review of miRNA biogenesis and function, we refer the reader to Krol *et al.* (145). Although widespread and important roles of miRNAs in the control of cellular function of eukaryotes (e.g. differentiation, proliferation, metabolism, and apoptosis) is well established (146–148), much less is known about how they regulate the immune system and inflammation linked to disease and infection (149, 150).

In 2009, we published a paper describing, for the first time, the role of miRNA in regulating allergic inflammation (151). At the time, we were interested in miRNA function because regulation of miRNA expression had been linked to TLR signaling (152–154). As mentioned above, we had identified an important role for TLR4 in the initiation of HDM-induced allergic disease (142). This suggested that a potential link may exist between the innate immune system and subsequent activation of inflammatory pathways and adaptive immunity, via miRNA function. Moreover, we wanted to assess the effect of targeting a miRNA linked to allergic inflammation, as a potential novel, broad anti-inflammatory approach. This approach seemed interesting as a single miR can target hundreds of mRNAs and modulate protein output from their respective genes (144). Therefore, a single or specific set of miRNAs may control discrete physiological processes by regulating the production of a few critical proteins that coordinate single or interrelated cellular events (e.g. cell proliferation, or in our case coordinated inflammation) (144, 146).

Role of miR-126

To assess potential functional roles of miRNAs in the regulation of allergic inflammation, we employed our HDM-induced model of AAD. Inhibition of miR-126 function in the lung with an antagomir (a cholesterol-linked single stranded antisense RNA) specifically designed to target miR-126 (ant-miR-126) significantly inhibited HDM-induced AHR, recruitment of eosinophils and neutrophils into the lung and reduced mucus hypersecretion (151). Notably, attenuation of allergic disease correlated with inhibition of the effector function of T_H2 cells, limiting their ability to produce IL-5 and IL-13 (151). Antagomir treatment also decreased the numbers of $CD4^+$ T cells and mDCs ($CD11b^+CD11c^+MHCII^{high}$) infiltrating the lung (151). We observed that ant-miR-126 treatment also increased expression of OBF.1/BOB.1 and PU.1, which are negative transcriptional regulators of GATA-3, a transcription factor which regulates T_H2 cell development and function (151). Thus, this investigation demonstrated that inhibition of miRNA function could attenuate the allergic inflammatory sequelae and that small non-coding molecules may be novel targets for anti-inflammatory therapy. miRNA have now been linked to the pathogenesis of a number of chronic inflammatory diseases of the lung (reviewed in (149, 150)).

The effect of blocking miR-126 function was less dramatic in a mouse model of chronic asthma, where allergic inflammation was induced by low-level exposure of the airways to OVA for 6 weeks (155). Although ant-miR-126 inhibited intraepithelial eosinophil accumulation (a predominant feature of asthmatic airways), there were no effects on the accumulation of other chronic inflammatory cells or on airway wall remodeling (e.g. sub-epithelial fibrosis and goblet cell hyperplasia/metaplasia) (155). The specific role of miRNAs in regulating aspects of AAD may be related to the stage of the disease process and the temporal expression of specific miRNAs. These data suggest that miR-126 is likely to regulate acute inflammatory events and other miRNAs may regulate the chronic aspects of disease. Alternatively, this observation may also be related to the mode of sensitization, as OVA is administered systemically with an adjuvant to induce T_H2 immune responses in the chronic model, without requirement for TLR4 (unlike the HDM model).

Role of miR-145

In a subsequent study, we also assessed the effects of antagomir treatment against miR-145, -21 and -let-7b in a HDM-induced model of AHR (156). Of these interventions, only ant-miR-145 treatment could suppress features of disease (156). Treatment reduced eosinophilic inflammation, mucus hypersecretion, T_H2 cytokine production (IL-5 and IL-13) and AHR (156). Inhibition of all disease features was similar to corticosteroid treatment alone (156). Interestingly, miR-145 has also been linked to the regulation of innate immunity by inhibiting IFN β production (157), and is one of the highly expressed miRs within the trachea and airway wall (158). Smooth muscle development and the differentiation of fibroblasts into smooth muscle cells have also been shown to be regulated by miR-145 (159). Thus, like miR-126, miR-145 is likely to inhibit allergic inflammation by targeting multiple transcripts and pathways.

Other investigators have also shown important roles for miRNAs in the regulation of allergic airways inflammation. For example, Lu *et al.* (160) identified miR-21 as an important regulator of the recruitment and activation of inflammatory cells in the lung, which may also play a role in the development of T_H2 responses by decreasing IL-12 levels.

We have also assessed the role of miRNAs in antimicrobial defense, with relevance to exacerbation of respiratory disease such as asthma and obstructive pulmonary disease (COPD). We demonstrated that miR-328 is reduced in neutrophils and macrophages, upon exposure to non-typeable *Haemophilus influenzae in vitro* (161). Antagomir-mediated inhibition of miR-328 enhanced bacterial killing, both *in vivo* and *in vitro*, without increasing inflammatory cytokine production (161). Importantly, ant-miR-328 treatment also improved bacterial clearance under conditions of corticosteroid-induced immunosuppression and in a model of chronic COPD (161). These findings provide a potential approach for the treatment of bacterial infections and/or exacerbations in respiratory disease such as asthma and COPD.

miRNA expression in inflammatory cell populations relevant to asthma

We have also undertaken a number of studies to determine the role of miRNAs in the differentiation, proliferation and activation of immune cells relevant to respiratory disease. In our first study, we identified 86 miRNAs with altered expression during the *in vitro* differentiation of bone marrow-derived mast cells (162). Target prediction analysis identified subsets of altered miRNAs with potential roles regulating key mast cell surface receptors, transcription factors, signaling molecules and secretory proteins (162). For example, *in silico* analysis predicted 7 specific miRNAs are directly associated with the expression of the mast cell surface receptors c-Kit and the high affinity IgE receptor (Fc ϵ RI α) (162). A further 18 miRNAs promote expression of MITF, GATA-1 and c/EBP α , three core transcription factors that direct mast cell differentiation (162). Furthermore, we identified 11 miRNAs that may regulate the expression of STAT-3, -5a/b, GATA-2 and GATA-3 during differentiation, along with 13 miRNAs that target transcripts encoding Ndst2, mast cell protease 4 (mMCP4) and mMCP6, and thus may regulate biosynthesis of mast cell secretory mediators (162).

In a similar approach, we identified 68 miRNAs that are changed during *in vitro* differentiation of eosinophils (163). Again by employing TargetScan and MeSH databases, we identified 348 transcripts involved in 30 canonical pathways that are potentially regulated by these miRNAs (163). Networks of miRNAs appear to target key eosinophil surface molecules and transcription factors, as well as potential regulation of TLR-4/13 expression (163). For example, 13 specific miRNAs were temporally associated with the expression of IL-5R α and the CCL11 receptor CCR3 and 14 miRNAs were associated with regulation of the transcription factors GATA-1/2, PU.1 and C/EBP ϵ . A further 17 miRNAs may regulate translation of TLR-4/13.

We also assessed miRNA expression profiles during differentiation of bone marrow-derived macrophages (BMDMs). We identified 112 miRNAs with altered expression (164). Employing network analysis databases, we identified 1267 transcripts involved in 30 canonical pathways linked to macrophage biology as potentially regulated by these specific 112 miRNAs (164). 18 miRNAs were temporally linked to the expression of CSF1R, CD36, MSR1 and SCARB1; 7 miRNAs linked to the regulation of the macrophage transcription factors RUNX1 and PU.1, and 14 miRNAs predicted to target the nuclear receptors PPAR α and PPAR γ (164). These are all factors linked to the development and activation of macrophages. Notably, miR-328 was highly upregulated by day 7 of differentiation and we have demonstrated its importance in regulating the pro-inflammatory status of macrophages (as discussed above (161)).

These are some of the first investigations to map changes in miRNA expression in whole bone marrow cultures during the differentiation of mast cells, eosinophils, and macrophages. They provide evidence of potential functional links between miRNA expression and their target mRNAs for the regulation of differentiation, cellular identity and function. Identification of the specific miRNA subsets employed in these processes offers potential avenues to attenuate the expansion and function of these immune cell populations at sites of inflammatory disease.

As an example, we extended these studies and have started to examine the functional relevance of the identified miRNAs in the regulation of macrophage differentiation and/or function (165). In this regard, we identified key roles for miR-487b as a negative regulator of macrophage activation (165). MiR-487b was increased during macrophage differentiation and we demonstrated that it directly binds to IL-33 mRNA, inhibiting IL-33 production in macrophages (165). IL-33 is known to play an important role in macrophage activation for innate host defense and proinflammatory responses. For example, IL-33 regulates macrophage foam cell formation, promotes the polarization of alternatively activated macrophages, and can enhance macrophage-regulated antibacterial immunity (166–168). In this study, exposure of macrophages to IL-33 increased MHC I/II, CD80/86 and iNOS expression and inhibition of miR-487b function promoted these responses (165). Stimulation with LPS to activate macrophage proinflammatory pathways suppressed miR-487b expression and increased expression of IL-33 and pro-inflammatory mediators (e.g. iNOS, IL-1 β , IL-6, and TNF α) (165). Ant-miR-487b administration also increased IL-33 expression and enhanced LPS-mediated induction of inflammatory gene expression (165). Collectively, our results indicate that miR-487b plays a negative regulatory role in

macrophages, by controlling the levels of IL-33 transcript and protein to fine-tune innate immune host defense and proinflammatory responses of these cells.

Modeling Chronic Asthma

A major focus in a long-term collaboration with Rakesh Kumar (University of New South Wales, Australia) was aimed at understanding the development of inflammation and lesions (remodeling) in chronic asthma by employing a chronic antigen exposure model. Here we highlight some of our investigations, which identify the complexity of the mechanisms leading to chronic disease, steroid-insensitivity and disease exacerbation. Interestingly, when we examined potential therapeutic interventions in this model, we often demonstrated dissociation between inflammation, tissue remodeling and AHR, and identified contributions of non- T_H2 -type responses to pathogenesis.

By contrast to our acute exposure models, in chronic models mice were sensitized and chronically challenged (6–8 weeks) with low concentrations of aerosolized OVA (169). Repeated OVA exposures caused low-grade airway specific inflammation, with intraepithelial eosinophils, lymphoid and mononuclear cells within the lamina propria, goblet cell hyperplasia, features of tissue remodeling (epithelial thickening and subepithelial fibrosis) and increased levels of IgE (169). Further, chronic OVA exposure induced AHR (169). In later studies (using Flexivent technology), we were able to demonstrate that AHR induced in response to methacholine exposure in this model was also airways-specific (as in asthma) and not derived from other lung compartments. Further, in contrast to acute models and other published models of chronic disease, there was no accumulation of immune cells in the alveolar spaces. This parallels observations in clinical disease, where pulmonary parenchymal inflammation does not typically occur. Thus, this model was the first to spatially reproduce many features of mild chronic asthma, including inflammatory cell localization and site-specific development of both tissue lesions and AHR.

This model of chronic asthma was subsequently used to define many novel aspects of pathogenesis. For example, we investigated the contribution of T_H2 cytokine-driven inflammation to the development of airway lesions and AHR by employing cytokine-deficient mice, and assessed the effects of chronic (6-week) exposure of OVA on chronic inflammation and epithelial changes (170). In $IL5^{-/-}$ mice, eosinophil numbers were decreased, but epithelial hypertrophy and subepithelial fibrosis were unaltered (170). However, $IL5^{-/-}$ mice were protected from the development of AHR (170). In contrast, $IL4^{-/-}$ mice had significantly more epithelial hypertrophy and subepithelial fibrosis and increased AHR, while airway inflammation was unaltered (170). Thus, we concluded that IL-5, but not IL-4, plays a central role in the development of chronic inflammation of the airways and the induction of AHR. Notably, however, chronic epithelial and fibrotic changes occurred independently of IL-5 and are not required for the development of AHR. Thus, airway wall remodeling is not simply a consequence of inflammation, nor does it directly correlate with AHR. The dissociation between airway wall remodeling and AHR has important implications for therapeutic approaches to asthma.

We also highlighted a key role for CD4⁺ T cells in the chronic challenge model (171). Depletion of CD4⁺ T cells decreased AHR and reduced features of airway remodeling (including subepithelial fibrosis, epithelial thickening and mucus cell hyperplasia) (171). Chronic inflammation was also reduced, with reduced accumulation of Ig-producing plasma cells (171). However, eosinophil accumulation and CCL11 expression were unaffected (171). These findings provided evidence for an important role of CD4⁺ T cells in regulating airway wall remodeling and AHR. Furthermore, they demonstrated that eosinophil accumulation could occur independently of CD4⁺ T cells, which are the major source of IL-5 (as demonstrated in acute models). Notably, in our chronic model we demonstrated that CCL11 expression is increased in epithelial cells and plasma cells (172) and that eosinophil recruitment was also almost completely ablated in *Il13*^{-/-} mice, despite maintenance of CCL11 expression (172). Thus, as with investigations with acute models, we show that tissue accumulation of eosinophils can occur by multiple pathways. Interestingly, Chu *et al.* have demonstrated plasma cells and eosinophils co-localize together in the bone marrow (173). Eosinophils were shown to provide key survival factors for plasma cells (173). In the absence of eosinophils, the numbers of plasma cells were much lower in the bone marrow both at steady-state and after immunization (173). Based on our studies in the lung, we speculate that eotaxin produced by plasma cells may play a key role in this spatial arrangement.

We extended these studies on the role of T_H2 cytokines and factors in our chronic model, to demonstrate a key role for IL-13 in eosinophil accumulation and airway remodeling (174). *Il13*^{-/-} mice exhibited decreased eosinophilia and inflammatory cell infiltrate into the lungs and reduced airways remodeling, compared to WT mice, but AHR was still induced (174). *Il-4Ra*^{-/-} mice had normal inflammatory responses and AHR, although some features of airway remodeling (epithelial hypertrophy and mucus cell hyperplasia) were reduced (174). We also assessed the role of STAT6 in our chronic asthma model. *Stat6*^{-/-} mice developed airway lesions, similar to wildtype mice, and exaggerated AHR (175). This is in marked contrast to observations made in acute models, where STAT6 is a critical factor for expression of disease. Further, in the absence of STAT6, we observed a shift in Ig production from a pattern typical of T_H2 disease, to a T_H1 pattern, as well as increased IFN γ expression (175). By contrast, *Il4*^{-/-}*Il13*^{-/-} mice had reduced eosinophil accumulation, airway remodeling and AHR (175). These studies highlighted the complexity of the mechanisms regulating disease with potential overlapping and redundant pathways. They also suggested that non-T_H2 type responses may also drive disease processes, particularly in the context of chronic disease.

In further investigations, we examined the therapeutic potential of phosphodiesterase inhibitors for the treatment of chronic asthma. We compared the effects of roflumilast (PDE4 inhibitor) or pentoxifylline (non-selective phosphodiesterase inhibitor) with steroid treatment (dexamethasone). Both roflumilast and dexamethasone inhibited eosinophil accumulation, inflammation, subepithelial collagen deposition and epithelial thickening and had minor effects on the level of AHR (176). Dexamethasone also reduced goblet cell hyperplasia, TGF β 1 levels and activation of NF κ B in the epithelium (176). By contrast, pentoxifylline only inhibited eosinophil accumulation and epithelial thickening, but also significantly reduced AHR (176). The differences observed between pentoxifylline and

roflumilast treatment might be attributed to the more effective inhibition of NF κ B signaling by nonspecific PDE inhibitors (pentoxifylline), than by PDE4-specific agents (roflumilast).

Collectively the above observations led us to look into the interplay between T_H2 (e.g. IL-13, IL-5) and T_H1-associated factors (IFN γ) and other inflammatory molecules (e.g. TGF β 1 and amphiregulin) in tissue remodeling. For example, we assessed the role of clinically-relevant growth factors derived from airways epithelial cells in regulating remodeling in our chronic model (177). At the time, the relationship between growth factor production by the airway epithelium and the subsequent development of remodeling remained poorly understood. We demonstrated that allergic sensitization increased the biologically active form of TGF β 1 and amphiregulin (177). Further, regulation of epithelial-derived TGF β 1 was linked to IL-13 production, as TGF β 1 expression was absent in *Il13*^{-/-} mice (177). These studies demonstrated an important role for airway epithelial cell-derived TGF β 1 and potentially other growth factors in the development of airway wall remodeling in chronic asthma.

Further, we assessed the impacts of neutralizing antibody treatment against IL-13, IL-5 and IFN γ in our chronic model. Anti-IL-5 treatment dampened inflammation and remodeling, but had no effect on AHR (178). Anti-IL-13 also suppressed eosinophil accumulation and partially suppressed airway remodeling, but had limited effects on AHR (178). Anti-IFN γ treatment suppressed AHR and the accumulation of inflammatory cells, but had no effect on eosinophils or tissue remodeling (178). This investigation highlighted that co-operative interaction between T_H2 and T_H1 type cytokines contributes to the pathogenesis of the lesions observed in chronic asthma. The observation that IFN γ could regulate AHR also helped shape our thinking about the development of AHR during pathogen-induced exacerbations (discussed below). In later investigations, by employing laser-capture microdissection techniques we demonstrated that the levels of T_H2 cytokines and IFN γ were significantly elevated in cells found within the airway wall (179).

Identification of steroid-resistant inflammatory pathways and their role in exacerbation of allergic inflammation

Exacerbations of asthma predispose to poor quality of life, are difficult to control with steroid and bronchodilator therapy and lead to further declines in lung function (see background above). One of our more recent research interests (see reviews (14, 180, 181)) has been in modeling the cellular and molecular mechanisms that lead to steroid-resistant inflammatory pathways (also see miRNA section below), and mechanisms linking respiratory infections to steroid-resistance and exacerbations of asthma. To unravel the complexity of asthmatic inflammation, we took a reductionist approach in a series of experiments designed to explore the mechanisms linking respiratory infection with steroid-resistant inflammation and AHR.

Role of IFN γ , LPS and IL-27 in regulating steroid-resistant inflammation

Increased IFN γ and LPS levels have been linked to the severity of asthma and both molecules are key activating signals of host defense pathways (182–184). During infection, a

primary mechanism regulating neutrophil recruitment into the airways and macrophage activation occurs via activation of TLR4 by LPS and subsequent activation of the MyD88 signaling pathway (182).

In our initial study, we demonstrated that the transfer of OVA-specific polarized T_H1 cells and subsequent challenge with both OVA and LPS were sufficient to induce neutrophil accumulation, macrophage activation and AHR, which was resistant to corticosteroid treatment (185). Development of AHR was dependent on IFN γ , inhibited by depletion of pulmonary macrophages (but not neutrophils) and abrogated in TLR4- or MyD88-deficient mice (185). The delivery of IFN γ and LPS (together but not individually) to the lungs also induced steroid-resistant inflammation and AHR (185). The steroid-resistant pathway operated independently of STAT6 (185). In contrast, transfer of OVA-specific polarized T_H2 cells induced eosinophilia and AHR, which could be suppressed by steroid treatment (185). This study demonstrated that cooperative signaling between IFN γ and TLR4/MyD88 constitutes a previously unrecognized pathway that regulates macrophage-dependent steroid-resistant AHR. This pathway may have an important role in the pathogenesis of airflow obstruction and AHR in asthma that is insensitive or unresponsive to steroid therapy.

To further investigate this novel interaction between IFN γ and LPS, and the mechanism regulating steroid-resistance, we performed gene arrays on isolated airway samples after intratracheal administration of IFN γ and/or LPS. We discovered that IL-27 was the most abundantly up-regulated transcript that was differentially regulated by combined administration of IFN γ and LPS (186). Next we demonstrated that exposure of pulmonary macrophages to IFN γ and LPS also induced IL-27 production. Importantly, integrated signaling by IL-27/IFN γ (but not LPS and IL-27) induced macrophage-dependent steroid-resistant AHR (186). IL-27/IFN γ administration also blocked glucocorticoid-induced translocation of the glucocorticoid receptor into the nucleus, in isolated macrophages (186). Furthermore, expression of both IL-27 and IFN γ was increased in induced sputum samples from steroid-refractory asthma patients (186). Collectively, our observations suggest that a novel IL-27- and IFN γ -driven pathway may be important in the regulation of steroid-resistant inflammation and AHR, and that manipulation of this pathway may be of therapeutic benefit.

Next we extended these observations into a mouse model of allergen-induced steroid-resistant AHR. In this study, we demonstrated that prolonged AHR was dependent on the presence of IFN γ and macrophages (187). The source of the IFN γ required for the development of steroid-resistant AHR appears to be resident pulmonary cells (187), possibly including activated natural killer (NK) cells. Significantly, prolonged AHR was independent of eosinophil-dominated pulmonary inflammation and the production of T_H2 cytokines, such as IL-5 and IL-13 (187). These findings further emphasized the contribution of innate host defense pathways to the manifestations of asthma.

Mechanisms of exacerbation induced by viral infections

Viral infections, the most common being caused by rhinovirus (RV) (188) and respiratory syncytial virus (RSV) (189, 190), are major triggers of asthma exacerbations (reviewed in (191–193)). In very recent studies, we have developed a model of RSV-induced steroid-

resistant exacerbation. RSV infection on the background of established AAD (OVA-sensitized/challenged BALB/c mice) resulted in significantly increased macrophage and neutrophil lung infiltration and AHR (194). Exacerbation was accompanied by increased levels of inflammatory cytokines in the lung, including TNF α , MCP-1/CCL2, and keratinocyte-derived protein chemokine [KC]/CXCL1 (194). Dexamethasone treatment of RSV-induced exacerbation only partially suppressed AHR and failed to inhibit macrophage and neutrophil infiltration or inflammatory cytokine production (194). Interestingly, we also observed increased TNF α levels in sputum samples from patients with neutrophilic asthma (194), a population that is more likely to exhibit steroid-resistant disease. Although RSV-induced exacerbation was resistant to steroid treatment, inhibition of either TNF α or MCP-1/CCL2 function or depletion of macrophages suppressed features of disease, including AHR and monocyte and neutrophil infiltration (194). High levels of TNF α have been directly associated with steroid-resistance, severe asthma, neutrophilic infiltration and viral-induced exacerbations (195–197). The therapeutic benefit however, would have to be judged against increased risk of infection, similar to that observed in anti-TNF α studies in humans (198, 199).

In this model, levels of IFN γ and IL-27 were also increased and again expression of these factors was resistant to steroid therapy (*Nguyen et al. J.I. manuscript in press*). Neutralization of either IFN γ or IL-27 could significantly suppress RSV-induced steroid-resistant airway inflammation and AHR. Further, stimulation of pulmonary macrophages with TNF α and/or MCP-1/CCL2 induced expression of both IFN γ and IL-27. These findings suggested critical roles for IFN γ and IL-27, downstream of TNF α and MCP-1/CCL2, in the mechanism of RSV-induced exacerbation. Collectively, these findings highlight critical roles for macrophages and inflammatory cytokines (including TNF α , MCP-1/CCL2, IL-27 and IFN γ) in viral-induced exacerbations of asthma and suggest examination of the pathways they regulate may provide insight into therapeutic approaches for disease management.

Role of miRNAs and transcriptional regulation in steroid-resistance

We have also explored a role for miRNAs in regulating pathways that mediate steroid-resistant inflammation and AHR. miR-9 levels were synergistically increased following IFN γ /LPS co-exposure in isolated macrophages and *in vivo*, and were also increased in sputum samples from neutrophilic asthmatics suggesting a role in the mechanism of steroid-resistance (200). Further, miR-9 production decreased the levels of target transcripts, protein phosphatase 2 regulatory subunit B(B56) d isoform (PPP2R5D), reducing PP2A activity and also inhibited steroid-induced glucocorticoid receptor (GR) nuclear translocation (200). Antagomir-mediated inhibition of miR-9 increased both PP2A activity and GR nuclear translocation in macrophages, and restored steroid sensitivity in isolated macrophages and in several mouse models of steroid-resistant AHR (200). Further studies with Hansbro and colleagues have also demonstrated a key role for miR-21 in a number of infection-induced steroid-resistant AHR models (201). Steroid-resistant disease was induced by infection with *Chlamydia*, *Haemophilus influenzae*, influenza virus or RSV, on the background of OVA-induced AAD (201). In each situation, miR-21 was increased and ant-miR-21 treatment could suppress features of disease (201).

Collectively the above studies highlight the importance of miRNA for the regulation of inflammation and immune responses to infection, relevant to asthma pathogenesis. Targeting these molecules in asthma is yet to be performed. However, differential expression has been observed in patients with different asthma phenotypes (202). Thus, analyzing miRNA expression may aid in developing new therapeutic and diagnostic approaches.

We have also targeted transcriptional regulation (by contrast to translational processes) by inhibiting bromodomain and extraterminal motif (BET) proteins during exacerbation (203). Histone acetylation has been shown to regulate inflammation, and may operate in various inflammatory diseases of the lung (204, 205). Histone acetylation is recognized by BET protein family members, including bromodomains (BRD)-2, BRD3, and BRD4 (206, 207). Treatment with I-BET-762 inhibitor was sufficient to broadly inhibit inflammation and AHR in both our reductionist (IFN γ /LPS administration) and RSV infection-induced exacerbation models (203).

Modeling allergen-induced exacerbation on the background of chronic disease

To gain insights into the mechanisms regulating exacerbations, we also developed an acute allergen-induced exacerbation model (208). We challenged mice with pre-existing chronic lesions of asthma with a single, moderate level, dose of OVA. Following allergen challenge, airway inflammation resembling an acute asthma exacerbation was rapidly induced and AHR developed (208). Notably, AHR was not dependent on eosinophils and it appeared to be related to distal inflammatory lesions (208). During acute exacerbation of chronic asthma, we also observed steroid-resistant neutrophilic inflammation (209). Dexamethasone suppressed inflammation associated with eosinophil and T-lymphocyte recruitment, but did not prevent neutrophil accumulation or development of AHR (209). Decreased HDAC2 activity and increased NF κ B activity observed after allergen challenge were also not altered by steroid treatment (209). Further, levels of TNF α , GM-CSF, IL-8 and p21waf were increased and not suppressed by steroids (209). Levels of lipid peroxidation and protein nitration products were also elevated in the acute exacerbation model (209). We concluded that impaired nuclear recruitment of HDAC2 could be an important mechanism of steroid-resistance of neutrophilic inflammation in exacerbations of asthma. Oxidative stress may contribute to decreased HDAC2 activity. In subsequent studies, we were able to demonstrate that during exacerbations of chronic asthma, alveolar macrophage (AM) and CD4⁺ T cell production of proinflammatory molecules (IL-1 β , IL-6, CXCL-1, and TNF α) and Type-2 cytokines (IL-4, IL-5 and IL-13) were enhanced, respectively (210). Moreover, we provided evidence that enhanced cytokine expression by AMs may play a critical role in triggering increased expression of cytokines by pulmonary CD4⁺ T cells.

Conclusion and Future Directions

In conclusion, we have demonstrated that inflammation driven by T_H2 cells and the cytokines they secrete play critical roles in regulating hallmark clinical features of asthma in experimental models (Figure 1). These include eosinophilia and recruitment of eosinophils into tissue, mucous hypersecretion and AHR. Orchestration of the inflammatory response by cytokines is complex, with individual cytokines (e.g. IL-4, IL-15 and IL-13) regulating

parallel and interlinked pathways. Signaling is integrated by crosstalk (bidirectional signaling) between T_H2 cells and eosinophils, which promotes allergic inflammation and AHR. Cooperative signaling between IL-5 and CCL11 underpin the elemental signals that regulate eosinophilia and recruitment of eosinophils into tissues. Signals from antigen-activated T_H2 cells also play key roles in the regulation of eosinophil activation and degranulation. One area of future research will be the relationship between T_H2 cells and ILC2s in the regulation of Type-2 inflammation, particularly in animal models (211) and samples from individuals undergoing disease exacerbation. Under these conditions, it is clear that Type-2 responses contribute to disease pathology, but the relative contribution of T_H2 cells and ILC2s in the exacerbation of established disease remains unclear.

Molecules derived from the airway epithelium, such as TRAIL and MID1, play critical roles in the development of T_H2 responses upstream of innate Type-2 cytokine production (e.g. IL-25 and IL-33) and STAT6-dependent pathways, and may be novel therapeutic targets for the suppression of inflammation. Furthermore, our studies suggest that microRNA and the pathways they regulate should also be explored as potential new anti-inflammatory approaches or as diagnostic markers of asthma phenotype.

Clinical trials targeting Type-2 factors (including IL-5, IL-13 and IgE) in specific patient groups have shown potential to improve disease outcomes and patient management. However, they are not effective in all patient groups. Although the T_H2 paradigm polarized experimentation in the field, these investigations also provided the realization that asthma is a complex, heterogeneous inflammatory disorder, where multiple inflammatory pathways may operate. These pathways may operate independently but also in parallel and be activated by various triggers (e.g. during respiratory infection, exacerbation and exposure to allergen). Further clinical characterization of non-allergic patient populations is now critical to improve our understanding of the mechanisms underlying pathogenesis in this patient subset. This in-depth knowledge is necessary to facilitate the development of novel animal models with relevance for this population, which in turn will allow for identification of new therapeutic approaches.

We have also demonstrated that innate immune responses play critical roles in the generation of steroid-resistant inflammation and AHR, particularly in the context of allergen- and pathogen-induced exacerbations (Figure 2). Macrophages play a central role in this exacerbation mechanism, but further research is needed in order to dissect out the fundamental mechanisms involved. Understanding the diversity and spatiotemporal role of inflammation and its interaction with resident airway cells will be key to further advancing knowledge on pathogenesis and for the development of new therapeutic approaches for the treatment of asthma.

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Figure 1. Type-2 Airway Inflammation and the Regulation of Eosinophil Recruitment

Following allergen re-exposure, T_H2 cells expand (supported by IL-4) and play a pivotal role in directing the local allergic inflammatory response, producing IL-5 and IL-13. IL-5 promotes proliferation and differentiation of eosinophils, while also enhancing CCL11/eotaxin-mediated eosinophil recruitment. Bronchial epithelial cells produce cytokines, including IL-25, IL-33, TRAIL, CCL11 and GM-CSF, at differing stages of disease. Interestingly, these factors can also activate ILC2s, although the relative importance of T_H2 cells versus ILC2s remains unclear. TRAIL production by epithelial cells, via MID1, has a key role in the promotion of Type-2 inflammation. These pathways can be modulated in part by miRNA expression (e.g. miR-126). A number of interventions (indicated in blue) have broad anti-inflammatory effects on Type-2 inflammation. Disease outcomes are indicated in red. *Abbreviations: βc = beta common receptor chain, Ant-126 = antagomir against miR-126, Eo(P) = eosinophil (progenitor), ILC2 = innate lymphoid cell type-2, GM-CSF = granulocyte/macrophage colony stimulating factor, IL = interleukin, T_H2 = CD4⁺ T helper Type-2 cell.*

Figure 2. Innate Immune Activation, Type-1 Airway Inflammation and Steroid-Resistant Disease Exacerbation

Exposure of airway epithelium and lung-resident alveolar macrophages to pathogens results in innate immune activation. This includes local pro-inflammatory cytokine production (e.g. MCP-1/CCL2, TNFα), which promote the recruitment and activation of tissue macrophages and induce steroid-resistant inflammation and AHR. Production of chemokines also recruits neutrophils into lung tissue and the airways. Further, differentiation of T_H1 cells also results in skewing to Type-1 inflammation. A number of interventions (indicated in blue) have broad anti-inflammatory effects on Type-1 inflammation. Disease outcomes are indicated in red. *Abbreviations: AM = alveolar macrophage, Ant-9/328 = antagomir against miR-9/328, I-BET = inhibitor of bromodomain and extra-terminal motif family proteins, iNOS = inducible nitric oxide synthase, KC = keratinocyte chemoattractant, MΦ = tissue macrophage, Mo = monocyte, Neu = neutrophil, TLR = Toll-like receptor*