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Regulatory T cells in many flavors control asthma

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That regulatory T cells (Tregs) have a crucial role in controlling allergic diseases such as asthma is now undisputed. The cytokines most commonly implicated in Treg-mediated suppression of allergic asthma are transforming growth factor-β (TGF-β) and interleukin (IL)-10). In addition to naturally occurring Tregs, adaptive Tregs, induced in response to foreign antigens, have been shown in recent studies. The concept of inducible/adaptive Tregs (iTregs) has considerable significance in preventing asthma if generated early enough in life. This is because cytokines such as IL-4 and IL-6 inhibit Foxp3 induction in naive CD4+ T cells and therefore de novo generation of Tregs can be expected to be less efficient when it is concomitant with effector cell development in response to an allergen. However, if iTregs can be induced, the process of infectious tolerance would facilitate expansion of the iTreg pool as suggested in the recent literature. It is tempting to speculate that there is a window of opportunity in early life in the context of a relatively immature immune system that is permissive for the generation of iTregs specific to a spectrum of allergens that would regulate asthma for lifelong. The focus of this review is the relevance of nTregs and iTregs in controlling asthma from early life into adulthood, the mechanisms underlying Treg function, and the prospects for using our current concepts to harness the full potential of Tregs to limit disease development and progression.

INTRODUCTION

Various lines of evidence indicate that T helper cells (CD4+ T cells) and their secreted products have a central role in orchestrating the unique inflammatory response in the airways of asthmatics. Local allergen challenge has been shown to induce an influx of CD4+ T cells and eosinophils into the airways of asthmatics.[1] In a mouse model, depletion of CD4+ T cells with a monoclonal antibody to CD4 antigen reduced airway eosinophilia and eliminated airway hyperresponsiveness.[2] T helper 2 type (Th2) cells are believed to augment the inflammatory response observed in asthma by expressing multiple cytokines such as interleukin (IL)-4, IL-5, IL-9, and IL-13, each of which could potentially augment the eosinophilic inflammation in asthma.[3–7] In addition to eosinophils, other cell types are also involved in asthma pathogenesis as reviewed recently.[8] For example, neutrophilic asthma is also well recognized and is associated with severe airflow obstruction, which is difficult to control by corticosteroids.[9] A more recently identified T helper subset, Th17, has been implicated in neutrophil-dominated asthma[10–12] and was shown to be responsible for steroid resistance in a murine model of asthma.[13] The effector function of all T helper cells, Th1, Th2, and Th17, is regulated by regulatory T cells (Tregs). This ensures protection from autoimmune diseases such as diabetes and multiple sclerosis as well as allergic diseases such as eczema and asthma. In this study we discuss recent findings that suggest an important role for Tregs in the induction of tolerance to environmental allergens and in limiting disease when allergic airway inflammation is induced. Also discussed are current concepts about development of Tregs and Treg-expressed mediators that have relevance in controlling asthma and how this knowledge might be exploited to enhance Treg function. **Table 1** enlists Treg-associated nomenclature commonly used in the literature to describe different types of Tregs and their regulatory role in controlling inflammation in the lung or the gut.

FROM BIRTH TO ADULTHOOD TREGS MAKE A DIFFERENCE

The phenomenon of tolerance was introduced by Owen in 1945 in his seminal observations of "mosaicism" (red cell chimerism) in adult cattle dizygotic twins.[14] A few years later in 1953, acquisition of tolerance to foreign allograft antigens *in utero* was shown in mice by Billingham *et al.*[15] Some 50 years later, the concept of acquired tolerance to foreign antigens is highly significant not only in the context of transplantation tolerance but also in the realm of allergic and autoimmune diseases. Enmeshed in the concept of acquired immune tolerance is the fabric of the "hygiene hypothesis," which was postulated to explain the increased prevalence of allergic diseases in developed

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Table 1 Treg nomenclature and relevance in immune regulation in the lung and the gut

Types of Tregs	Phenotype and original identification	Mucosal relevance	
		Lung	Gut
Thymically derived nTregs	CD4 + CD25^{hi} Foxp3 + (mice)[221]	Ref. [44,54,55,73,90]	
	CD4 + CD25^{hi} Foxp3 + CD49d^{-} (human)[222]	Ref. [33,35,36,202]	Ref. [76]
Peripherally induced adaptive Tregs (iTregs)	Peripherally induced from CD4 + CD25 – cells. The regulatory T cells could be Foxp3 + or Foxp3 –		
(i) Foxp3 + iTreg	CD4 + CD25^{hi} Foxp3 + (mice)[53]	Ref. [48,53,51, 61,144]	
(ii) Foxp3 – Tr1 cells	IL-10-expressing CD4 + CD25^{hi} Foxp3 – [34]	Ref. [42,43,45, 46,49,118,158,159]	Ref. [24,25, 34,40]
(iii) Foxp3 – Th3 cells	TGF-β-expressing CD4 + T cells[223]	Have yet to be associated with immune suppression in the lung or the gut	
ExFoxp3	CD4 + CD25^{hi} Foxp3 + Treg cells that have spontaneously lost Foxp3 expression and secrete pro-inflammatory cytokines[93,94]		Ref. [94]

IL-10, interleukin-10; TGF-β, transforming growth factor-β.

countries in recent years. The basic tenet of this hypothesis is that early childhood exposures to pathogen-associated products inversely correlates with the incidence of allergic diseases in adulthood. Initially postulated in 1989,[16] the underlying mechanism associated with this hypothesis was Th1/Th2 cross-regulation. However, after epidemiological studies showed that Th2-inducing parasitic infections could protect from atopic diseases that are also engendered by Th2 cells, it became clear that additional mechanisms are needed to be invoked to explain the protective effect of microbial exposures.[17,18] The need for a more general immunoregulatory mechanism was realized when the same beneficial effect of microbes on Th1-induced diseases such as autoimmune diseases was also evident. With the rapid progress of research in the field of Tregs in recent years, it seems that microbial infections are conducive to the development of these cells,[19,20] which are then able to exercise their immunosuppressive functions to dampen unwarranted immune responses against foreign or self-antigens.

There is significant interest in understanding the development of the immune system from the fetus to adulthood to help define the triggers and brakes in the etiology of allergic diseases. It is now well recognized that microbes can induce the production of the suppressive cytokines transforming growth factor-β (TGF-β) and IL-10.[17,19,21–23] It is noteworthy that IL-10 knockout mice develop colitis and a recent study has shown that it is the secretion of this cytokine from myeloid-derived cells stimulated by microbiota that prevents the induction of colitis.[24,25] Similarly, mice bearing a dominant-negative mutation in the TGF-β receptor on CD4 T cells show profound inflammation in various organs, including the lungs, liver, and the pancreatic islets, suggesting impaired development of Tregs.[26]

Recent studies in humans and mice show the importance of Tregs in the promotion of tolerance to foreign antigens transmitted through the placenta to the fetus.[27] The mother during pregnancy is exposed to several antigens from the environment and these antigens, termed "non-inherited maternal antigens," constantly traverse the placenta and have the capacity to evoke an immune response. How is the mounting of immune response to these foreign antigens stumped in the developing fetus? A study in humans showed that maternal cells traffic in large numbers to fetal lymph nodes to induce CD4 + CD25 + Foxp3 + cells with suppressive functions that prevent antimaternal immunity lasting until early adulthood. This study also challenges the preconceived notion that fetal lymphocytes are incapable of mounting an immune response[28] (**Figure 1**). In fact, the data from this study show that fetal lymphocytes secrete high levels of cytokines in response to non-inherited maternal antigens when Tregs have been selectively depleted. In a study in mice in which the protocol of aerosolized ovalbumin (OVA) was used to induce airway tolerance in mothers, transfer of OVA and TGF-β through breast milk into suckling neonates induced suppressive CD4 + T cells whose generation depended on a functional TGF-β receptor[29] (**Figure 1**). Similarly, when pregnant female mice were tolerized to antigen, the offspring were also tolerized to the same antigen, which was sustained only when the infants were nursed by the tolerized mothers.[30] These studies suggest that foreign antigens can be transferred across the placenta or through breast milk to fetuses or infants, respectively, who can induce Foxp3-expressing Tregs in the presence of TGF-β. The findings not only underscore the importance of Tregs in tempering immune responses in the developing fetus but also highlight a window of opportunity for the development of novel therapeutics in early life to ward off disease.[31,32] Thus, it may not be too fanciful to propose administration of minute doses of a cocktail of allergens to expectant mothers or neonates to develop antigen-specific Tregs that would provide life-long protection from developing allergic diseases such as asthma (**Figure 1**).

WHICH MECHANISM IS RELEVANT—FOXP3 + TREGS OR IL-10—IN EXPRESSING TR1-LIKE CELLS?

It is clear that individuals with defective or suboptimal Foxp3 expression due to mutations in the *Foxp3* gene (immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome) or in genes that promote Foxp3 expression, such as STAT5b, are susceptible to allergic disease.[33] This section will review studies in both humans and rodents that suggest the importance of both Foxp3-expressing Tregs and IL-10—the source of the latter not necessarily being Foxp3-expressing

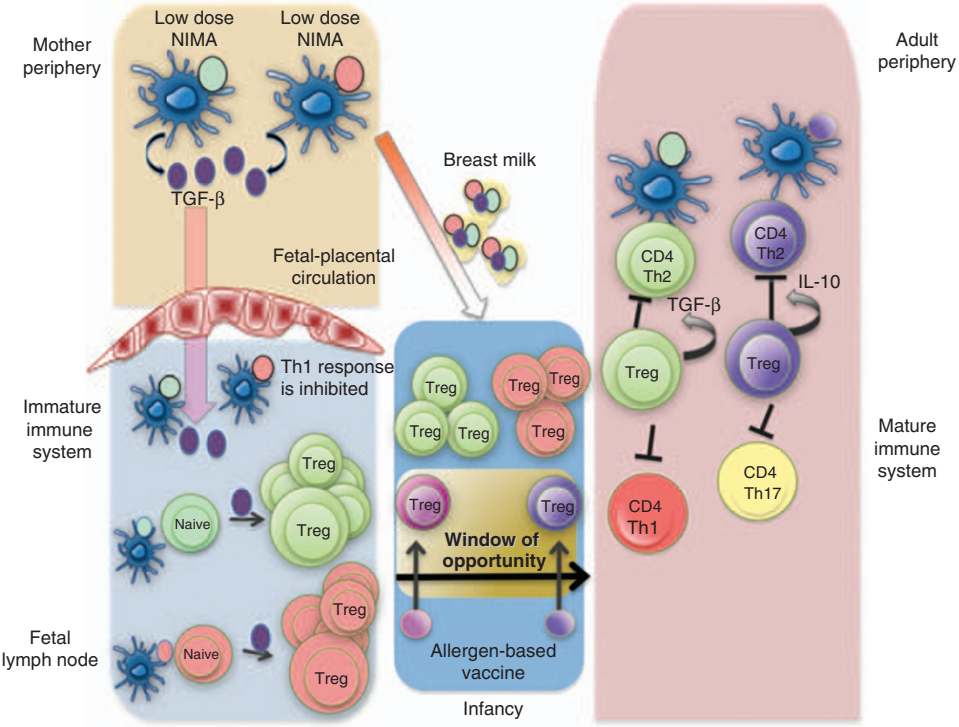

Figure 1 Induced Tregs specific to allergens in early life may be the elixir of asthma-free life. As shown recently,[28] the fetus is constantly exposed to non-inherited maternal antigens (NIMA) that traverse the placenta and trigger the development of Tregs in the fetal lymph nodes. Tregs constitute a high percentage of total lymphocytes in the fetal lymph nodes. This pool of Tregs aids in tempering fetal immune responses against maternal antigens. During or after pregnancy, transfer of antigen and transforming growth factor-β (TGF-β) across the placenta or through breast milk induces antigen-specific Tregs.[29,30] The immune system's ability to induce antigen-specific Tregs in early life suggests a unique window of opportunity to administer allergen-based vaccines to infants for the induction of protective allergen-specific Tregs.

cells—in regulating allergic asthma. CD4+ T cells that do not express Foxp3 but secrete IL-10 and suppress effector functions of T helper cells are known as Tr1 cells.[34]

In humans, along with typical disease manifestations such as elevated immunoglobulin E (IgE) levels, eczema, and insulin-dependent diabetes mellitus, intestinal inflammation, asthma, and other recurrent respiratory disorders have also been associated with IPEX.[35–37] Although eczema is a common feature of IPEX deficiency, there is less documentation of asthma in IPEX patients. The most likely reason for this is that there is inherently significant uncertainty in the diagnosis of asthma in young children. Although wheezing is a phenotype of asthma, it is not a definitive diagnosis of this disease in young children. A vast majority of infants and children of <6 years of age show wheezing during recurrent respiratory infections, but the children outgrow the phenotype with age.[38] Because of the difficulty in diagnosing asthma in early life, asthma is not as commonly associated with IPEX as is eczema, which manifests in infants and is easily diagnosed. For example, in one study of 14 patients, all of whom were diagnosed early with severe disease symptoms including eczema, only one was diagnosed with asthma. This patient underwent spontaneous remission of early diarrhea and all traces of autoimmunity at age 4, but was left with mild eczema, relatively high serum IgE levels, and occasional upper respiratory tract infections.[39] Whether asthma also persisted is not documented in the report. This patient had two mutations in Foxp3—one a silent mutation at the intron 4/exon 5 boundary that presumably influences RNA splicing and another non-conservative mutation in the FKH domain. It is possible that these mutations allowed some level of expression of functional Foxp3 in later life that was adequate to suppress autoimmunity but not allergic disease. In another study, CD25 deficiency was associated with reduced IL-10 expression and the patient developed symptoms indistinguishable from those observed in IPEX patients with the patient progressing to develop asthma.[40] As it is now well recognized that IL-2 is required to maintain Foxp3+ Tregs in the periphery,[41] it is not surprising that this patient showed an IPEX-like phenotype. Most importantly, the development of asthma in this suggests an important regulatory role for Foxp3+ Tregs in asthma. In another study, maintenance of tolerance to beestings in beekeepers by seasonal exposure to bee stings was found to be associated with increased bee venom antigen-specific IL-10-producing CD4+ Tr1-like cells.[42,43] Hence, what are the sources of IL-10 and TGF-β and their contribution in suppression of allergic asthma when expressed from a Foxp3-expressing cell or a Foxp3-negative cell? Interestingly, two types of nTregs that develop in the thymus have been recently described—ICOS$^-$ and ICOS$^+$—that use TGF-β and IL-10, respectively, for their suppressive functions.[44]

A review of animal studies on the role of Tregs in regulating allergic asthma reveals an interesting difference in the involvement of IL-10 vs. Foxp3-expressing Tregs, depending on whether or not the animals were sensitized through the airways. In multiple studies that used the intraperitoneal mode of antigen

sensitization, in which non-airway dendritic cells (DCs; such as splenic DCs) would be involved in priming naive T helper cells, a dependence on IL-10 for suppression of effector T-cell responses and airway pathology was noted. An exception to this general trend is the results of one study that used intranasal delivery of adjuvant-free OVA, in which an involvement of IL-10 in suppressing allergic airway inflammation was noted[45] that involved the ICOS–ICOS ligand pathway.[46] There does not seem to be an agreement in the different studies with respect to the source of IL-10. In one study, the source was found to be not the Foxp3-expressing cells but actually bystander CD4+ T cells.[47] In contrast, a different study showed that deletion of IL-10 in Foxp3-expressing CD4+ T cells promoted allergic airways disease.[48] In a recent study, in which peptides derived from the cat allergen, Fel d 1, were delivered intradermally to promote immunotherapy in mice previously immunized by the intraperitoneal route, IL-10-producing cells but not TGF-β-expressing Foxp3+ Tregs were found to mediate immune suppression.[49] In our own studies of tolerance induction through repeated exposure to a low dose of aerosolized antigen, a protocol initially established by Holt and colleagues,[50] an involvement of Foxp3+ Tregs expressing membrane-bound TGF-β in mediating resistance to development of antigen-induced airway inflammation was identified.[51,52] In another study of oral tolerance, Foxp3+ Tregs were shown to inhibit allergic airway inflammation that was dependent on TGF-β and not IL-10.[53] Similarly, when animals were immunized by intratracheal delivery of the allergen house dust mite, depletion of $CD25^{hi}$ population of cells induced airway hyperresponsiveness in a normally resistant mouse strain C3H.[54] In this study, no involvement of IL-10 was apparent.[54] Thus, it is clear that no single mechanism of Treg-mediated suppression of allergic airways disease has emerged from multiple studies of experimental asthma reported to date.

As a dependence on IL-10 for suppression was not evident in models in which the antigen was delivered through the respiratory route,[52–54] which was the opposite when a non-airway mode was adopted,[43,47–49,55] it seems that the cell types that are engaged as suppressive cells in the two settings of antigen exposure are distinct. When animals were repeatedly exposed to inhaled antigen without any adjuvant such that sufficient maturation of airway DCs would not occur, Foxp3+ Tregs expressing membrane-bound TGF-β were found to mediate inhibition of allergic inflammation in the airways.[52] Conversely, conversion of a normally resistant mouse strain susceptible to development of an asthma phenotype by depletion of $CD25^{hi}$ cells was a direct effect of loss of attenuation of lung DC costimulatory functions by Tregs promoting unopposed inflammatory effects of T helper cells.[54] On the other hand, if effector cells and their regulators were primarily induced at a distant site, such as the skin[49] or the spleen,[55,47] IL-10 seemed to be an important negative regulator of pulmonary disease development, as was also suggested in the study of bee keepers.[42,43] The primary reason for this difference maybe that when Tregs are activated locally in the lung-draining lymph nodes, particularly in the presence of a low dose of antigen, IL-10 gene expression is not favored either from the Tregs or from bystander cells in the tissue. However, if IL-10 gene expression is induced when priming occurs elsewhere, the source of the cytokine maybe the Foxp3-expressing cell[48] or a bystander cell.[47] It seems likely that IL-10 production is determined by the dose and T-cell receptor (TCR) affinity of the antigen, the adjuvant used, or other properties for a complex allergen, such as ligands, for pattern recognition receptors. A topic for future exploration is differential distribution of Foxp3+ Tregs that use TGF-β vs. IL-10 in the periphery.

Another possible explanation for the involvement of Foxp3+ Tregs expressing membrane-bound TGF-β rather than IL-10 in mediating tolerance in the airways is the relative enrichment of Foxp3+ Tregs in mucosal tissues and in lymph nodes draining the lung and the gut. Although a proportion of these Tregs may be induced *de novo*, polyclonally expanded locally residing $ICOS^-$ nTregs may also be involved in disease suppression (discussed in the following section). However, when Tregs are induced after immunization with antigen and adjuvant delivered through the skin or spleen, IL-10 and not TGF-β produced by Foxp3+ Tregs (probably $ICOS^-$)[44] or Foxp3− Tr1-like cells (probably $ICOS^+$)[44] limit the severity of inflammation. It is clear, however, that Foxp3 deficiency promotes allergic disease.[35,36] Therefore, regardless of whether or not IL-10 or TGF-β is the mediator of suppression of asthma or other allergic diseases such as eczema, Foxp3-expressing Tregs are important negative regulators of allergic disease. It will be useful to determine in future studies what conditions induce IL-10 production from a Foxp3-expressing cell even when it is not innately programmed to express IL-10.[44] If such conditions could be established in situations in which Foxp3 is mutated or its expression is suboptimal, as observed in IPEX patients, IL-10 could potentially substitute for Foxp3-dependent suppressive mechanisms. As eczema/atopic dermatitis develops early in life in most IPEX patients, such strategies may need to be directed to the skin, which may also protect the lung (peptide immunotherapy).

ROLE OF THYMUS-DERIVED TREGS (NTREGS) VS. PERIPHERALLY INDUCED TREGS (ITREGS) IN REGULATING ALLERGIC ASTHMA

The generation of Foxp3+ Tregs *in vitro* by activation of CD4+ T cells in the presence of TGF-β showed that acquisition of Foxp3 is possible in a non-thymic environment.[56] Although the identification of CD4+CD25+Foxp3+ T cells after adoptive transfer of naive CD4+ T cells into recipient TCR transgenic mice devoid of nTregs also suggested similar *de novo* generation in the periphery, the conversion of CD25–Foxp3+ cells into CD25+Foxp3+ cells could not be ruled out in these experiments as the naive cells were not depleted of Foxp3+ cells before adoptive transfer.[57] Close on the heels of these findings, it was more definitively shown using genetic approaches that naive CD4+ T cells in the periphery can indeed be induced to express Foxp3 under appropriate conditions and that the cells have suppressive function similar to nTregs.[53,58–60] Using OVA-specific TCR transgenic mice crossed to scurfy mice that harbor a mutated *Foxp3* gene, it was shown that a protocol of oral tolerance induced by supplying OVA in the drinking water of mice induced Tregs in the periphery that were important to

control allergic sensitization.[61] The findings in this study suggest that the induction of airway tolerance by delivery of aerosolized antigen[50,52] most likely also involves iTregs, although the involvement of nTregs activated in a bystander manner cannot be ruled out.

Induction of tolerance by delivery of antigen through the airways[51,52] or the oral route[53,61] almost completely thwarts the development of effector T cells if animals are subsequently immunized with antigen and adjuvant. Using TCR transgenic mice, it was shown that iTregs are also induced along with effector T cells after sensitization and antigen challenge and these Tregs limit the severity of allergic airway inflammation and also minimize its dissemination to extrapulmonary locations.[61] The question that arises from the results of this study is whether the iTregs were induced during the challenge phase or not during priming in the inflammation model. This is because the presence of IL-4 and IL-6 during priming in the spleen should have inhibited Foxp3 induction. However, during challenge by inhaled antigen without adjuvant, low cytokine levels would have been conducive to iTreg generation in the lung-draining lymph nodes. In humans and in specific animal models, in which sensitization and challenge both occur by inhalation of allergen, iTreg generation may be compromised by the inhibitory effects of IL-4 and IL-6. However, these same cytokines would permit polyclonal proliferation of nTregs as discussed below.

It has been suggested that TCR specificity guides peripheral Treg development that is also influenced by the location.[62] Thus, the contribution of iTregs induced by allergens vs. nTregs in the regulation of allergic asthma would be dictated by both the allergen and the local microenvironment. In this regard, a possibility that can be entertained is that iTregs that regulate allergic airway inflammation are peripherally induced at sites other than the lung to be subsequently recruited by antigen-induced inflammation to the lung. A likely mucosal site for this is the gastrointestinal tract in which Tregs are required to maintain intestinal homeostasis by upholding tolerance to food antigens and gut microflora, the absence of which could lead to experimental colitis.[63–66] This speculation is based on knowledge derived from various studies that show intestine and associated lymphoid tissue as a site for proliferation[66] and induction of Foxp3+ Tregs from naive precursors,[21,67] and the observations that changes in gut microflora[68] and probiotic treatments influence airway hyperresponsiveness and allergies.[69–71] When viewed in combination, it seems likely that the gut serves as a "hub" or "depot" for inducing Tregs, which then home to various sites of infection/inflammation to exert suppressive activity. The repertoire of these induced Tregs would be mainly governed by the nature of the intestinal microflora, the subtle changes in which could lead to susceptibility or resistance to a particular antigen locally or more distally in the lung. Needless to say, an interplay between genetics and the immune mechanisms would drive the net outcome in every individual. So, how do iTregs or nTregs elicit immune suppression?

The process of infectious tolerance was recently shown to involve Foxp3+ Tregs expressing membrane-bound TGF-β coupled to latency-associated peptide.[72] These membrane-bound TGF-β latency-associated peptide-expressing Tregs induced Foxp3 expression in naive Foxp3− CD4+ T cells when stimulated by plate-bound anti-CD3, which in turn acquired suppressive function.[72] Given that Tregs have been shown to inhibit the immunogenic potential of pulmonary DCs by downregulating expression of major histocompatibility complex class II and costimulatory molecules,[54,55] an expanded population of Tregs expressing TGF-β may also serve to downregulate DC function in both draining lymph nodes and the tissue. This raises the question of the involvement of antigen-specific Tregs in regulating allergic asthma. It is difficult to imagine that the large pool of Tregs that infiltrate the airways during allergic inflammation comprise only antigen-specific iTregs. Even if the process of infectious tolerance is operative in the induction and activation of many of these Tregs, a substantial fraction of the Tregs likely consists of polyclonal nTregs. Thus, in the first study that generated Tregs *in vitro* from naive CD4+ T cells in the presence of TGF-β, the adoptive transfer of a polyclonal population of Tregs into mice was highly efficient in suppressing house dust mite-induced allergic airway inflammation.[56] Another recent study of experimental asthma also suggests that suppression of allergic inflammation in the airways involves locally activated polyclonal Tregs.[73] Activation of Tregs is important for efficient suppression as freshly isolated Tregs from naive mice have weak suppressive function, unless activated to boost their potency.[74] Taken together, it seems reasonable to conclude that the most efficient Treg-mediated suppression of allergic asthma can be achieved by establishment of mucosal tolerance to a range of allergens in early life. This would involve induction of antigen-specific Tregs enhanced by the process of infectious tolerance that would last lifelong to limit generation of allergen-specific effector T cells. However, the potential to induce such Tregs would be less after antigen sensitization because of cytokine inhibition.

Any discussion on the efficiency of iTreg generation should also consider the context. For example, in one study, when CD4+ Foxp3− T cells were adoptively transferred into transgenic mice with TCR specificity for myelin basic protein and the mice were subsequently immunized with myelin oligodendrocyte glycoprotein peptide to induce experimental autoimmune encephalomyelitis, *de novo* conversion of Foxp3− to Foxp3+ cells was minimal in the draining lymph nodes.[75] As this model is a driver of intense Th17 differentiation, it illustrates the point that specific pro-inflammatory cytokines produced in the lymph nodes exercise a strong influence on iTreg development. Somewhat conterintuitively, these same cytokines that block iTreg development promote the proliferation of nTregs and help maintain their Foxp3 expression. This would explain why in our study as well as in the experimental autoimmune encephalomyelitis and multiple other studies in humans and animals, Tregs can be found in large numbers at the site of inflammation. As can be concluded from the study of Lafaille *et al.*,[61] the Tregs that accumulate at the site of inflammation help to temper the inflammation as well as to contain it. Lastly, an important question that needs to be addressed in future studies is whether the efficiency of iTreg development is dissimilar in different lymph nodes.

As discussed previously, there maybe a window of opportunity in early life to develop life-long tolerance to harmful antigens, such as allergens, through development of antigen-specific Tregs before sufficient maturation of the immune system and generation of effector T cells has taken place (**Figure 1**).[31,32] Interestingly, a recent study of colorectal cancer patients has shown that Tregs specific to only a limited set of tumor-associated antigens control antitumor effector responses to the same set of antigens.[76] Thus, knowledge of antigen specificity of Tregs in disease would undoubtedly be immensely helpful to promote antitumor responses or to suppress effector responses in allergic and autoimmune diseases.

BLOCKADE OF TREG DEVELOPMENT BY PRO-INFLAMMATORY CYTOKINES AND EXFOXP3 TREGS

It is clear that both Th2 and Tregs coexist at the site of allergic inflammation.[61,77] Therefore, a logical question that arises is what effects the pro-inflammatory cytokines secreted by the Th2 cells exert on Tregs. We focus here on two cytokines, IL-6 and IL-4, both of which promote Th2 but block Treg development. IL-6 also promotes Th17 development in combination with TGF-β.[78–81] The IL-6 receptor exists in both membrane-bound and soluble forms (sIL-6R) and the complex of IL-6 with its soluble receptor upon interaction with gp130 expressed on cells induces downstream signaling in a process called IL-6 trans-signaling.[82] sIL-6R is found in large quantities in chronic inflammatory diseases. The IL-6/sIL-6R complex has been associated with inhibition of development of CD4 + CD25 + Foxp3 + cells and also impairment of their suppressive function in models of asthma and inflammatory bowel disease.[83,84] In T cells, loss of STAT3 (signal transducer and activator of transcription 3), which is activated by IL-6, upregulated Foxp3 expression.[85] Thus, targeting IL-6 signaling should improve Treg-mediated suppression in asthma. IL-4-induced signaling in T cells promotes Th2 differentiation but inhibits Treg development. STAT6 is activated by IL-4 in naive CD4 + T cells and upregulates GATA-3 expression, which is the master regulator of Th2 cell differentiation.[86,87] Both STAT6[88] and GATA-3[89] can bind to the Foxp3 promoter to inhibit Foxp3 gene transcription. Together, the cytokines IL-4 and IL-6 would not only drive T helper cell differentiation but would also block iTreg development.

As discussed above, it can be assumed that a large fraction of Tregs that accumulate in the airways during allergic inflammation are nTregs that are induced to proliferate and express CCR4 in the lung-draining lymph nodes, resulting in their recruitment to the site of inflammation.[90] An additional level of complexity recently identified by us is that chronic STAT6 signaling in the effector cells imparts resistance to suppression by Tregs.[77] Thus, although IL-4 promotes nTreg proliferation and maintains Foxp3 expression, Tregs are unable to completely block Th2 effector function *in situ*. The opposing actions of IL-4 and IL-6 on T helper vs. Treg development illustrate an important role of cytokines in regulating the dynamics of inflammation. At the same time a cytokine would allow T helper cell differentiation and induce resistance to Treg-mediated suppression, it would inhibit iTreg development and infectious tolerance. However, under these conditions, nTregs would actually be allowed to proliferate and traffic to the tissue as IL-4 is a proliferative factor for already developed Tregs.[77,91] This would ensure that when the effector response eventually contracts, the Tregs are poised to take over and restore homeostasis. In a similar manner, in sarcoidosis, a granulomatous disease of the lung driven by Th1 cells, spontaneous IL-2 secretion by lung effector T cells has been implicated in Treg expansion around the granulomas,[92] which was also noted in the study of experimental autoimmune encephalomyelitis.[75] The competing effects of IL-4 and IL-2 on Tregs vs. T effector function likely determine the magnitude of the induced inflammation. Accumulation of a large pool of Tregs in the inflamed lung may also be undesirable in light of the recent unexpected finding that Tregs are susceptible to loss of Foxp3 at inflamed sites[93,94] and that along with Foxp3 they coexpress transcription factors induced in effector T cells.[95–97] Whether these factors promote the stability of Tregs will be interesting to determine in the future.

Recently, some unexpected features of Tregs have been identified. Tregs in inflamed tissues were shown to lose Foxp3 (resulting cells termed exFoxp3) and secrete pro-inflammatory cytokines.[93,94] Second, Tregs were shown to express transcription factors characteristic of T helper cells such as T-bet and interferon regulatory factor 4 (IRF4).[96,97] IRF4 promotes IL-4 gene expression in Th2 cells and T-bet is a Th1-specific transcription factor. It has been proposed that expression of specific effector T-cell transcription factors in Tregs helps limit the function of the effector T cell in which the same factor is expressed.[95–97] The mechanism by which this is achieved is unclear. If this dual-expressing Treg then loses Foxp3 but retains the other transcription factor such as IRF4, it may cause expression of pro-inflammatory cytokines from that cell, as was observed for exFoxp3 cells recovered from different tissues of diabetic animals.[93] It is, however, unclear whether such a cell would indeed be capable of producing all of the Th2 cytokines, as IRF4 alone is not enough for the expression of Th2 cytokine genes. Given that both STAT6 and GATA-3, which are expressed specifically in Th2 cells, inhibit Foxp3 expression,[88,89] it is unlikely that a Foxp3 + Treg would be replete with the full spectrum of Th2 factors. Tregs can coexpress T-bet and Foxp3, whereas retinoic acid-related orphan receptor-γt and Foxp3 have a reciprocal relationship.[98–100] Therefore, loss of Foxp3 in Tregs in a model of diabetes might promote secretion of interferon-γ or IL-17A. This, however, does not necessarily imply that Foxp3-deficient Tregs would behave as Th2 cells. Whether these would instead develop into Th17 cells in the context of allergic inflammation is an interesting possibility, given that IRF4 is also important for the Th17 phenotype and loss of Foxp3 may promote retinoic acid-related orphan receptor-γt expression. This is of significant concern as Tregs express CCR4, which is also expressed by Th2 cells,[101] and thus the Tregs with lost Foxp3 would still coexist with Th2 cells at the site of inflammation. These exFoxp3 cells may secrete IL-17A to promote neutrophilic asthma, which is a characteristic of severe asthma. In fact, it may not be too far fetched to consider the possibility that retention of the exFoxp3 cell by virtue of CCR4 expression in the lung, and stimulation

by self antigen-derived peptides resulting in IL-17A secretion, is one of the recipes for the development of non-atopic/intrinsic asthma.

MECHANISMS BY WHICH TREGS INHIBIT EFFECTOR T-CELL FUNCTION IN ASTHMA

As discussed above, the two molecules that have received the most attention with respect to Treg-mediated suppression of allergic airway inflammation are TGF-β and IL-10. Our investigations showed that membrane-bound TGF-β expressed by Tregs in mice tolerized by inhaled antigen[52] activates Notch inducing expression of its downstream target gene Hes1 (hairy and enhancer of split 1) in naive CD4+ T cells.[51] Hes1 is a potent repressor of gene expression.[102,103] Simultaneous engagement of TCR, CD28, and Notch was shown to inhibit T-cell activation, which was associated with upregulation of Hes1 expression.[104] Given that membrane-bound TGF-β, originally identified on activated Tregs,[105] has now been implicated in infectious tolerance,[72] Hes1 may have an important role in the induction and/or stabilization of Foxp3 expression in the CD4+ T cell destined to become an iTreg. Indeed, Notch activation in Tregs was recently shown to promote Foxp3 expression and stabilize Tregs *in vivo*.[106] **Figure 2** merges all of these concepts in the context of mucosal tolerance. iTreg development is at a disadvantage during allergen-induced T helper cell differentiation that promotes nTreg proliferation (**Figure 2**).

Although Notch activation has been also associated with T helper cell differentiation,[107] it is likely that the strength of Notch ligation and duration of expression of its target genes such as Hes1 differs when T cells are activated vs. when they are suppressed. IL-10 induces T cell anergy and it is believed to be due to inhibition of costimulation of T cells.[108,109] IL-10 also has effects on Ig isotypes promoting IgG4 and inhibiting IgE switching.[110] With regard to other mechanisms used by Tregs in curbing asthma, a recent study showed that Treg-expressed OX40 upon ligation of OX40L on mast cells inhibited their degranulation.[111] Although IDO (indoleamine 2, 3 dioxygenase) has been associated with Treg-mediated suppression,[112] our studies and that of others suggest an involvement of IDO in promotion of Th2 responses using the model allergen, OVA.[113,114] In mice infected with *Aspergillus fumigatus*, a fungus associated with development of allergic bronchopulmonary aspergillosis, a role for IDO in control of fungal burden, allergic response, and Treg function late in infection was noted.[115] More studies are needed in different animal models and in humans to elucidate the role of IDO in regulating effector responses in the airways.

With the demonstration of a specific role for Treg-expressed CTLA-4 (cytotoxic T-lymphocyte antigen 4) in suppressive

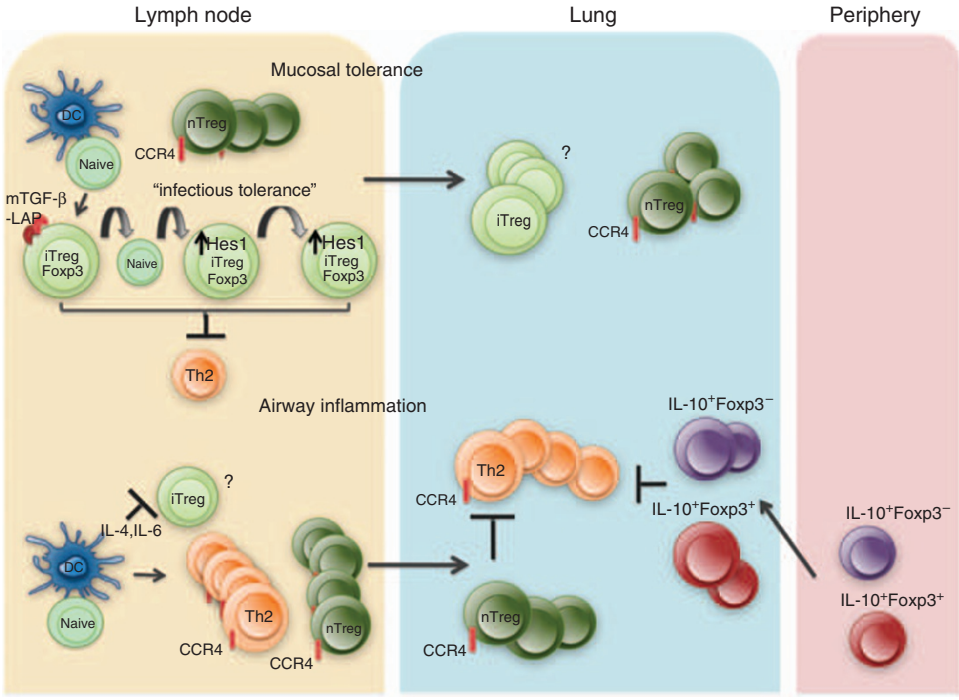

Figure 2 Mucosal tolerance favors inducible/adaptive Treg (iTreg) development in lung-draining lymph nodes that have free reign in tolerance but compete with nTregs during inflammation. Latency-associated peptide (LAP)-associated membrane-bound transforming growth factor-β (mTGF-β) on iTregs exerts an effect through infectious tolerance to increase its pool size.[72] mTGF-β induces Notch activation and upregulation of the downstream repressor Hes1(hairy and enhancer of split 1) in naive CD4+ T cells.[51] Hes1 has been shown to stabilize Foxp3 expression in Tregs.[106] The iTregs efficiently suppress effector cell development (shown is Th2) in antigen-tolerized animals.[51–53,61] In allergen-sensitized animals, the cytokines interleukin (IL)-4 and IL-6 released during priming events suppress Treg induction.[83,88,89,220] However, the same cytokines promote nTreg proliferation while inducing Th2 differentiation[77,83,220] (Th17 cells are also induced by allergens). The Th2 cells and nTregs traffic to the tissue in recall response to inhaled allergen in which they downmodulate costimulatory molecules on dendritic cells (DCs)[54,55] (not shown). If priming occurs at a distant site (skin, spleen), IL-10-expressing Tregs are favored,[47–49,55] which are recruited to the lung in response to allergen challenge to suppress effector T-cell functions.

Table 2 Treg-mediated suppression of effector CD4+ T-cell induction or function in allergic disease

	Regulation of effector T-cell	
Treg type	**Induction**	**Function**
Foxp3+	Ref. [29,51–53,61]	Ref. [54,55,73,77,83,97,118]
Foxp3–	Ref. [43,45,46]	Ref. [47–49]

function through downregulation of CD80/CD86 on DCs,[116] CTLA-4 may have a key role in inhibition of expression of costimulatory molecules by lung DCs in allergic airway inflammation.[54,55] Another cytokine, IL-35, secreted by Tregs has been implicated in the suppressive effects of these cells.[117] In a recent study, a complex of IL-2:anti-IL-2 monoclonal antibody was administered into mice sensitized through the intraperitoneal route with Schistosome antigen or OVA/alum and subsequently challenged with antigen.[118] The regimen of cytokine-antibody complex diminished allergic airways disease and required IL-10-producing Tregs.[118] Interestingly, an increase in IL-35 production was observed in IL-10-competent but not deficient animals in this study.[118] This observation was in line with previous findings that observed IL-10-promoting effects of IL-35. **Table 2** is a summary of the literature that associated either Foxp3+ or Foxp3– Tregs with either induction of effector T cells or their function.

MICRORNA CONTROL OF TREGS

The role of microRNAs in Treg function in the periphery is not sufficiently understood at the present time. Treg-specific deletion of Dicer, the processing enzyme for precursors of microRNAs, had only minimal effect on Treg development, seeding, proliferation, and survival in the periphery. However, splenomegaly and lymphadenopathy was developed in >5-week-old mice and they died at 6–8 weeks of age.[119,120] The role of a specific microRNA, miR-155, in Treg development and function was addressed by using miR-155$^{-/-}$ mice as it is expressed at higher levels in nTregs when compared with that in double-positive thymocytes or CD4+ T cells. Although the frequency and number of Tregs in miR-155$^{-/-}$ mice were reduced by two- to threefold in the thymus and spleen, their suppressive function was not impaired.[121,122] miR-155-deficient mice show a bias toward Th2 development and produce more IL-10-producing CD4+ T cells.[123,124] Thus, generation of IL-10-producing Tregs may involve downregulation of miR-155 expression.

USE OF VARIOUS TREATMENTS TO EXPAND TREGS IN THE CONTEXT OF ASTHMA

Instability of *in vitro*-generated Tregs

All of the compelling evidence in the literature suggests that Tregs are ideal candidates for developing effective therapies to treat diseases such as asthma. We have already discussed about the possibility of developing allergen-specific Tregs in neonates for life-long protection from atopic asthma (**Figure 1**). What about treating asthmatics who already have allergen-specific effector cells? One possible strategy that has been most studied is Treg cell transfer-mediated immunotherapy in which antigen-specific Treg cells are expanded/induced *in vitro* and transferred back into the host. The major hurdles in expanding the existing antigen-specific cells from the repertoire were (i) low frequency of antigen-specific Treg cells, especially in tissues, (ii) poor proliferation *in vitro*,[125,126] and (iii) expansion of contaminating effector cells in the presence of IL-2.[126,127] Given that polyclonal Tregs have been also shown to exercise suppressive effects,[56] a possibility exists in using *in vitro*-expanded polyclonal Tregs. However, it was shown that *in vitro*-generated human Foxp3+ CD4+ T cells lack suppressive function and produce effector cytokines.[128] In addition, in the absence of exogenous TGF-β, the majority of *in vitro*-induced Tregs lose their Foxp3 expression.[129,130] As stable Foxp3 expression is quintessential for the maintenance, sustenance, and suppressive functions of Treg cells,[131] the efficacy of TGF-β-induced Foxp3+ CD4+ T cells in comparison to nTregs is doubtful. The following section discusses the role of various agents that have been shown to promote Treg generation and/or function and the relevance of these findings in suppressing allergic responses in the lung.

Retinoic acid

In vitro, TGF-β-induced conversion to Foxp3+ Tregs has been shown to be enhanced and stabilized by the vitamin A metabolite, retinoic acid.[67,88,132–138] Retinoic acid was shown to induce *de novo* conversion to Treg cells even at high levels of costimulation, suggesting that by some unknown mechanism, retinoic acid overrides the inhibitory effect of costimulation on the induction of FoxP3 expression.[138] Recently, it was shown that promotion of Foxp3+ iTregs by retinoic acid is not due to its direct effect on the responder cells but because of inhibition of cytokine production by CD44^{hi} effector memory T cells.[135]

Similar synergistic relationship between TGF-β and retinoic acid in inducing, expanding, and maintaining Tregs has been observed *in vivo*.[135,139] To understand the function of retinoic acid in the generation of Tregs *in vivo*, we need to understand its metabolism. Retinoic acid production from retinol is a sequential process that ultimately involves retinal to retinoic acid conversion by retinal dehydrogenases (RALDH). Among the various biologically active retinoids, all-*trans* retinoic acid (ATRA) is the most potent known metabolite.[140] Extensive research has characterized ATRA as the physiological ligand for the retinoic acid receptor family of nuclear hormone receptors.[141,142] Multiple studies have recently shown production of retinoic acid or expression of retinal dehydrogenases (RALDH1 and/or RALDH2) in different cell types in the gut promoting Treg development. Even though both vitamin A intake and its metabolism to retinoic acid have been shown to be essential in lung development and maintaining pulmonary homeostasis, the cellular participants responsible for generating retinoic acid in the lungs are still unclear. In one study,[143] isolated lung lipid interstitial cells were found to be capable of converting all-trans retinol to an acidic retinoid with properties that are similar and possibly identical to those of ATRA. Most of the retinoic acid produced by the lipid interstitial cells was secreted in the lung alveoli, in which it could be taken up by other lung cells to exert immunodulatory effects. Recently, airway epithelial

cell-expressed MMP-7 (matrix metallopeptidase 7) was shown to regulate RALDH1, which promoted the development of immunosuppressive Tregs causing attenuation of allergic responses.[144] This finding was in contrast to reports that showed exacerbated allergic responses after *in vivo* administration of ATRA.[145,146] It should be noted that in the steady state, retinoic acid is bound to albumin and circulates in plasma in nanomolar concentrations with a typical half-life, in rodents, of less <1 h.[147] Most studies that have shown the biological activities and importance of retinoic acid in regulating immune responses have used supraphysiological concentrations added either to cultured cells or, less frequently, administered to animals *in vivo*. The dose of ATRA used in the *in vivo* studies has been variable, depending on the mouse model used. Although such experiments have shown the potential scope of retinoic acid-mediated induction of Tregs and suppression of inflammatory responses in the gut, the suppressive effect of retinoic acid in the lung is clearly not uniformly observed. The reasons for the divergent results need to be determined in future studies, especially because there are studies showing Th2-inducing potential of retinoic acid through retinoic acid–retinoic acid receptor-mediated signaling.[145,146,148–150] Iwata *et al.*[150] suggest that this effect of retinoic acid on Th2 differentiation is stage dependent and retinoic acid enhances Th2 responses only if it is added in initial stages of T helper cell differentiation.

Vitamin D

Independently, several investigations have shown a direct relationship between vitamin D and asthma.[151–154] 1,25-dihydroxyvitamin D3 [1,25(OH)2D3)], the active metabolite of vitamin D3, is a lypophilic molecule that exerts its actions through a nuclear receptor, the VitD3 receptor (VDR).[155–157] A number of recent studies have consistently shown the ability of vitamin D3 and VDR agonists to exert an effect as strong immunosuppressors.[158,159] Vitamin D3 has also been shown to directly enhance suppressive function of Tregs.[160] As an immunosuppressant, $1,25(OH)_2D3$ has been implicated in inhibition of Th1 differentiation, as studied in Th1-dominated animal models.[158,161,162] Interestingly, Vitamin D3-VDR signaling and VDR agonists have been shown to either promote or inhibit proliferation and differentiation of Th2 cells.[154,158,163–165] Such opposite effects of vitamin D3 in regulating Th2 cell responses were also observed in models of experimental allergic asthma, in which 1,25(OH)2D3 treatment was either beneficial,[153,154,166] without effect,[167] or deleterious.[153,165,167] As with retinoic acid, the discrepancies in the findings could be due to variations in dose, route of administration, duration of exposure, and the choice of experimental model.

Allergen-specific immunotherapy

Another strategy to expand antigen-specific Treg cell repertoire *in vivo* involves exposure to low doses of antigen without adjuvants. Such protocols induce antigen-specific immunosuppression and are designated allergen-specific immunotherapy (SIT) pioneered by Noon in 1911.[168,169] SIT involves the administration (usually subcutaneous) of increasing doses of allergen to achieve hyporesponsiveness to it. Various effects of SIT have been documented, including induction of Tregs secreting IL-10 and TGF-β.[49,170–173] In the clinical setting, SIT is the only treatment that induces specific Treg cells in human subjects.[174] Although administration of specific allergens has been shown to be effective in rhinitis[175] and insect venom allergy,[176,177] the role of this intervention in allergic asthma remains controversial.[178–180] SIT has proven efficacious in treating mild asthma,[181] as well as for preventing the progression to asthma in patients suffering from rhinitis.[182,183] However, it is not yet recommended for treatment of moderate-to-severe asthma.[184] Furthermore, the major drawbacks of this treatment are the risks for inducing rare but life-threatening systemic reactions mediated by IgE in patients requiring long-term administration of SIT.[179,185]

Strategies to improve the safety of allergen immunotherapy were initially focused on reducing the allergenicity of the preparation by using modified allergens (allergoids), novel adjuvants, and use of alternate routes of administration.[186–188] Along these lines, peptide immunotherapy, involving use of peptide fragments from allergens corresponding to T-cell epitopes to induce immunologic tolerance, has met with success in experimental models of allergic disease.[189] Peptide immunotherapy is an advantage over SIT as it does not result in adverse IgE responses because of their relatively small size and therefore inability to cross-link allergen-specific IgE.[190,191] Peptide treatment suppressed allergen-specific T-cell proliferation and production of cytokines IL-4, IL-13, and interferon-γ but induced IL-10 production[192–194] and IL-10-dependent functional Tregs.[195] Because of the successful induction of tolerance and suppression of inflammatory responses, peptide immunotherapy has emerged as an ideal alternative to SIT. Currently, clinical data are available for two allergens that have been targeted with this approach, which are the cat allergen Fel d 1[192,193,196] and the bee venom allergen Apim1 (phospholipase A2).[176,197,198] Despite accumulation of considerable evidence showing a beneficial role of allergen-derived peptide immunotherapy, there have been instances in which late asthmatic reactions followed by bronchial hyperresponsvieness to the peptides were reported in patients.[192] These adverse effects could be related to peptide dose and immunogenicity. Further attempts are being made to fine-tune the existing protocols that can provide maximum benefit at lower doses of peptides. Recently, linked epitope suppression, a key immunologic mechanism of peptide immunotherapy was shown, in which treatment with selected epitopes from a single allergen, Fel d 1, resulted in suppression of immune responses to other "linked" epitopes within the same molecule.[49] Additional evaluation of appropriate immunogenic peptides from different allergens would increase the applicability of peptide immunotherapy to severe asthmatics.

Corticosteroids and rapamycin

Corticosteroids or glucocorticoids are by far the most routinely used and most effective anti-inflammatory therapy for both acute and chronic allergic diseases and asthma. Among the various mechanisms identified for glucocorticoid-mediated suppression, promotion of Foxp3 expression and suppressive function in CD4+ T cells was also noted.[199] The increase in Foxp3 mRNA subsequent to glucocorticoid treatment, although

transient, was tightly correlated with increase in IL-10 mRNA, although the expression of TGF-β remained unchanged. Similar increases in IL-10 synthesis and reduction in pro-inflammatory cytokine production subsequent to inhalation of corticosteroids have been reported in asthma patients.[200,201] A more conclusive study recently showed that an inhaled corticosteroid could reverse the observed poor numbers of CD4 + $CD25^{hi}$ cells in the bronchoalveolar lavage fluid of asthmatic children.[202] However, treatment with corticosteroids (dexamethasone) has been also shown to inhibit induction of IL-10 and development of Tregs.[203] The researchers have cautioned against long-term use of corticosteroids, especially in treating chronic conditions. They suggest that the inhibitory effect of corticosteroids on Treg development may cause excessive pro-inflammatory responses when the individual is re-exposed to the allergen. In contrast, Barrat *et al.*[158,159] have shown that dexamethasone, in synergism with 1α, 25-dihydroxyvitamin D3 (calcitriol), the active form of vitamin D, directly induces IL-10 secreting regulatory T cells (Tr1 cells).

Rapamycin, a small molecule drug, chemically defined as a macrocyclic lactone, routinely used as an immunosuppressive drug in organ transplantation, has been found to promote *in vitro* expansion of both murine[204,205] and human Tregs,[206–209] which could be highly significant in generating clinically relevant quantities of Tregs.[210] The mechanisms underlying the selective and preferential expansion of Tregs and maintenance of suppressive activity in the presence of rapamycin are unclear. Some studies attribute it to selective survival advantage,[209] whereas others show induction of Treg-like phenotype in T effector cells in the presence of rapamycin.[211–213] Recently, a study showed that the molecule Pim2, which is selectively upregulated in Foxp3-expressing cells, is responsible for providing the survival signal.[214] This preferential expansion or survival of Treg cells has also been shown after *in vivo* administration of rapamycin.[215] In independent studies, rapamycin has been shown to inhibit asthma and airway remodeling in experimental models,[216–219] thereby highlighting another drug that can be used to expand antigen/allergen-specific Tregs.

CONCLUDING REMARKS

Both human and animal studies show that Tregs are regulators of allergic airways disease. There does not seem to be a unique mechanism that underlies their regulatory function in this disease as has been realized in other diseases as well. It is clear that thymus-derived nTregs are not the only Tregs that are involved in suppression of allergic disease. iTregs have been identified in early life and the challenge in future years is to capitalize on this realization to induce allergen-specific Tregs. However, multiple issues and controversies need to be resolved before Treg cell-based immunotherapy becomes routine practice. More knowledge about the molecular mechanisms driving Treg cell proliferation, activation, and survival is required. Investigations pertaining to stability of Foxp3 in *ex vivo*-manipulated and later adoptively transferred induced/expanded Treg population is critical, especially in light of the concept of exFoxp3 cells.[93,94] Given the intense interest in these cells in the field of immunology as a whole, it is hoped that the basic knowledge gained about these cells would translate into dependable Treg-mediated patient care in the not too distant future.

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DISCLOSURE

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