

Role of Treg in immune regulation of allergic diseases

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Allergy is a Th2-mediated disease that involves the formation of specific IgE antibodies against innocuous environmental substances. The prevalence of allergic diseases has dramatically increased over the past decades, affecting up to 30% of the population in industrialized countries. The understanding of mechanisms underlying allergic diseases as well as those operating in non-allergic healthy responses and allergen-specific immunotherapy has experienced exciting advances over the past 15 years. Studies in healthy non-atopic individuals and several clinical trials of allergen-specific immunotherapy have demonstrated that the induction of a tolerant state in peripheral T cells represent a key step in healthy immune responses to allergens. Both naturally occurring thymus-derived CD4⁺CD25⁺FOXP3⁺ Treg and inducible type 1 Treg inhibit the development of allergy via several mechanisms, including suppression of other effector Th1, Th2, Th17 cells; suppression of eosinophils, mast cells and basophils; Ab isotype change from IgE to IgG4; suppression of inflammatory DC; and suppression of inflammatory cell migration to tissues. The identification of the molecules involved in these processes will contribute to the development of more efficient and safer treatment modalities.

Key words: Allergy · Allergen-specific immunotherapy · Tolerance · Treg

Introduction

The immune system is a complex interactive network with the capacity to protect the host from a broad range of pathogens while keeping a state of tolerance to self and innocuous non-self antigens. Immune tolerance-related diseases such as allergy, autoimmunity, tumor tolerance and rejection of organ transplants arise as a direct consequence of dysregulated immune responses. The main clinical manifestations of allergy encompass allergic rhinitis, allergic asthma, food allergy, atopic eczema/dermatitis and anaphylaxis. Currently, allergen-specific immu-

notherapy (allergen-SIT) by administration of increasing doses of allergen extracts remains as the single curative treatment of allergic diseases with the potential to modify the course of the disease [1].

Adoptive transfer experiments in mouse models of allergy and asthmatic inflammation have shown that Treg are essential for the induction and maintenance of immune tolerance to allergens [2]. In humans, studies on immune responses to allergens in healthy individuals have demonstrated the existence of dominant Treg subsets specific to common environmental allergens [3]. In addition, allergen-SIT represents the only clinically established treatment that induces antigen-specific Treg and peripheral tolerance with the capacity to restore homeostasis in human subjects [3–8]. Accordingly, active immune regulation through allergen-specific Treg emerges as a potential therapeutic option

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in the prevention and cure of allergic diseases. The aim of this review is to discuss the immune regulation mechanisms operating in allergic diseases with a focus on the role of Treg in the generation of tolerance against allergens in healthy immune responses and allergen-SIT.

Immune mechanisms underlying allergic diseases

The immune mechanisms underlying allergic diseases can be divided into two main phases: (i) sensitization and memory, and (ii) effector phase, which can be further subdivided into immediate and late responses [1]. During the sensitization phase of allergic diseases, the differentiation and clonal expansion of allergen-specific CD4⁺ Th2 cells producing IL-4 and IL-13 is essential for the induction of B-cell class-switch to the ϵ -immunoglobulin heavy chain and the production of allergen-specific IgE Ab. Allergen-specific IgE binds to the high-affinity Fc ϵ RI on the surface of mast cells and basophils, thus leading to the patient's sensitization. During this step, a memory pool of allergen-specific T and B cells is also generated. The effector phase is initiated when a new encounter with the allergen causes cross-linking of the IgE-FcRI complexes on sensitized basophils and mast cells, thus triggering their activation and subsequent release of anaphylactogenic mediators responsible for the classical symptoms of the immediate phase (type 1 hypersensitivity).

Late-phase reactions are triggered in the continuous presence of allergen, leading to T-cell activation. Activated allergen-specific Th2 cells produce IL-4, IL-5, IL-9 and IL-13, which play a key role in the maintenance of allergen-specific IgE levels, eosinophilia, recruitment of inflammatory cells to inflamed tissues, production of mucus and decreased threshold of contraction of smooth muscles [5, 9]. As a consequence of these events, the more severe clinical manifestations of allergy, such as chronic persistent asthma, allergic rhinitis, atopic dermatitis, and in extreme cases, systemic anaphylactic reactions appear. Recently, newly identified cytokines such as IL-25, IL-31 and IL-33 have been shown to participate in the Th2 response and inflammation [10–12]. Additionally, other effector T-cell subsets can contribute to ongoing allergic reactions. Depending on the specific disease model and stage of inflammation, Th1 cells can either exacerbate the effector phase, for example, by inducing apoptosis of the epithelium in asthma and atopic dermatitis [13, 14], or dampen allergic inflammation [15]. Recently, it has been shown that IL-32 induced by IFN- γ and TNF- α is an essential player in keratinocytes apoptosis in atopic dermatitis, which leads to eczema formation [16]. An increase in activation-induced cell death of high amounts of IFN- γ -producing Th1 cells, as determined by intracellular staining and flow cytometry, also contributes to the predominant Th2 profile in atopic diseases [17]. It has also been demonstrated that neutralization of IL-17 and Th17-related functions in an experimental asthma model reduces neutrophilia, while increasing eosinophil infiltration in the

lung [18]. In addition, recently, two new subsets of effector Th cells have been identified according to their cytokine signature, Th9 and Th22 cells. Although Th9 and Th22 cells' potential contribution to allergic inflammation requires further investigations, Th9 cells may represent an IL-9- and IL-10-producing subset that lack suppressive function and promote tissue inflammation [19], while Th22 cells contribute to epidermal hyperplasia in inflammatory skin diseases [20, 21].

Treg and immune deviation in allergic diseases

In addition to the above-mentioned effector Th cell subsets, T cells with immunoregulatory properties exist and these are broadly referred as Treg. Other cell subsets with suppressive capacity include CD8⁺ T cells, $\gamma\delta$ T cells, CD4⁺CD8[−] T cells, IL-10-producing B cells, IL-10-producing NK cells, IL-10-producing DC and some macrophage subsets [9, 22]. The main role of all these cell subsets is to maintain integrity of the body through avoiding misguided or excessive immune responses that may result in harmful immune pathology, as well as to keep a state of tolerance to innocuous substances.

Treg have the ability to control and modify the development of allergic diseases altering the ongoing sensitization and effector phases *via* several major pathways (Fig. 1). Treg impair the inflammatory properties as well as the capacity of DC to prime effector Th1, Th2 and Th17 cells while promoting the development of tolerogenic DC phenotypes [23]. In addition, Treg directly inhibit the activation of allergen-specific Th2 cells, thus minimizing the production of IL-4, IL-5, IL-13 and IL-9, which are essential cytokines during the effector phase of allergic reactions [3, 6, 8]. Treg also suppress allergic inflammation through direct action on mast cells, basophils and eosinophils and Treg play an important role in tissue remodeling by interacting with resident tissue cells [24, 25]. Treg can also block the influx of effector T cells into inflamed tissues through a cytokine-dependent rather than a cell–cell contact-dependent manner [26]. As an additional mechanism, Treg also impair the induction of Th0/Th1 cells, thus abrogating apoptosis of keratinocytes and bronchial epithelial cells, which prevents tissue injury [13, 27]. Importantly, Treg exert a direct effect on B cells, suppressing the production of allergen-specific IgE and inducing IgG4 [28]. Recently, it has also been demonstrated in a mouse model that antigen-specific natural Treg (nTreg) suppress Th17-mediated lung inflammation, thus regulating lung neutrophilic inflammation, B-cell recruitment and the levels of polymeric IgA and IgM in the airways [29]. To execute all of these functions, Treg employ a broad range of soluble and membrane-bound suppressor factors, such as IL-10, TGF- β , CTLA-4, program death-1 or histamine receptor 2 [3, 7, 30].

As discussed, compelling experimental evidence indicates that Treg play a central role in controlling allergic diseases. These aspects together with various epidemiological studies have led to new interpretations of the hygiene hypothesis. It has been

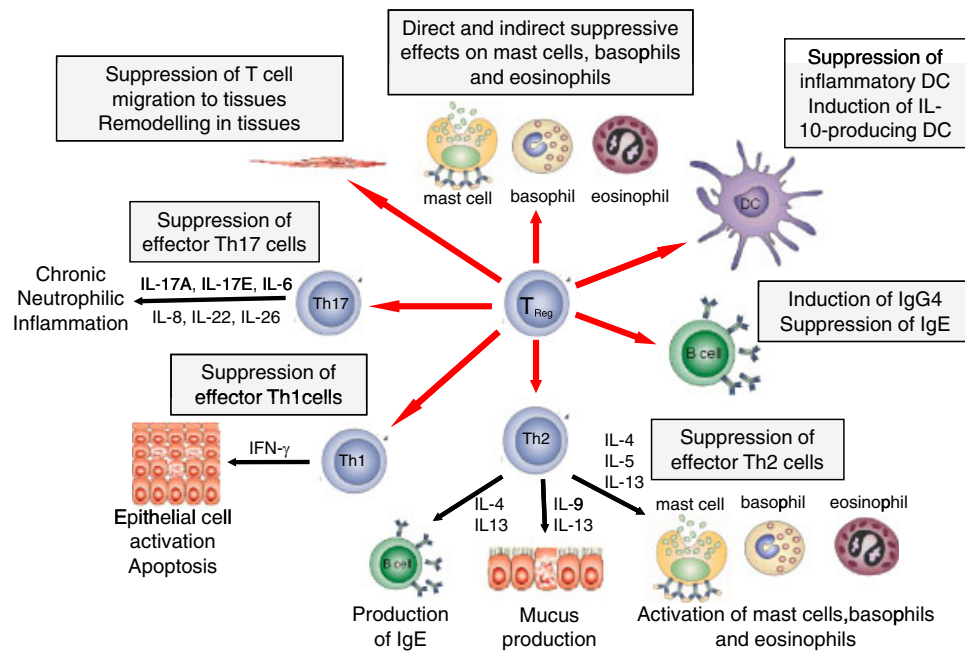


Figure 1. Treg in allergic diseases. Treg suppress allergic Th2 immune responses and contribute to the control of allergic diseases through several pathways. Red arrows indicate the regulatory and suppressive effects that Treg exert directly or indirectly on effector cells (mast cells, basophils and eosinophils), DC, B cells, effector Th2, Th1 and Th17 cells and tissues. Black arrows show the contribution to allergic diseases of activated effector Th2, Th1 and Th17 cells.

proposed that as a consequence of excessive hygiene and lower microbial burden, Treg activity is impaired (Fig. 2), which results in increased Th1 and Th2 responses (reduced immune suppression) accounting for the observed increment of prevalence not only for Th2-mediated allergic diseases but also for Th1-mediated autoimmune disorders [31].

On the other hand, it is noteworthy to mention that over the past 20 years, a large number of studies have contributed to support the original explanation of the hygiene hypothesis, postulating that the outburst of allergic diseases in Western countries is the consequence of a decreased microbial exposure that leads to a missing immune deviation from Th2 to Th1 responses [32, 33]. The lack of microbial stimulation leads to a decreased production of Th1-polarizing cytokines by innate immune cells, which in turns result in a reduced Th1 polarization and increased Th2 response (Fig. 2). Several *in vitro* studies have shown that microbial components or synthetic adjuvants can directly act on innate immune cells such as DC and NK cells triggering the production of IL-12, IFN- α and IFN- γ , thus leading to the switch of allergen-specific Th2 cells toward a Th1 phenotype [34, 35]. *In vivo*, various studies have also demonstrated that stimulation of innate immune cells with microbial or synthetic compounds hamper the development of allergic diseases by promoting Th1 phenotypes. For example, a mouse model of asthma has demonstrated that the administration of the major allergen of ragweed (*Ambrosia artemisiifolia*), Amb a 1, linked to CpG ODN reverses airway hyperresponsiveness [36]. Two common bacterial species identified in farm cowsheds have been shown to induce a Th1-polarizing program in DC that result in an impaired induction of allergic reactions in mice [37]. Evidence

also exists from human studies, which support the hypothesis that a balance of Th1/Th2 responses plays an important role in the development of allergy. For example, children with peanut allergy display predominant allergen-specific Th2 responses, whereas children who outgrow their allergy and children without allergy, show a predominant allergen-specific Th1 phenotype [38]. Several clinical trials have also shown that vaccination with Amb a 1 conjugated to CpG ODN inhibited Th2 responses in peripheral blood, eosinophil infiltration in the nasal mucosa and significantly reduce allergic rhinitis symptoms and the need for medication [39, 40]. Recently, a new molecular mechanism that explains how DC polarize T-cell responses toward a Th2 or Th1 phenotype has been described [41]. The Notch ligand Jagged-1 is constitutively expressed by immature DC and plays an important role in polarizing Th2 responses. Maturation of DC after TLR-triggering by microbial compounds leads to the downregulation of Jagged-1 and upregulation of Delta-4, another Notch ligand playing an important role in the polarization of Th1 immune responses.

CD4⁺CD25⁺FOXP3⁺ Treg

Over the past 15 years, an extensive effort has been performed in the phenotypic and functional characterization of nTreg. Nowadays, it is well established that FOXP3 acts as master switch transcription factor for nTreg development and function [42]. In humans, the *in vivo* relevance of FOXP3 was recognized after the discovery of the X-linked immune dysregulation, polyendocrinopathy syndrome [43]. Patients with X-linked immune

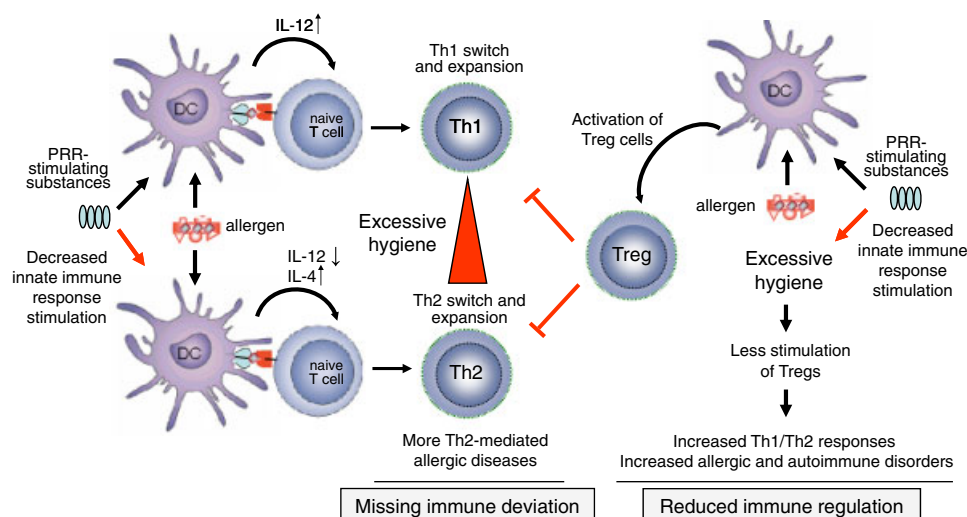


Figure 2. Reduced immune regulation and missing immune deviation in allergic diseases. Deprivation of PRR-stimulating substances due to excessive hygiene leads to decreased stimulation of Treg. Due to the lack of immune suppression by Treg, excessive Th1 and Th2 immune responses occur, which explain the increased prevalence of allergic and autoimmune diseases (reduced immune regulation). Stimulation of DC with allergens and microbial products, simultaneously, induce the activation of Th1-polarizing programs. A lower microbial burden results in the activation of Th2-polarizing programs by DC due to the lack of Th1-polarizing cytokines such as IL-12. As a consequence, immune responses to allergens are shifted toward a Th2 phenotype resulting in an increased prevalence of allergic diseases (missing immune deviation).

dysregulation, polyendocrinopathy syndrome present a typical allergic and autoimmune phenotype due to mutations in FOXP3 leading to non-functional nTreg. Similarly, scurfy mice present a deletion in the forkhead domain of FOXP3, which results in an impaired capacity to develop thymus-derived nTreg [42, 45]. These mice are characterized by a lymphoproliferative disease, hyper-IgE levels and eosinophilia without a Th2 skewing, with a life-span of approximately 3 weeks. Although there is no direct evidence that allergy is due to impaired function and defects of the FOXP3 pathway, a recent study has shown that single-nucleotide polymorphisms of FOXP3 are associated with allergy development in childhood [44]; however, further studies are needed to firmly demonstrate this association.

Recently, it has been shown that nTreg FOXP3 can directly interact with the Runt-related transcription factor 1 (RUNX1), which impairs the expression of IL-2 and IFN- γ and exerts suppressive activity [46]. Although the mechanisms regulating the expression of FOXP3 at the transcriptional level and the molecular pathways involved in the control of sustained high levels of FOXP3 in nTreg is not well understood, new exciting data in this area are emerging. A recent study in mice has shown that the RUNX transcription factors are essential for maintaining high FOXP3 expression, thus ensuring Treg lineage identity [47]. In this context, a new molecular mechanism linking TGF- β and FOXP3 expression in humans has been reported [48]. This study shows that the induction of RUNX1 and RUNX3 by TGF- β play an essential role in the generation and suppressive function of induced Treg. RUNX1 and RUNX3 bind to the FOXP3 promoter and activate the induction of FOXP3-expressing functional Treg. The study demonstrates that these events take place *in vivo* in human tonsils with high expression of RUNX3 in circulating and tonsil Treg. In humans, glycoprotein-A repetitions predominant

has been identified as a key receptor controlling FOXP3 levels in nTreg through a positive feedback loop [49, 50]. Several cytokines (including IL-2, IL-10 and TGF- β), as well as various surface markers such as CD25, CTLA-4, CD103, glucocorticoid-induced TNF family receptor, neuropilin-1 and latency associated peptide are also involved in the thymic development, peripheral maintenance and suppressive function of nTreg. In human adult peripheral blood, two populations of FOXP3⁺ nTreg displaying either a naïve-like (CD45RA⁺) or a memory-like (CD45RO⁺) phenotype have been identified [51]. Recently, the existence of two subsets of nTreg in human thymus and the periphery, defined by the expression of ICOS, has also been reported [52]. ICOS⁺FOXP3⁺ nTreg use IL-10 and TGF- β to suppress DC and T-cell functions, respectively, whereas ICOS⁻FOXP3⁺ nTreg express TGF- β . Interestingly, it appears that the alpha-chain of the IL-7 receptor (CD127) is a definitive surface marker distinguishing between human regulatory and activated effector T cells, thus facilitating both Treg purification and functional characterization in human diseases [53].

Mechanisms of Treg generation

Compelling experimental evidence has demonstrated that the immune system has the ability to induce peripheral mechanisms of immune tolerance to allergens. In these processes, DC play a pivotal role as DC have the dual capacity to mount strong immune responses against invading pathogens and also to keep a state of tolerance to innocuous substances, thus ensuring the integrity of the body in an environment full of pathogens and potential allergens.

The generation of Treg constitutes an essential mechanism in the establishment and maintenance of peripheral tolerance. Certain circumstances and particular microenvironments favor the generation of Treg. For example, specific DC subsets promote the generation of Treg in a microenvironment of tumors and chronic infections. The consequences of this conversion are the maintenance of immune tolerance to tumor antigens/microbial persistence, and limitation of collateral tissue damage. The intestinal lamina propria is constantly exposed to high antigenic pressure (commensal bacteria, food-derived antigens and pathogens) and represents a suitable microenvironment for the generation of Treg that contribute to homeostasis [54].

The tolerogenic capacity of DC depends on certain maturation stages and subsets of different ontogeny and can be influenced by immunomodulatory agents. For a long time, it has been accepted that immature or partially mature DC have the ability to induce peripheral tolerance through the generation of Treg [55] and that fully mature DC prime naïve T cells to different effector Th cell subsets depending on the encounter stimulus [56]. Related to prevention of asthma development, it has been shown that DC distributed throughout the lung capture allergens and migrate to mediastinal lymph nodes within 12 h of activation [57]. These DC express an intermediate array of costimulatory molecules and induce T-cell tolerance. Antigen presentation by partially mature IL-10-producing DC induces the formation of inducible type 1 Treg (T_{R1}) that downregulates subsequent inflammatory responses [58]. It is generally accepted that myeloid DC and plasmacytoid DC (pDC) are different functional subsets that play distinct and complementary roles in innate and adaptive immunity [59]. Maturing pDC have the ability to generate Treg in humans, thus indicating that pDC constitute a unique DC subset exhibiting intrinsic tolerogenic capacity [59, 60]. In support of this concept, depletion and adoptive transfer of pulmonary pDC in mice have revealed that pDC play an essential role in the prevention of allergy sensitization and asthma development [61]. Although further investigations are needed, especially in humans, the application of this concept to allergic diseases may well open new strategies aimed at specifically targeting pDC to generate peripheral tolerance to allergens. The capacity of DC to generate new populations of Treg can also be conditioned by FOXP3⁺ Treg [62]; pathogen-derived molecules, such as filamentous hemagglutinin [63]; and exogenous signals, such as histamine [7], adenosine [64], vitamin D3 metabolites [65], or retinoic acid [66].

Although the molecular mechanisms of Treg generation *in vivo* remain to be fully elucidated, some recent studies have contributed to better a understanding of these processes. A counter-regulation of Th2 and Treg was first described *in vivo* in healthy subjects and in patients with allergy [3]. Recently, a novel mechanism for the inhibition of tolerance induction by a Th2-type immune response has been reported showing that GATA3 directly binds to the promoter region, thus inhibiting the expression of FOXP3 [67]. An interesting dichotomy in the generation of pathogenic Th17 and protective Treg responses have been demonstrated in autoimmune disease models,

whereby TGF- β has been shown to contribute to the generation of both Th17 and Treg. TGF- β directs the peripheral conversion of effector T cells into FOXP3⁺ Treg [68]; however, in the presence of IL-6, TGF- β promotes the generation of Th17 from naïve T cells [69]. Retinoic acid also plays a key role in the balance of inflammatory Th17 cells and suppressive Treg by inhibiting the formation of Th17 cells and enhancing the expression of FOXP3 through a STAT3/STAT5-independent signaling pathway [70].

Role of allergen-specific Treg in healthy immune responses

Several studies in humans have demonstrated that in healthy individuals, if an immune response to common environmental allergens is detectable, T_{R1} cells specific for such allergens represent the dominant subset [3, 6–8]. Both healthy and allergic individuals display allergen-specific Th1, Th2 and T_{R1} cells that recognize the same T-cell epitopes. Accordingly, depending on the predominant subset and the balance between Th2 and T_{R1} cells, the individuals may develop allergy (Th2 predominance) or recovery (T_{R1} predominance). Two human models have demonstrated that high-dose exposure to the offending allergens lead to tolerance induction [7, 71]. Beekeepers are naturally highly exposed to bee venom allergens during the beekeeping season due to an increased number of bee stings. A reduction in T-cell-related cutaneous late-phase reactions and impaired capacity of allergen-specific T cells to proliferate and produce Th1 and Th2 cytokines is observed throughout the beekeeping season, reaching initial levels within 2 to 3 months after initial venom exposure. This regulation correlates with a clonal switch of venom antigen-specific Th1 and Th2 cells toward IL-10-secreting T_{R1} cells. In this model, histamine receptor 2 is upregulated on specific Th2 cells and plays a dual role in the suppression of allergen-stimulated T cells and contributes to increased IL-10 production [7]. In another model of high-dose exposure to cat allergens, IgG4 Ab responses and IL-10-producing T_{R1} cells are induced without subsequent development of new sensitizations or asthma development [71]. Supporting the protective role of Treg in allergy development, a recent study in mice has demonstrated that breast milk-mediated transfer of antigens to the neonate results in oral tolerance induction in an antigen-specific manner preventing allergic airway inflammation [72]. This effect is mediated by Treg and depends on TGF- β signaling. Similarly, it was previously shown in humans that children who outgrew their milk allergy present a higher frequency of Treg and decrease *in vitro* proliferative responses to specific allergens than children who did not tolerate milk and displayed clinical symptoms of allergy after consumption [73].

Role of allergen-specific Treg in allergen-SIT

Allergen-SIT represents the single curative treatment in allergic diseases. It has been used for almost a century as a

desensitization strategy by the repeated administration of increased doses of the causative allergen to induce a state of tolerance. The induction of peripheral T-cell tolerance through the generation of allergen-specific Treg and suppressed proliferative and cytokine responses against the offending allergen represent an essential step in successful allergen-SIT. Several clinical trials have demonstrated that allergen-SIT induces functional Treg with the capacity to modify the course of allergic diseases [4, 8, 74]. Recently, it has been shown that the increased number of FOXP3⁺CD25⁺ Treg in nasal mucosa after grass pollen immunotherapy correlated with clinical efficacy and suppression of seasonal allergic inflammation, thus supporting the role of Treg in the induction of allergen-specific tolerance in human subjects [4]. Several mechanisms involving Treg in tolerance induction after allergen-specific SIT has been documented. Such mechanisms include increased capacity of Treg to suppress Th1 and Th2 cells [75, 76], induction of IL-10 and TGF- β [75, 77], decreased allergen-stimulated T-cell proliferation [77] or suppression of effector cells [78]. Although, in some cases, immunological changes have not been detected [79], similar findings have been also reported in sublingual-specific immunotherapy, in which a sublingual application of the allergen extracts is employed. Classical events associated with the down-regulation of allergic responses such as induction of IL-10 in T cells, suppression of Th2 cells, decreased eosinophil infiltration to nasal mucosa or increased serum allergen-specific IgG4 levels have also been reported in sublingual-specific immunotherapy [9]. Another alternative that has been successfully employed for the induction of peripheral tolerance to allergens is peptide immunotherapy. Mixtures of short peptides derived from the major cat allergen Fel d 1 and the bee venom allergen phospholipase A2 induced downregulation of systemic Th1 and Th2 cell responses to allergens [80] together with concomitant induction of IL-10 production [81, 82].

Concluding remarks

Our understanding of the mechanisms underlying allergic diseases as well as those operating in healthy immune responses to allergens and allergen-SIT has significantly increased over the past decade. Peripheral T-cell tolerance to allergens represents an essential mechanism not only in healthy immune response to allergens but also in successful allergen-SIT. Both CD4⁺CD25⁺FOXP3⁺ Treg and IL-10 and/or TGF- β -secreting T_R1 cells play an essential role in the establishment of a healthy well-balanced immune response to allergens. Recent advances in the field of Treg biology have partially delineated the mechanisms involved in the *in vivo* generation of functional Treg. The identification of new molecules implicated in these processes is emerging. These aspects, together with a better understanding of the role that specific DC subsets play in the generation of functional Treg, will contribute to the design of more efficient and safer immunotherapy against allergic diseases in the near future.

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Abbreviations: Allergen-SIT: allergen-specific immunotherapy · nTreg: natural Treg · pDC: plasmacytoid DC · RUNX: Runt-related transcription factor · T_H1: inducible type 1 Treg

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