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An Update on Lymphocyte Subtypes in Asthma and Airway Disease

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Inflammation is a hallmark of many airway diseases. Improved understanding of the cellular and molecular mechanisms of airway disease will facilitate the transition in our understanding from phenotypes to endotypes, thereby improving our ability to target treatments based on pathophysiologic characteristics. For example, allergic asthma has long been considered to be driven by an allergen-specific T helper 2 response. However, clinical and mechanistic studies have begun to shed light on the role of other cell subsets in the pathogenesis and regulation of lung inflammation. In this review, we discuss the importance of different lymphocyte subsets to asthma and other airway diseases, while highlighting the growing evidence that asthma is a syndrome that incorporates many immune phenotypes. CHEST 2017; 151(5):1122-1130

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The Global Asthma Network estimates that approximately 334 million individuals around the world have asthma.[1] Early definitions of asthma referred exclusively to shortness of breath. Today, asthma is recognized as a disease of reversible airflow obstruction, accompanied by chronic airway inflammation, airway hyperresponsiveness, and airway remodeling. Classically, allergic asthma, constituting approximately 50% of all asthma cases, has been viewed as a disease resulting from an unwarranted type 2 inflammation that arises after exposure to common environmental allergens.[1] However, rapid advancements in murine modeling and disease phenotyping have demonstrated roles for other T-cell subsets in asthma that can in some cases synergize with T helper (Th)2 responses. These advances have also revealed a broadened role for Th2 T cells in asthma pathogenesis than was first thought. Here, we will review new functions of Th2 T cells and the role of new T-cell subsets in the context of a Th2 disease.

Th2 T-Cell-Mediated Immunity in Asthma

The identification of IL-2, IL-4, and IL-5 enabled the segregation of T cells producing these cytokines into Th1 or Th2 cells.[2] The ability of IL-4 and IL-5 to facilitate B-cell class switching and eosinophil recruitment established a plausible link between T cells expressing these cytokines and allergic

ABBREVIATIONS: CD = cluster of differentiation; CXCR5 = CXC chemokine receptor 5; *EBI3* = Epstein-Barr virus-induced gene 3; FoxP3 = forkhead box P3; IFN = interferon; ILC2 = type 2 innate lymphoid cell; iTr35 = IL-35-generated regulatory T cell; OVA = ovalbumin; Tfh = follicular B-helper T cell; TGF = transforming growth factor; Th = T helper; Treg = regulatory T cell

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disease. Many studies have implicated enhanced levels of T cells producing type 2 cytokines (IL-4, IL-5, and IL-13) in asthma; these cells are known to be enriched in the BAL and lung tissue of people with atopic asthma.[3,4] Moreover, strategies attempting to deplete Th2 T cells through the reduction of GATA3 expression, a required Th2 transcription factor,[5] through the use of a DNAzyme that targets and cleaves GATA3 messenger RNA, has been demonstrated to ameliorate airway inflammation in murine models of allergic airway disease,[6] and, in a randomized, double-blind, placebo-controlled trial, to attenuate allergen-induced early and late-phase asthmatic responses in subjects with allergies.[7] These, and many other studies, established the belief that asthma was primarily the result of a Th2-mediated immune response. Further confirming this belief was the establishment of numerous murine models wherein the selective ablation or blockade of one or more of these cytokines led to complete resolution of disease.[8-11]

Since these findings, numerous antibodies have been developed, with the goal of treating allergic disease by selectively depleting IL-4, IL-5, and IL-13. Some notable successes have been made with anti-Th2 cytokine antibodies,[12,13] supporting the importance of Th2 cytokines in allergic asthma. Importantly, success of these trials has been dependent on identifying a target patient population, through disease phenotyping, that is amenable to therapy; specifically, this entails identifying subjects with asthma with a predominantly Th2 asthmatic phenotype, through the use of high eosinophils or periostin as enrollment or patient stratification criteria.[14] In trials with less stringent patient stratification, anticytokine strategies have led to little improvement in disease. Although it is plausible that there have been confounding factors associated with potentiation of cytokine activity by treatment with anticytokine antibodies,[15] it is also possible that these strategies were less efficacious than predicted because of the heterogeneity of clinical asthma. Careful clinical phenotyping of patient populations, by cluster analysis, has led to the establishment of numerous asthma endotypes, or known subtypes of disease, with distinct pathologic mechanisms.[16,17] These studies, in the context of failed anti-Th2 cytokine strategies have firmly established that asthma can no longer be considered a disease constrained to exaggerated Th2 responses, but rather, a heterogeneous syndrome that culminates in airflow obstruction through a variety of mechanisms.

Th2 T Cells: Old Dog(ma), New Tricks

Classically, the Th2 component of asthma was thought to be exclusively due to effector cytokines produced by Th2 cells. However, the discovery of type 2 innate lymphoid cells (ILC2s) in 2010[18] has challenged this framework. ILC2s lack lineage surface markers and an antigen-specific receptor but act similarly to Th2 T cells in that they facilitate the rapid propagation of type 2 immune responses through secretion of IL-5 and IL-13 following stimulation with IL-33, IL-25, and IL-2, or thymic stromal lymphopoietin.[18,19] ILC2s are critical effectors of the Th2 immune response to allergens,[20] proteases,[19] and helminth infection,[21] while also mediating tissue repair following virus infection.[22] ILC2s were first identified in the mesenteric lymph node,[23] but they have since been detected in the lungs of mice and humans and in human nasal polyps.[24,25] ILC2s also have been implicated in asthma.[26,27] Although the development of ILC2s is beyond the scope of this review, it is intriguing that they share many commonalities with T cells, evolving from the common lymphoid progenitor and sharing several transcription factors with Th2 T cells, including inhibitor of DNA binding 2, Bcl11b,[28] and GATA3.[29]

The activity of ILC2s in allergic asthma has driven the development of numerous therapeutic strategies. Neutralization of IL-25 and IL-33 in murine models of allergic airway disease have protected mice from airway inflammation and alleviated airway hyperresponsiveness, similarly demonstrating the importance of ILC2s activating cytokines.[30,31] In 2013, lipoxin A4, a suppressive lipid mediator, was found to inhibit ILC2 function in vitro.[32] Finally, a study examining the blockade of thymic stromal lymphopoietin, an ILC2-activating cytokine, in people with mild allergic asthma provided encouraging results, suggesting that targeting ILC2s will be an important feature of evolving antiallergy therapies,[33] while also serving to highlight the importance of these cells to the disease.

Interaction with ILC2s represents a new function for Th2 T cells, as evidence of cross talk between these cell types has rapidly been accumulating. A number of studies have shown that ILC2s can play a role in T-cell sensitization and disease propagation. In mice, ILC2s facilitated T-cell sensitization to papain, in an IL-13-dependent fashion, by activating dendritic cells within the lung and stimulating their homing to the lung-draining lymph nodes.[34] ILC2 facilitation of T-cell

responses has also been confirmed in vitro and in a model of *Nippostrongylus brasiliensis* expulsion, wherein antigen uptake and presentation by major histocompatibility complex class II molecules by ILC2s were critical to the development of robust type 2 responses.[35,36] These studies also demonstrated that T-cell-derived IL-2 drives the activation and proliferation of ILC2s[36] and that ILC2s can also potentiate Th2 T-cell responses.[37] The clinical relevance of T-cell/ILC2 cross talk will require extensive study. Of particular importance will be identifying what factors lead to the termination of this T-cell/ILC2 positive feedback loop. Intriguingly, evidence from a trial of subcutaneous grass pollen immunotherapy, a treatment known to induce regulatory T cells (Tregs), has demonstrated regulation of ILC2 recruitment,[38] suggesting that T cells may be capable of regulating ILC2 function. Whether this is through the function of immunosuppressive cytokines or consumption of IL-2 remains to be determined.

Lymphocytes (T cells and ILC2s) have been recognized as contributors to the maintenance and restoration of tissue homeostasis following exposure to epithelial-derived cytokines. IL-33, in addition to its noted inflammatory capacity, induces the differentiation and maintenance of Tregs at epithelial surfaces[39] and within muscle.[40] These Tregs, in addition to dampening immune responses, have been found to produce amphiregulin by some[41,42] but not by others.[43] Amphiregulin is an epidermal growth factor that acts to restore epithelial integrity following acute injury by inducing the proliferation and differentiation of epithelial cells and keratinocytes.[44-46] ILC2s also produce amphiregulin following activation, which can mediate epithelial integrity in the absence of T cells.[47] Finally, IL-33 can also induce the recruitment and differentiation of alternatively activated macrophages, an innate cell that can contribute to the resolution of inflammation and restoration of tissue homeostasis.[48,49] Thus, as our focus moves beyond inflammation, evidence indicating the importance of lymphocytes interacting with the epithelium and innate immune cells continues to mount.

T-Cell Plasticity and Asthma Heterogeneity

As mentioned, allergic asthma has long been thought to be primarily driven by Th2 responses. However, evidence indicates that Th1 and Th2 responses may coexist in asthma. A study examining the Th1 and Th2 cytokine-producing capabilities of peripheral blood mononuclear cells and BAL cells from subjects with asthma and control subjects who had atopic or nonatopic conditions revealed that T cells obtained from subjects with asthma produced significantly more interferon (IFN)-γ on stimulation, with no differences in IL-4 production noted.[50] This observation led to the suggestion that there may be a superimposition of Th1 responses onto allergen-specific Th2 responses in asthma. Similarly, in a study of 34 subjects with steroid-resistant asthma, 14 subjects were nearly devoid of eosinophils, but the remaining subjects had expectedly high levels of eosinophils and mast cells.[51] In this report, it was suggested that perhaps the asthmatic disease in the patients without eosinophils had begun as typical Th2-driven eosinophilic disease and progressed to the observed eosinophil-deficient phenotype. In accordance with these findings, the transfer of Th1-polarized T cells into mice with preexisting Th2 allergic lung disease did not inhibit disease but rather enhanced airway dysfunction.[52]

In a study comparing mild to moderate asthma against severe asthma, analysis of BAL fluid revealed that subjects with severe asthma possessed fourfold more IFN-γ-positive T cells, significantly enhanced IFN-γ levels in the BAL, and enhanced secretion of IFN-γ by ex vivo cultured T cells.[53] Intriguingly, this study identified low levels of secretory leukocyte protease inhibitor, a serine proteinase inhibitor involved in wound healing,[54] produced by airway epithelial cells, in addition to high levels of IFN-γ, to be a marker of severe asthma in both humans and mice.

The dose of the allergen may affect the asthmatic phenotype. A murine model of allergy to fungi has supported this claim, as repeated low-dose exposure of mice to fungi yielded eosinophilic lung inflammation, but high-dose challenge led to neutrophilic and Th1-dominated lung inflammation.[55] Studies such as these have led to the hypothesis that asthma is a fluid immune disorder wherein Th2- and Th1/17-mediated mechanisms represent polar extremes of disease, with patient phenotypes developing and fluctuating between these poles as a result of environmental pollutants and allergen exposure doses.[56]

Th17

IL-17-producing cells are capable of inducing marked inflammation, predominantly neutrophilic, and mediating the progression of both autoimmune disease and allergic asthma.[57] The possibility of a blended

Th2/Th17 cell also has arisen. In 2010, cluster of differentiation (CD)4^+ Th2 T cells that produced IL-17 were found in the peripheral blood mononuclear cells of people with allergic asthma.[58] Elsewhere, a study of 52 subjects with asthma and 25 control subjects found that the number of dual positive Th2/Th17 cells in the BAL correlated with reduced airway function and increased blood eosinophilia.[59] These, and murine, studies suggest Th2 cells may acquire a Th2/Th17 phenotype. Exposure of people with asthma to environmental pollutants that enhance IL-6 may facilitate differentiation from Th2 to Th2/17.[60] The ability of Th2 T cells to acquire Th17-like function and synergize with Th1 cells in the lungs of people with asthma demonstrates how Th2 T-cell plasticity could facilitate diverging disease mechanisms.

Th9

In 1993, IL-9-expressing $CD4^+$ T cells were identified, forming the basis for the Th9 subset.[61] Although the precise role of these cells in lung pathogenesis is unknown, murine models have demonstrated that IL-9 can mediate airway inflammation and remodeling (reviewed elsewhere[62]). In a murine model of allergic airway inflammation, blockade of IL-9, using a monoclonal anti-IL-9 antibody, protected mice from airway remodeling and airway dysfunction.[63] In contrast, IL-9 blockade, in humans, has been an ineffective treatment of allergic asthma, failing to enhance pulmonary function or reduce medication use or asthma exacerbation rates, in populations with mild[64] and moderate to severe asthma.[65] Taken together, it appears as though IL-9 can propagate allergic inflammation, but the inability of IL-9 blockade to reduce symptoms indicates that it may not do so independently. These findings align with early research wherein IL-9 was found to potentiate Th2 inflammation but be insufficient to drive inflammation independently.[66,67]

In 2013, the ability of IL-9 to augment Th2 responses was revisited. During infection with *N brasiliensis,* IL-9 receptor-deficient mice were found to have impaired ILC2 accumulation and survival that led to deficient Th2 cytokine production and impaired lung repair.[68] Further examination revealed that IL-9 led to the upregulation of BCL-3 in lung-derived ILC2s, an antiapoptotic protein required for ILC2 survival. Thus, through interaction with ILC2s, IL-9 contributes to the persistence and magnitude of Th2 inflammation. The development of Th9-augmented Th2 responses may also signal the worsening of disease and represent an important diagnostic tool. In 2014, expression of IL-9 by T cells was used to differentiate between subjects with peanut allergy and individuals sensitized to peanut (IgE positive) who tolerate peanut challenge.[69] Cumulatively, these data highlight the ability of Th9 cells to augment existing type 2 inflammation and indicate that the induction of IL-9 expression may demarcate a worsening of disease, making IL-9 expression a potentially useful biomarker.

Th22

IL-22 is member of the IL-10 family whose receptor is composed of two subunits, IL-10R2 and IL-22R1. IL-10R2 is expressed by a wide range of cell types, but IL-22R1 is expressed by cells at barrier surfaces, such as the lungs, gut, and skin[70]; perhaps surprisingly, IL-22R1 is not expressed by leukocytes.[70] Importantly, the production of IL-22 is not restricted to Th22 cells, and it can be produced by Th1 cells, natural killer cells, innate lymphoid cells, and Th17 cells. What separates Th22 cells from other IL-22-producing cell types is that Th22 cells do not produce any other lineage-specific cytokines (eg, IL-17, IFN-γ, or IL-4).[71]

Many studies have investigated the effect of IL-22 at barrier surfaces. The conclusion of these studies is that IL-22 can be proinflammatory or tissue protective. The proinflammatory nature of IL-22 is demonstrated by its ability to enhance the production of antimicrobial peptides, chemokines, and inflammatory cytokines in addition to enhancing the effects of inflammatory cytokines such as tumor necrosis factor-α, IL-1, and IL-17.[70-72] In contrast, IL-22 can be tissue protective by enhancing the epithelial barrier, promoting wound healing via enhanced proliferation and antiapoptosis, reducing the amount of IL-25 produced by the airway epithelium, and inhibiting symptoms of airway inflammation in mice.[71,73-76] Whether IL-22 exhibits proinflammatory or protective effects appears to depend on the other cytokines coproduced by the cell in question.[76] In a bleomycin lung injury model, Th17 cells were found to be the predominant producers of IL-22 and IL-17. Blockade of IL-22 during bleomycin administration ameliorated airway inflammation, suggesting that IL-22 played a proinflammatory role; however, in IL-17-deficient mice whose T cells still produced high levels of IL-22, there was also a significant reduction in airway inflammation, demonstrating that in the absence of IL-17, IL-22 played a protective role. Therefore, the proinflammatory function of IL-22 was dependent on synergizing with

existing IL-17. IL-22 may also have different effects during different phases of disease.

In an ovalbumin (OVA) asthma model, neutralizing IL-22 during the sensitization phase, which is analogous to the bleomycin study just mentioned, significantly reduced airway inflammation resulting from intranasal challenge with OVA. In contrast, neutralizing IL-22 after sensitization, during the intranasal OVA challenge led to increased airway inflammation.[75] The majority of studies have relied on blocking IL-22 by using neutralizing antibodies or administering exogenous IL-22 to investigate the role of IL-22. Because there are many sources of IL-22, it is difficult to interpret the findings in terms of the role of Th22 cells. Studies that directly examine Th22 cells are required to begin to determine the role of Th22 cells in airway inflammation. The use of adoptively transferred Th22 cells in models of airway disease, as has been done with enteropathogenic bacteria,[77] might be particularly enlightening in this respect.

Follicular B-Helper T Cells

A population of T cells expressing CXC chemokine receptor 5 (CXCR5) was identified that could home to B-cell follicles and proficiently help B cells produce high-affinity antibodies. These cells were termed "follicular B-helper T cells" (Tfhs).[78] Tfhs are potent inducers of immunoglobulin class switching and antibody production by B cells. Furthermore, the prototypical Tfh cytokine, IL-21, and surface markers (CD40L, inducible costimulator, and so forth) support the survival, activation, and differentiation of B cells. Current evidence suggests that Tfh can differentiate as a distinct T-cell lineage, evolving directly from naïve T cells in the presence of IL-21,[79] or acquire Tfh functionality after previous commitment to Th1[80,81] or Th2.[82]

To date, Tfh function has predominantly been linked to autoimmune diseases such as systemic lupus erythematosus and Sjögren syndrome,[83] with little information about their role in allergic disease. The difficulties identifying a role for Tfhs in allergic asthma may arise from their apparent ability to impair IgE production through secretion of IL-21.[84] However, a study of mild and severe asthma found that the frequency of circulating Tfhs was significantly greater in those with severe asthma and correlated positively with IgE titers.[85] Similarly, in a study of individuals with allergic rhinitis, circulating Tfh populations were notably skewed toward a Th2-like Tfh.[86] Intriguingly, evidence from two murine models of house dust mite allergy suggests that the Tfh-derived cytokine IL-21 may directly play a role in the development of type 2 immune responses in the lung, as IL-21 potently enhanced Th2 cell cytokine production and function.[87,88] Thus, it appears that Tfh, through helping B cells and IL-21 secretion may propagate lung inflammation. Elsewhere, it has been suggested that Tfhs may precede and govern subsequent type 2 responses as intranasal administration of house dust mite was found to induce the generation of IL-4-positive Tfhs (IL-21+CXCR5+BCL-6+GATA3^{lo}) detectable in the mediastinal lymph nodes, but not the lung, that could differentiate into effector Th2 T cells (IL-21^{lo}CXCR5^{lo}BCL-6^{lo}GATA3^{hi}) and migrate to the lung in response to subsequent house dust mite challenges.[89] Thus, it is possible that the plasticity of Tfh enables the development of Th2 responses. Indirect evidence for a role of Tfh in asthma comes from studies of allergen immunotherapy in which effective therapy was linked to reductions in circulating Tfh.

Tregs

The demonstration that the adoptive transfer of CD4^{+} CD25^{+} T cells could prevent autoimmune disease elicited a renaissance in the study of Tregs.[90] Following this demonstration, T cells with immunosuppressive capabilities have been described as Tregs that are predominantly split into two categories: forkhead box P3 (FoxP3)$^{+}$ thymus-derived natural Tregs and peripherally induced, FoxP3$^{+/-}$, cytokine-secreting (IL-10 or transforming growth factor [TGF]-β) inducible Tregs. Problematically, the inability to rigorously differentiate inducible Tregs from natural Tregs has limited the ability to identify their relative contributions to the maintenance of peripheral tolerance and progression of allergic asthma. Several putative markers have been used to delineate these cell populations, such as Helios[91] and neuropilin-1.[92,93] However, evidence has suggested that neither is a specific marker of natural Tregs. An elegant adoptive transfer study by Gottschalk et al[94] observed that adoptively transferred T cells upregulated Helios before FoxP3 following intravenous peptide administration to recipient mice, thereby demonstrating the expression of Helios by inducible Tregs. Similarly, high neuropilin-1 expression was found in mice that predominantly generate inducible Tregs.[95] Thus, we continue to lack a reliable marker that can differentiate inducible Tregs from natural Tregs; such a tool would enable better

understanding of disease cause and the design of therapies that rely on expansion of Treg subsets. Intriguingly, neuropilin-1-positive Tregs were found to possess impaired suppressive capacity compared with Helios$^+$ Tregs,[96] a feature attributed to reduced expression of IL-10, cytotoxic T-lymphocyte-associated protein 4, and Epstein-Barr virus-induced gene 3 (*EBI3*).

IL-10 and cytotoxic T-lymphocyte-associated protein 4 are well known effectors of immunosuppression. In contrast, *EBI3* is a relatively new suppressive molecule that defines a new subset of suppressive Tregs. IL-35 is a heterodimeric cytokine from the IL-12 family, consisting of *EBI3* and IL-12α/p35.[97] IL-35 was found to be exclusively, and constitutively, secreted by murine FoxP3$^+$ Tregs. The robust regulatory activity of IL-35 was demonstrated in *Ebi3*$^{-/-}$ and *Il12a*$^{-/-}$ Tregs, as these cells lacked the ability to regulate in vitro immune responses and failed to cure inflammatory bowel disease.[97] Similarly, recombinant IL-35 suppressed T-cell proliferation, and ectopic expression of IL-35 conferred regulatory capacity to T cells.

Of particular interest is the ability of IL-35 to generate a population of Tregs called "iTr35s." Expression of IL-35 by Tregs is sufficient to induce the production of IL-35 by effector T cells. In this manner, IL-35 facilitates infectious tolerance, the spread of antigen-specific regulation, as effector cells that acquire the capability to produce IL-35 become potent regulatory cells as evidenced by the observations that iTr35 cells generated in this manner possess the capability to regulate lethal autoimmunity (*Foxp3*$^{-/-}$ mice), experimental autoimmune encephalomyelitis, and inflammatory bowel disease and contribute to the regulatory milieu that fosters tumor growth.[98] In a murine model of allergy to OVA, regulation of disease and airway hyperresponsiveness were associated with the presence of IL-35-producing T cells, as *Ebi3*$^{-/-}$ deficient mice failed to develop OVA tolerance; IL-10 and TGF-β were dispensable.[99] Similarly, transfecting pulmonary cells with an IL-35 DNA construct protected mice from the development of allergen-specific IgE and eosinophil accumulation in a model of house dust mite allergy.[100] In a study of newly diagnosed asthma, IL-35 was significantly reduced in patients with asthma compared with that in healthy control subjects.[101] In contrast to these studies, in a murine model of allergic sensitization, challenge, and peptide immunotherapy, we were unable to detect altered levels of IL-35 in the blood, lung, or BAL of mice following effective therapy.[102] Taken together, these studies indicate that IL-35 and iTr35 possess robust immunomodulatory functions, but the role of IL-35 in human asthma and its therapeutic potential remain to be examined.

Conclusion

Historically, allergic lung disease has been considered the result of excessive, aberrant Th2 T-cell responses. This outlook provides a reasonable foundation for understanding allergic disease, accurately conveying many of the dominant mechanisms engaged in allergic asthma. As disease phenotyping tools become more sophisticated and clinical disease is better understood, we must begin to consider how cross talk between different Th subsets may augment established Th2 disease. In a similar manner, resolution of disease through T-cell-mediated regulation can no longer be viewed as exclusively the role of IL-10$^+$, TGF-β$^+$, and FoxP3$^+$ cells. To obtain maximal efficacy in the clinic, strategies such as immunotherapy that attempt to expand Tregs will need to consider new targets, such as iTr35 Tregs. In conclusion, established paradigms in the immune-mediated pathogenesis and regulation of lung disease have evolved because of a preponderance of evidence; new subsets and functions of T cells should be entwined with these existing concepts to reflect the spectrum of disease.

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