

Review article

Regulatory T cells in bronchial asthma

The main focus of this review was the role of a specific subset of T cells with immunomodulatory or immunosuppressive activities, termed regulatory T cells (Tregs), in the pathogenesis and treatment of bronchial asthma. Evidence that these cells are important in maintaining immune homeostasis in health and exhibit impaired activity in active disease will be discussed. Their therapeutic potential is perhaps best highlighted by evidence that therapies with demonstrated efficacy in allergic and asthmatic disease are associated with the induction or restoration of regulatory T-cell function, e.g. glucocorticoids, allergen immunotherapy. Strategies to improve the safety and efficacy of these treatments and that induce or boost Tregs in bronchial asthma are discussed.

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Asthma is a chronic inflammatory disorder of the airways associated with airway hyper-responsiveness (AHR) leading to recurrent episodes of wheezing, breathlessness, chest tightness and coughing. These episodes are usually associated with airflow obstruction within the lung that is often reversible either spontaneously or with treatment (1). The aetiology of asthma is multifactorial. There is a strong genetic influence, illustrated by the phenomenon of atopy, where individuals have a hereditary predisposition to produce IgE antibodies against common environmental allergens (2). Genes have also been identified that influence bronchial hyper-responsiveness independently of atopy (3).

The environment also affects the asthmatic phenotype, particularly during early development. Breastfeeding in the neonatal period may have a protective effect against atopy (4), promoting tolerance through chronic oral administration of antigen (5). Allergen exposure later on in infancy, by contrast, promotes atopy (2). Another important environmental factor is infection, which has variable influence. It may be beneficial, as demonstrated by the low prevalence of atopy in countries with a high incidence of infectious disease. Atopy in this instance may be prevented by infection promoting the development of immunoregulatory responses that dampen down inflammation (6). There is an association between birth order and atopy, which probably represents protection from atopy secondary to cross-infection between siblings (7).

However, infection can also promote the development of asthma, as exposure to respiratory viruses may promote asthma in atopic infants (8). Pollution can precipitate asthma exacerbations (9), but limited evidence also suggests that it may be a risk factor for development of allergic sensitization/asthma for children living in urban environments – both particulate matter and nitrous oxide found in traffic fumes have been associated with the development of allergic sensitization and persistent wheezing in infants (10–12). Dietary intake can also have an effect on allergic airway disease. Nutritional deficiencies, such as lack of the fat-soluble vitamins D (13) and E (14) have also been associated with asthma, particularly if the maternal diet is poor.

A number of different cells are involved in asthmatic inflammation (Fig. 1). In patients with asthma, inhaled allergen activates mast cells following cross-linking of allergen-specific IgE and binding to high affinity IgE receptors on the cell surface (15). This promotes release of preformed mediators such as histamine and cysteinyl leucotrienes, triggering acute symptoms (early allergic response). Further infiltration of the airway by eosinophils and Th2 lymphocytes with release of the cytokines IL-4, IL-5, IL-9 and IL-13, occurs a few hours later leading to the late allergic response which is more prolonged (2). Eosinophilic inflammation is also triggered by viral infection (16) which is a frequent precipitant of acute exacerbations. Evidence for this pattern of

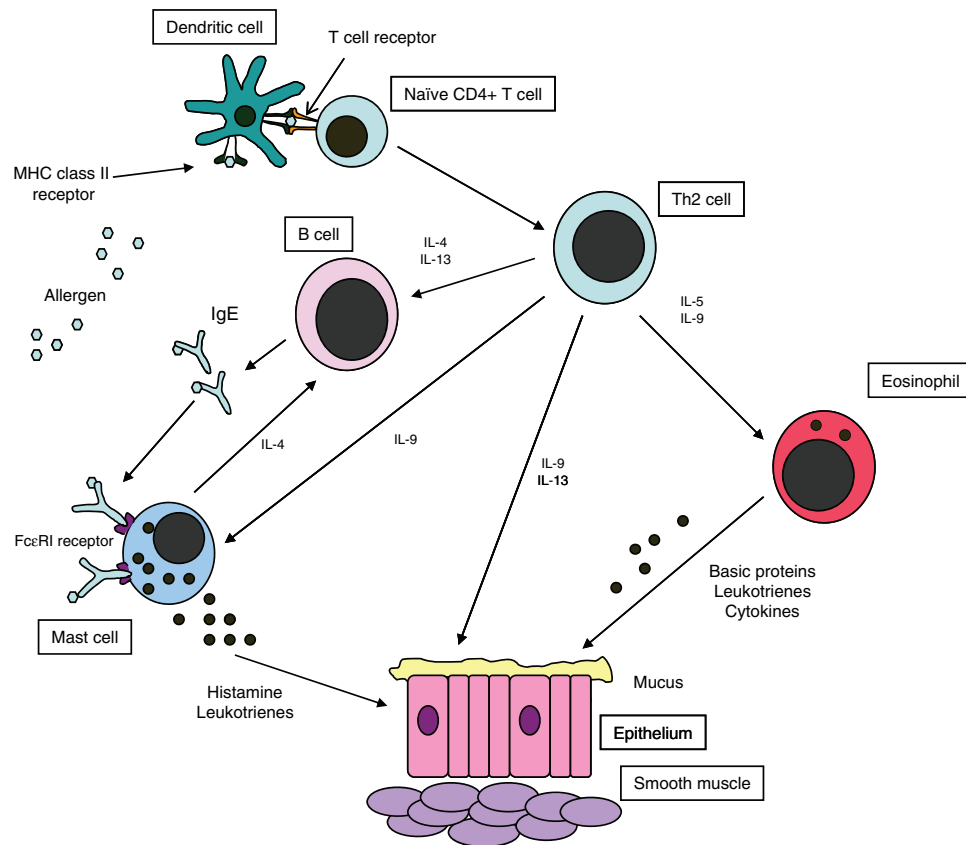


Figure 1. Mechanism of allergic asthma. Sensitization in atopic asthmatics occurs when allergen is presented to T cells, which differentiate into Th2 cells and release cytokines that stimulate B cells, leading to the production of allergen-specific IgE. When a person is challenged with the same allergen, IgE binds to FcεRI receptors on mast cells, resulting in degranulation and release of histamine and leucotrienes. This phenomenon results in bronchial hyper-responsiveness and airway constriction (early allergic response). A few hours after the initial allergen challenge, Th2 cells secrete cytokines that stimulate eosinophil and B-cell production, as well as cytokines that have direct effects on airway epithelium and smooth muscle. The activated Th2 cells and eosinophils promote further inflammation and bronchoconstriction (late allergic response).

inflammation has been seen in autopsy specimens from patients with fatal asthma, as well as bronchial biopsy specimens (15). Eosinophil infiltration, Th2 cytokines and cells with markers of T-cell activation (such as CD25) have been identified in bronchial biopsy specimens from patients with allergic disease, nonatopic asthma and occupational asthma (17, 18).

Structural remodelling of the airway occurs in addition to inflammation, with changes including damage to the epithelium, recruitment and activation of myofibroblasts, and hyperplasia of goblet cells, smooth muscle and fibroblasts. Increased deposition of collagen and other extracellular matrix proteins is found in the lamina reticularis of the basement membrane and throughout the bronchial mucosa in association with airway angiogenesis (19). There is a dynamic interaction between the cellular infiltrate and the damaged epithelial and mesenchymal tissues as a result of direct effects and induction of pro-fibrotic growth factors (19–21).

Current therapies and the unmet clinical need

The traditional pharmaceutical approach to control bronchial asthma has focused on use of potent anti-inflammatory drugs, particularly steroids (often in conjunction with long acting β_2 agonists), which have a broad-spectrum inhibitory activity against a range of effector cells and their products (1). Glucocorticoids are effective in the majority of patients but fail to provide sustained relief of disease symptoms without repeated administration, even after many months or years, and they do not provide a cure. Long-term, high-dose usage is generally unpopular with patients and can be associated with side-effects. Furthermore, glucocorticoids fail to control the disease fully in a significant number of asthma patients. These individuals are most at risk of hospitalization and death from their asthma. These data highlight the need for more specific therapies with the potential to provide long-term relief from clinical

symptoms and to control the increasing prevalence of asthma.

Uniquely in allergic disease, a commonly available antigen-specific therapy exists with the potential to provide specific and long-term relief from disease symptoms. Allergen immunotherapy involves the injection of increasing amounts of allergen to which the patient is allergic under carefully controlled clinical conditions, resulting in immunological 'desensitization' and some clinical benefit. Although greatly encouraging this treatment is most effective in certain subgroups of patients, e.g. those with high levels of IgE, it needs to be administered over several years for maximal efficacy. Studies have indicated a potential benefit of this therapy in asthma (22), although immunotherapy carries a significant risk of adverse events and even on occasion fatality. Improving the safety and efficacy of antigen-specific therapies, especially for allergic asthma, would be of great benefit and represents a major research initiative (23).

A number of novel therapeutic approaches that modulate effector mechanisms associated with Th2-mediated responses have been investigated in a series of clinical trials, including antagonist-specific for IL-4, IL-5 and IgE (24). However, these studies in general highlight that targeting a single immune mediator (e.g. a cytokine) may be insufficient to control the disease fully (25). DNA vaccines and other molecular methods of down-regulating antigen-specific Th2-mediated responses have also been studied (26–28).

Immune homeostasis in the lung

The mucosal milieu of the lung represents a unique immunological environment. Functionally, it needs to protect the lung from inhaled pathogens, whilst minimizing inflammation and damage to the fragile structure of the lung tissue essential for the primary function of gaseous exchange. For this purpose, potent phagocytic and antimicrobial pathways exist, and the alveolar macrophage appears particularly important for this (29). However, studies from animal models and human studies also suggest that inhibitory pathways exist in parallel, which dampen down pro-inflammatory and adaptive immune responses (30). Important mechanisms proposed to preserve the functional integrity of the lung in health include the anti-inflammatory cytokine IL-10 and regulatory T cells (Treg) (31).

Regulatory T cells – introduction

T cells with immunosuppressive or immunomodulatory activity, generally termed Treg, prevent the generation of immune responses to self-antigens and innocuous environmental antigens, including allergens, in the periphery. They also likely limit immune responses to pathogens,

preventing excessive tissue damage following pathogen clearance. Treg inhibits T lymphocyte, antigen-presenting cell and innate cell functions via a variety of mechanisms including cell contact-dependent pathways, competition for essential growth factors, cytotoxicity and the secretion of inhibitory cytokines such as TGF β and IL-10 [reviewed in (32); Fig. 2]. The immunomodulatory properties of IL-10 and TGF β relevant to asthmatic disease are the subject of several recent reviews (33–35). The majority of published studies have described the regulatory functions of CD4 $^{+}$ T lymphocytes. However, it is clear that NK and NKT cells, CD8 T cells, $\gamma\delta$ T cells, B cells and even mast cells may possess important regulatory activities. Indeed, any cell with the capacity to secrete inhibitory cytokines has 'regulatory' potential. Nevertheless considerable interest remains in CD4 $^{+}$ Treg cells given the capacity to generate or expand inhibitory cells with unique and disease-relevant antigen specificity.

The investigation of regulatory T cells is hampered by the lack of specific markers to identify this population in patients. For example, constitutive expression of the CD25 antigen, the IL-2 receptor alpha chain, is most commonly used to identify CD4 $^{+}$ Treg. However, CD25 is also expressed by all T cells upon activation, which has led at times in generation of confusing data from patient studies attempting to describe Treg function in conditions mediated by aberrant immune activation. Additional markers identified include CD62L, CTLA-4, GITR (36) and the folic acid receptor (32), but most are also expressed by activated effector T cells. The transcription factor Foxp3 was identified as a marker of Treg following the discovery of scurfy mice with autoimmune disease (37) and immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), an X-linked chromosome disorder with a similar phenotype in humans (38). However, in humans Foxp3 expression can be induced, at least transiently, upon activation of effector T cells. Despite these difficulties, there is enough functional data to suggest that the use of Foxp3 and CD25, together with the CD127-negative phenotype, identifies a significant proportion of Treg.

In broad terms thymus-derived Foxp3 $^{+}$ Treg, thought to be specific for self-antigens, and adaptive Treg (Foxp3 $^{+}$ and Foxp3 $^{-}$) induced in the periphery have been described (Fig. 2). However, the relative importance of thymus-derived *vs* peripherally induced Foxp3 $^{+}$ Treg in allergic airway disease is unclear and difficult to ascertain. Peripheral induction may represent an important mechanism to generate cells with specificity for exogenous or environmental antigens, including allergens and to maintain the Foxp3 Treg population with age (39) and a recent study in mice provides evidence to support a role for peripheral induction of Foxp3 $^{+}$ Treg to control allergic airway disease (40). In addition, a number of antigenic stimulation regimes have been described that promote the generation of adaptive Treg secreting inhibitory cytokines [reviewed in (34)], which are also generally CD25 $^{+}$. It is thought that

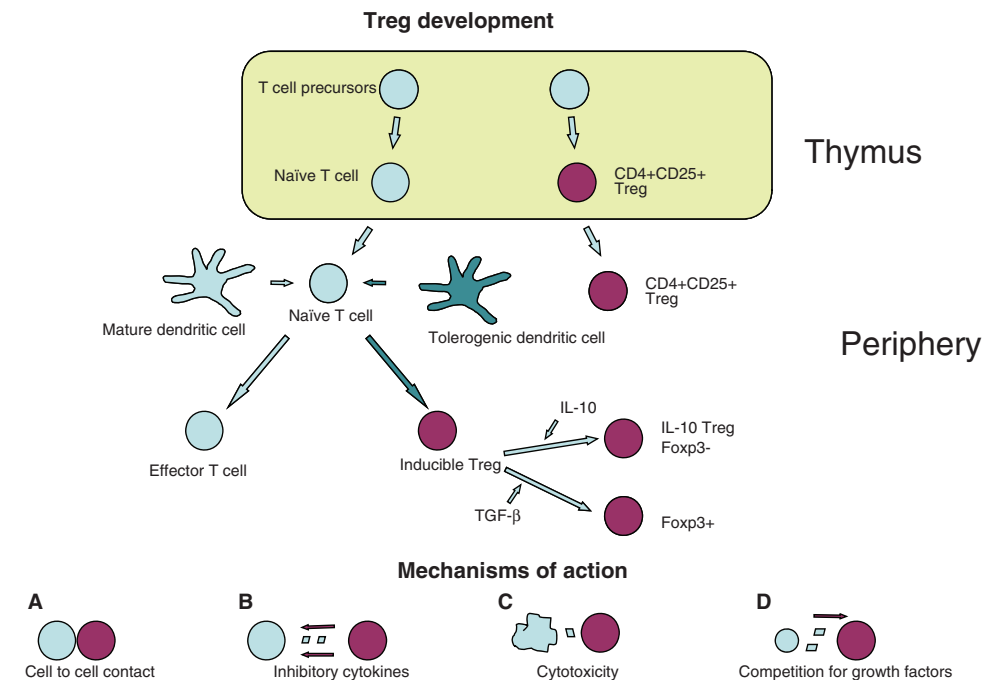


Figure 2. Natural Treg (CD4+CD25+) are derived from the thymus, and are thought to be responsible for deletion of self-reactive T cells that have escaped negative selection and moved into the periphery. Treg can also be induced in the periphery from naïve T cells. Both natural and inducible Treg attenuate inflammation by acting on effector T cells. Treg exert their actions on effector cells through several pathways - cell to cell contact (A), inhibitory cytokines such as IL-10 and TGF- β (B), cytotoxicity mediated by perforins and granzymes (C) and competition for T cell growth factors such as IL-2 (D).

IL-10 secreting Treg, frequently termed Tr1 or IL-10-Treg, are likely to be of specific relevance in immune homeostasis in the lung and asthma (31, 33). Treg secreting TGF β may be more relevant in the gut (35, 41), but they also possesses the potential to promote pathology in the lung through the capacity of TGF β to promote wound healing, fibrosis and structural remodelling. We do not yet fully understand the relationship between these different Treg populations and their relative importance in disease control, a major stumbling block representing lack of markers that distinguish these populations. Although earlier studies focused on the induction of Treg from naïve T-cell populations, a newly emerging concept, beyond the scope of this review, is the plasticity of T-cell phenotype and the potential to generate Treg from effector T-cell populations (39).

Experimental evidence for the role of Treg in health and asthmatic disease

Although numerous publications have compared the function of Treg in the peripheral blood of healthy and asthmatic subjects, it is clear that it is both more difficult, but essential, to study cells at the active site of disease, the respiratory tract. Important clues about Treg function are also emerging from animal models of allergic airway disease.

In vivo experimental models

A number of different animal models of allergic airway disease or 'asthma' have been described, although none captures all aspects of human disease. Many have used alum-induced sensitization via the peritoneum and acute respiratory challenge with allergens (which until recently were rarely representative of common human aeroallergens), to study the development of airway inflammation and AHR in mice and few attempts to study the structural remodelling in the airways associated with human disease. The majority of studies, although generally not contradictory, all identify slightly different aspects of Treg function, based in part on differences in animal strains, allergens, adjuvants, route of antigen administration, the duration and dose of allergen, sensitization protocols used and therapeutic strategies investigated. The different aspects of these models, their use to study tolerance induction, the role of regulatory T cells and the relevance to human disease have been the subject of several recent reviews (31, 42–44) and in general support the paradigm that a failure of tolerance plays a prominent role in the development of allergic disease. Most models readily demonstrate the capacity to tolerate before sensitization of naïve mice to allergen; however, more therapeutically relevant are those studies that address the capacity to modify the course of established disease and to induce or restore tolerance to

allergen at this stage. Studies in animal models of allergic airway disease have focussed on three pathways of regulation, which are by no means mutually exclusive: IL-10, TGF β and CD25^{high} Treg.

A number of studies have highlighted the importance of IL-10 in maintaining immune homeostasis in the lung [reviewed in (34)]. Interleukin-10 gene knockout mice (IL-10^{-/-}) were used to examine the role of endogenous IL-10 in the control of allergic lung responses and demonstrated that IL-10 is a natural suppressor of cytokine production and inflammation in a murine model of allergic bronchopulmonary aspergillosis (45). More recently, conditionally disrupted IL-10 expression in Foxp3⁺ Treg cells resulted in spontaneous colitis and immune inflammation in the lungs and skin. The authors concluded that IL-10 is important at environmental interfaces for limiting excessive immune responses (46). Intranasal instillation of recombinant IL-10 at the time of allergen challenge (47) and IL-10 gene delivery to the lung suppressed cellular recruitment and airway inflammation (48, 49). Adoptive transfer of IL-10-secreting Treg inhibited a Th2-specific response *in vivo* (50), whilst CD4⁺ T cell engineered to express IL-10 controlled airway hyperactivity and inflammation (51). A number of, but not all, studies of inhalational tolerance induction in the airways also support a role for IL-10 in therapeutic efficacy (52, 53). Together, these and other studies provide strong support for the role of IL-10 in maintaining immune homeostasis in the lung and its therapeutic potential in allergic airway disease.

The evidence for a role of TGF β in maintaining immune homeostasis in the lung is more complex as this cytokine exhibits both regulatory, but also pro-fibrotic actions. TGF β is produced by many different cells and inhibits a wide range of immune cells and functions (35, 41). TGF β -expressing T cells have been shown to control allergic airway inflammation (54). Studies in TGF β 1 heterozygous mice expressing lower levels of TGF β demonstrated exacerbated airway disease compared with wild-type animals, suggesting the role of endogenous TGF β in suppressing the development of airway disease (55). Blocking TGF β signalling in T cells also resulted in enhanced airway hypersensitivity, airway inflammation and increased Th2 cytokine production (56). Whilst these models provide evidence that TGF β can block allergic airway inflammation in mice, they predominantly represent models of acute airway inflammation. The role of TGF β in wound repair and fibrosis is also well recognized. Importantly in a chronic allergen challenge model of airway disease administration of neutralizing monoclonal antibody to TGF- β , led to a reduction in airway remodelling, but not in airway inflammation (57). However, in a different model of chronic airway disease TGF β was shown to regulate house dust mite (HDM)-induced chronic airway inflammation but not remodelling, with the authors arguing for caution against the use of therapeutic strategies aimed at interfering with TGF β activity in the treatment of this disease (58). TGF β has the capacity to control airway

inflammation. It may be important in the repair of asthmatic airway, but also has the capacity to induce fibrosis and structural remodelling in the airways. Existing data from animal models argue for a role of endogenous TGF β in preventing the induction of allergic airway disease and the requirement for TGF β in the peripheral induction of Foxp3⁺ Treg may be relevant to this. However, the consequences of targeting TGF β in disease are likely to be complex and further work is required to allow full interpretation of the results from the different experimental systems. Finally, it is worth highlighting that TGF β is a member of a super family of molecules (59) which are also likely to impact on pulmonary function, e.g. activin A (60).

Whilst most studies identify the capacity of CD4⁺CD25⁺ Treg to control allergic airway inflammation, these studies do not always agree on the mechanisms involved, specifically the importance of IL-10 and TGF β . For example, the prophylactic administration of CD4⁺CD25⁺ Treg cells has been shown to inhibit disease. In one study, systemic administration of purified antigen-specific CD4⁺CD25⁺ Treg cells, before challenge of immunocompetent, previously sensitized mice inhibited AHR, eosinophil recruitment and Th2 cytokine production (61). The Treg cell-mediated inhibition was dependent on the induction of IL-10 from host T cells in the lung (61). However, a separate study (62) demonstrated that intratracheal, but not intravenous, administration of naïve lung CD4⁺CD25⁺ T cells before allergen challenge of sensitized mice resulted in reduced AHR and inflammation, and that both IL-10 and TGF- β were necessary for the suppressive function of the CD4⁺CD25⁺ Tregs. In a complimentary approach, depletion of CD25⁺ cells was used to examine the capacity of CD4⁺CD25⁺ Treg cells to control allergic airway disease (63). Depletion of CD25⁺ cells before delivery of HDM allergen led to significantly increased Th2 cytokine responses, IgE levels, eosinophilia and AHR in allergy-resistant mice (C3H strain), but had comparatively little effect in the allergy-sensitive mice more commonly used by other groups. Another study using a natural protein allergen, Der p1 (64), showed that CD4⁺CD25^{high} Treg depletion exacerbated allergic airway inflammation, whilst adoptive transfer of naïve CD4⁺CD25⁺Foxp3⁺ Treg abolished airway inflammation and concluded that Treg control disease resolution, but in an IL-10 independent manner. The maintenance of protective Treg activity is dependent on the continuing presence of allergen stimulation (65), which is likely to be due to the continuing presence of necessary chemokines for Treg recruitment and retention in the airways (66). Recently, the therapeutic transfer of CD4⁺CD25⁺ Treg has been shown to not only resolve established allergen-induced pulmonary inflammation (eosinophilia, Th2 infiltration, IL-5, IL-13 and TGF β), but also prevent the development of airway remodelling, and reduce mucus hypersecretion and peribronchial collagen deposition (66). These data are encouraging with respect to control of established and chronic disease.

A number of therapeutic strategies to inhibit allergen-induced airway disease have been investigated in mice. The most widely studied allergen in the therapeutic context is OVA, which has been administered via aerosol, intranasally or intraorally to induce tolerance. TGF β predominates in models using comparatively lower allergen doses (67, 68), whereas IL-10 may be more relevant to studies with higher doses of allergen used for tolerance induction (52, 53).

Oral antigen administration of allergen can induce TGF β and reduce lung inflammation as well as Th2 cytokine and IgE production (69). Verhasselt (5) demonstrated breast milk-mediated transfer of an antigen to the neonate-induced tolerance and protection from allergic asthma in the offspring that was shown to be TGF β -dependent, in line with a significant body of earlier data highlighting the role of TGF β in the induction of oral tolerance (41). Additional strategies used to modify the course of the disease successfully have been described, including the use of pathogens or their products (70–72) with evidence for the role of the Treg and the induction of inhibitory cytokines [reviewed in (73)]. Several studies have also investigated other potential adjuvants. Taher et al. (74) described a model of allergic asthma where the combined effects of allergen-specific immunotherapy (SIT) with the active form of vitamin D (1 α ,25-dihydroxyvitamin D3) were examined and demonstrated that both IL-10 and TGF- β were important in suppressing asthma. Others have focused on the role of dendritic cells in promoting regulatory cells (52, 53). Although fewer animal studies to date have investigated Treg induction in asthma by allergens known to cause disease in humans, the HDM allergen *Dermatophagoides pteronyssinus* (Der p)2 has been incorporated retrovirally into dendritic cells (75), which then conferred a regulatory phenotype on T cells following transfer to wild-type mice and challenge with mite.

As discussed above, whilst there are many limitations in these experimental models, they remain a crucial tool to understand mechanisms promoting lung inflammation and remodelling relevant to endobronchial asthma and to investigate novel therapeutic strategies.

IL-10, TGF β and Treg in human disease

Whilst studies in animal models provide evidence for a role of IL-10, TGF β and Treg in preventing the development of allergic airway disease and encouraging evidence regarding therapeutic strategies targeting these pathways to control disease, ultimately evidence in humans is essential in realizing novel and improved treatments for asthma.

The anti-inflammatory cytokine IL-10 has important immunomodulatory properties relevant to the maintenance of human pulmonary health [reviewed in (33, 34)]. For example, IL-10 inhibits the synthesis of pro-inflam-

matory cytokines, DC maturation, causes overall dampening of Th1- and Th2-type immune responses, inhibits mast cells and eosinophils mediators of the early and late-phase asthmatic response and favourably modulates IgE to IgG4 ratios (34). Furthermore, decreased IL-10 is observed in allergic and asthmatic disease. IL-10 production is reduced in the lung of severe asthma patients as compared with healthy control subjects (76), whilst genetic polymorphisms in the IL-10 promoter are associated with more severe disease (77). Overall, an inverse association between IL-10 levels and the severity of allergic and asthmatic disease has been reported (78–81). Furthermore, as discussed below, therapies associated with amelioration of disease, including glucocorticoids and allergen immunotherapy, are associated with the induction of IL-10 synthesis by T cells (80, 82).

In contrast, the importance and role of TGF β , as in mice, is considerably more complex and difficult to evaluate in humans. TGF β appears to be instrumental in influencing airway repair and remodelling in addition to its immunomodulatory properties (35). TGF β 1 is pro-fibrotic and increased levels of TGF β 1 are associated with myofibroblast differentiation and deposition of extracellular matrix proteins (83). An increase in TGF β 2 has been associated with airway remodelling in acute allergen challenge in asthmatics (84). TGF β is increased following experimental infection of bronchial epithelial cells with rhinovirus, which is an important respiratory pathogen in asthma exacerbations (85). However, it seems likely that low levels of endogenous TGF β production may be important in the induction or maintenance of peripheral Foxp3+ Treg, prevention of sensitization and inhibition of existing airway inflammation. Although some reports of TGF β induction following allergen immunotherapy exist (86), this is much less common in comparison with associations with IL-10 (23, 87).

Studies of Treg in human bronchial asthma

Phenotypic studies have evaluated the presence of Treg in healthy subjects and in asthma patients. However, the majority of studies have assessed CD4+CD25+ or CD4+CD25^{high} Treg in the peripheral blood, which is obviously distal from the active site of disease. These studies have also been hampered by the lack of suitable markers to distinguish Treg from activated effector T cells and to identify distinct Treg subsets. This latter point is particularly pertinent as although most contributors describe CD4+CD25+ 'Treg' it is unclear whether these represent Foxp3+ Treg or IL-10 secreting Treg, which can occasionally also express Foxp3. Most *in vitro* studies of Treg function state that cell contact is required for suppression, although the same populations frequently inhibit via inhibitory cytokines *in vivo* (31). Emerging data may provide an explanation for these discrepancies (88), but is clear that an understanding of the relationship

between these subsets and markers to distinguish them is still urgently required for patient studies.

Studies of Tregs in bronchial asthma, as opposed to allergy are much more limited, but there is evidence to suggest that they are associated with the disease in both paediatric (89–92) and adult (93–97) patients. Probably, the most convincing evidence for a role of Foxp3⁺ Treg in preventing the development of atopic disease stems from studies of the X-linked autoimmunity-allergic dysregulation syndrome, also termed immune dysregulation, poly-endocrinopathy, enteropathy, X-linked (IPEX) syndrome. These represent rare X-linked multisystem disorders caused to a genetic abnormality – mutation(s) in the gene coding for the transcription factor Foxp3 on the X chromosome. Young boys affected by this mutation present with enteropathy, endocrinopathy and severe infections, as well as a number of additional autoimmune disorders, and marked allergic manifestations (38, 98), which include food allergies, atopic dermatitis, hyper-IgE and eosinophilia (99). Skewing towards Th2 is seen in humans as part of the disease process and cell analysis has shown a substantial decrease or absence of CD4⁺CD25⁺Foxp3⁺ cells. Foxp3 mutant mice similarly developed an intense multiorgan inflammatory response associated with allergic airway inflammation, a striking hyperimmunoglobulinaemia E, eosinophilia and dysregulated Th1 and Th2 cytokine production albeit in the absence of overt Th2 skewing (100). It is difficult to quantify the number of infants that develop allergic airway inflammation as a result of IPEX, as the manifestation in patients with the most severe forms of the disease tends to be in the form of diabetes, gastrointestinal illness, haematological disturbance and infection within a few months and they die without bone marrow transplantation or immunosuppression. However, reactive airway disease and asthma have been noted in children who have defects in Foxp3 resulting in less severe forms of the disease and live to an older age (101, 102). Interestingly, a variant of IPEX with allergic asthma can be caused by CD25 deficiency alone, with defective IL-10 expression, underlining the importance of this receptor and cytokine in the disease (103).

Data on the number of regulatory T cells present in asthmatic patients are conflicting with many studies failing to see a reduced numbers of CD4⁺CD25⁺ Treg in the periphery. In one of the most comprehensive studies to date, Hartl et al. (90) demonstrated that CD4⁺CD25^{high} T cells are enriched in bronchoalveolar lavage compared with peripheral blood. In the asthmatic children studied pulmonary, but not blood, CD4⁺CD25^{high} T cells were decreased in number; however, this was restored following inhalation of corticosteroids. The number of CD25^{high} cells correlated with clinical parameters such as lung function ($r = 0.85$). In a second independent study (91), a slight decrease in these cells in the peripheral blood of paediatric patients with bronchial asthma and allergic rhinitis was observed. These findings relate to the use of the CD25 marker to identify Treg. On analysis of Foxp3 expression,

both studies found a lower level of Foxp3 mRNA in peripheral blood from asthmatics as compared with control subjects (90, 91). The level of Foxp3 protein was not evaluated in these studies but one might assume that it would be decreased in line with the other findings. Shi (95) identified a higher proportion of regulatory T cells in stable and unstable asthmatics, but the study only measured the total CD25⁺ population, so this population may have included effector cells. Additional studies noted increased numbers of CD25^{high} cells in the peripheral blood of allergic asthma patients despite the lower expression of Foxp3 in comparison with nonatopic control subjects (92) or no significant difference in Treg numbers, based on CD4⁺CD25⁺ phenotype, between atopic asthmatic vs and nonatopic subjects (94). Analysis of additional surface markers has shown that CD25⁺ Tregs isolated in the peripheral blood of asthmatics are CD127^{low} (97) and have higher levels of expression of CTLA-4 (93, 94) and GITR (94). One study demonstrating equivalent numbers of peripheral CD4⁺CD25⁺ Tregs in patients with atopic asthma and healthy subjects, reported that the Treg preferentially expressed CTLA-4, GITR, latency-associated peptide (LAP/TGF- β 1) and Foxp3 (94). Decreased numbers of CD4⁺CD25^{high}LAP⁺ T cells was observed in the acute asthmatic but not other groups studied. Inhaled corticosteroid enhanced the percentage of Treg expressing LAP and this reversal could be reproduced *in vitro*. In conclusion, reduced numbers of pulmonary CD4⁺CD25⁺ Treg have been noted in BAL fluid form of asthmatic subjects, but variable results are observed in studies of peripheral blood. However, the fewer studies additionally measuring Foxp3 suggest this marker of Treg may be reduced in the periphery of asthmatic compared with healthy subjects.

A paediatric study (89) investigated regulatory cells in bronchial tissue. The subjects were young (<2 years) with chronic symptoms suggestive of reversible airway obstruction. Immunohistochemistry was performed on bronchial biopsies from the central airways. CD3⁺Foxp3⁺ cells were identified in bronchial-associated lymphoid tissue but there was no correlation with lung function or atopy. Little is known about regulatory T cells at this stage of lung development so it is difficult to say how this would translate to adults – further biopsy studies (ideally combined with BAL) across the spectrum of age would be useful in this regard.

Treg function in asthma and the effects of therapeutic intervention

The study of suppressive function and the capacity of immunomodulatory interventions to alter Treg numbers and function have been pursued in many fewer studies. Moniuszko (97) studied the response to acute bronchial challenge with HDM allergen and observed that bronchial allergen inhalation in sensitive patients (unex-

pectedly this occurred predominantly in those who did not develop significant bronchoconstriction) was associated with a decrease of proportion of peripheral lymphocytes with a regulatory T-cell phenotype. Several earlier studies that assessed the function of CD4+CD25+ Treg in the peripheral blood of atopic patients in culture proposed that cells in patients with active disease or robust Th2 responses showed less suppressive capacity than those from nonatopics; however, these studies did not assess Foxp3 expression and these results may reflect increased numbers of activated effector T cells in their study samples (93, 104, 105).

Several studies show that there is functional impairment of Tregs in asthmatic patients (90–92, 95). In the Hartl study (90), both blood and pulmonary cells from nonasthmatic children were competent to suppress CD4+CD25– T-cell proliferative, cytokine and chemokine responses. In asthmatic children pulmonary, but not blood, CD4+CD25^{high} T cells were both decreased in number and were less suppressive. Importantly, this inhibitory function was restored following inhalation of corticosteroids. Lin (92) also showed a decrease in efficacy of Tregs in allergic asthmatics. In this study, the expression of Foxp3 and its cell-induced suppressive activity were significantly lower in Der p1-sensitive asthmatic children when compared with nonatopic controls. The expression of Foxp3 and the functional activity of Treg cells were reversed in allergic asthmatics who received SIT. Of particular interest, the addition of recombinant tumour necrosis factor (TNF)-alpha directly downregulated Foxp3 expression *in vitro* and abrogated the cell-induced suppressive function of Treg cells, whilst the TNF-alpha antagonist, etanercept, restored the functional activity and Foxp3 expression of CD4+CD25^{high} Treg derived from allergic asthmatics. This is of particular note as early clinical trials with TNF antagonists have recently been demonstrated to show some efficacy in severe asthma (106, 107). Furthermore, the beneficial effects of TNF antagonists, first pioneered in rheumatoid arthritis, have been linked with a beneficial impact on Treg populations in these patients (108). Treg activity can be dampened by the presence of pro-inflammatory signals, such as IL-6 (109, 110) and a study in a mouse model of allergic airway disease has shown that blocking local IL-6 led to an expansion of Foxp3+ Treg in the lung (111). Thus, studies in asthma that isolate Treg away from the active site of disease and the associated milieu may not fully evaluate impaired regulatory function.

Pharmacological treatments for asthma are able to modify the Treg compartment

Several groups have demonstrated that glucocorticoid treatment beneficially influences Treg, both Foxp3 expression and IL-10. Adult asthmatic patients receiving glucocorticoids had higher levels of CD4+CD25+ Treg

and Foxp3 mRNA expression in peripheral blood cells compared with untreated asthmatic patients (96), whilst Hartl obtained similar results in paediatric blood and BAL following treatment (90). Several studies document the capacity of steroids to promote IL-10 synthesis *in vitro* (112) and following treatment of patients (96, 113). Two studies described the restoration of Treg numbers or suppressive activity following glucocorticoid treatment (90, 114). The data from these studies provide evidence for the theory that steroids lead to immunosuppression via a dual effect on both effector and regulatory T cells.

Evidence that induction of IL-10 production may be therapeutically relevant in the efficacy of glucocorticoid treatment of asthma patients comes from a study comparing the *in vitro* response to steroids of asthma patients who are clinically sensitive or refractory to steroids for improvement of their lung function. Patients with strong clinical responses demonstrated good induction of IL-10, whilst those who demonstrated steroid insensitivity showed poor IL-10 induction (80, 81). The combination of glucocorticoids and 1,25-dihydroxyvitamin D3 or calcitriol, the active form of vitamin D, have been shown to induce IL-10-producing Treg (115–120). As steroid insensitivity in asthma is an acquired defect generally associated with disease severity, the capacity of calcitriol to enhance the steroid-induced IL-10 response in these patients was investigated (81). In a small proof of concept study, ingestion of calcitriol at standard formulary doses for just 1 week restored the subsequent *ex vivo* response of CD4+ T cells from these patients to steroids for IL-10 production (81). A clinical trial to test whether the capacity of calcitriol to restore IL-10 synthesis correlates with a clinical steroid enhancing effect of calcitriol in patients is now awaited. Other drug combinations also clearly promote IL-10 secreting Treg populations. For example, glucocorticoids in combination with long-acting β_2 agonists can also induce allergen-specific regulatory T cells when used in culture of naïve T cells from the peripheral blood of asthmatics (121), although this drug combination was not as effective as the original dexamethasone plus calcitriol combination. These data further suggest that vitamin D may be important in immune homeostasis in the lung. Epidemiological studies demonstrate that vitamin D deficiency is associated with poor respiratory health, including poor control of asthma, and this may be due, in part, to the failure to appropriately induce or maintain appropriate regulatory pathways (13, 120).

Several studies have tried to identify the mechanism of suppression of Treg in asthma patients; however, we believe caution is required in the interpretation of *in vitro* studies and their extrapolation to mechanisms operating *in vivo*. Bellinghausen (93) and Hartl (90) found that Treg activity was not dependent on IL-10. Hartl showed that there was diminution of the Treg response in response to blocking TGF β . However, *in vitro* assays of suppressive function may well be misleading. This is first highlighted by animal studies, reviewed above, where most *in vitro* studies

of inhibition by CD4⁺CD25⁺ Treg demonstrate a requirement for cell contact, whereas *in vivo* the same cells variously inhibit via cell contact, and/or inhibitory cytokine (IL-10, TGF β or both). A very recent study may shed light on this apparent contradiction. The authors demonstrated two phenotypic populations of Foxp3⁺ Treg, based on the lack or presence of inducible co-stimulatory molecule (ICOS), which were both suppressive *in vitro*. The ICOS⁺ Foxp3⁺ Treg population inhibited via IL-10, but survived poorly in culture suggesting some inhibitory mechanisms maybe lost in culture (88).

Allergen-specific immunotherapy is a more specific therapeutic regimen tailored to the needs of individual patients, but it is not without its risks in asthma. It usually requires years of administration and it can only be suitable for certain cases of allergic asthma. The capacity of allergen immunotherapy to promote and act via the induction of IL-10 secreting Treg cells in allergic patients has been widely reported and reviewed elsewhere (23, 87). Fewer studies of IT in asthma have been reported. Of interest, repeated challenge with aerosolized allergen peptides in asthmatic patients has led to the development of late-phase allergic reactions with an increase in AHR (122). Peripheral administration of allergen immunotherapy appears to reduce, rather than provoke, episodes of bronchoconstriction in asthmatic patients (22, 123). Encouragingly, a recent meta-analysis of treatment with sublingual immunotherapy in paediatric asthma (123) showed improvement in a number of clinical scores, although the number of studies included was small. The timing of exposure in reducing the incidence of airway disease seems to be important as evidenced by the preventive allergy treatment study (124), which showed that adults aged 16–25 had decreased rates of asthma 10 years after treatment with immunotherapy for rhinitis. Trials are in progress in the paediatric setting to look at primary prevention of asthma using sublingual therapy with a mixture of inhalant allergens in children who are already sensitized to food (125).

Clinical application of regulatory T cells to control bronchial asthma

There is considerable interest in the therapeutic application of Treg to allergic and asthmatic patients with the

aim of providing a specific, more effective, safer and long-lasting treatment to control disease symptoms. Strategies under consideration are protocols that induce or boost endogenous Treg frequency and/or function in patients, and the infusion of *in vitro* expanded autologous Treg back into patients. In both, it is obviously highly desirable to promote allergen-specific Treg to minimize potential side-effects.

Protocols for the generation of antigen-specific T cells and selective Treg expansion have formed a major sphere of interest in other immune-mediated pathologies (126–128). Although exciting, overall it represents a complicated, time-consuming protocol that could potentially prove difficult to accomplish in humans, with major ethical considerations. Additional issues that need to be resolved (118) include identifying signals that direct Treg homing to the disease site, that control stability of the Treg phenotype, as well as survival and longevity of these cells *in vivo*. Although trials are planned of cell transfer in transplantation and other immune pathologies, it is difficult to envisage this as a major therapeutic strategy in asthma, given the efficacy, if not longevity of current treatments, and concerns relating to the invasiveness, practicality, safety and expense of this strategy to treat human asthma.

From our perspective, the capacity to induce or boost antigen-specific Treg populations in patients, both Foxp3⁺ and/or IL-10 secreting Treg is a more realistic and exciting prospect for the long-term improved and safe treatment of allergic and asthmatic disease. Although many protocols for the induction of different Treg populations are nonspecific, it should be possible to harness the effects of drugs, pathogen components, hormones, modified dendritic cells, cytokines or other agents, in conjunction with an allergen desensitization regime, which uniquely already exists in allergic disease, for this purpose.

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