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Immunological mechanisms in specific immunotherapy

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Abstract Specific immunotherapy (SIT) represents the only curative treatment of allergy and is, therefore, of particular interest for immunological and pharmacological research. The current understanding of immunological mechanisms underlying SIT focuses on regulatory T cells (T regs), which balance Th1 and Th2 effector functions. This ensures that allergens are recognized, but tolerated by the immune system. There is clear evidence that SIT restores the disturbed balance of T regs and effector cells in allergic patients. Current efforts are focused to improve SIT regimens to make them more applicable in atopy and asthma. The current review provides an overview on the mechanisms of SIT and possible adjuvant treatment strategies on the background of the T reg concept.

Keywords Specific immunotherapy · Regulatory T cells · Allergy

Introduction

Allergies are mainly characterized by IgE-mediated immediate hypersensitivity against environmental antigens (allergens), which are normally tolerated by healthy individuals and which are non-pathogenic. The fact that the disease-relevant allergens are often known allows the specific peripheral tolerance to be restored by repeated injections of high doses of allergen over a long period of time. This specific immunotherapy (SIT) treatment, also termed allergen vaccination, is most successfully applied to allergies against insect venom [1] or to rhinitis [2]. It currently represents the only specific curative treatment of allergy. Ongoing studies are focusing on the molecular mechanisms, but also on improved treatment strategies, including the treatment of asthma and juvenile respiratory disease (Table 1). Studies in recent years have shown a significant treatment success and recommend SIT for treatment of allergies (Table 1). SIT of allergic rhinitis also reduces the risk of developing allergic asthma [3], and was shown to improve clinical symptoms of established disease [4, 5, 6]. Intensive research in the field of SIT has revealed molecular mechanisms

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Table 1 Selection of recent publications on SIT, showing the general positive treatment success of SIT and the increasing attempts to treat or prevent asthma already in children (*SIT* specific immunotherapy)

Title	Therapy	Success	Juvenile	Asthma	Side effects	Mechanistic	Reference
The effect of specific immunotherapy on T-cell receptor repertoire in patients with allergy to house-dust mite	SIT	+				+	[113]
TH2 lymphocytes from atopic patients treated with immunotherapy undergo rapid apoptosis after culture with specific allergens	SIT	+				+	[114]
Variations in serum levels of interleukin (IL)-1β, IL-2, IL-6, and tumor necrosis factor-α during specific immunotherapy	SIT	+		+		+	[115]
IL-10 and TGF-β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy	SIT	+		+		+	[18]
Can HLA typing predict the outcome of grass pollen immunotherapy?	SIT	+				+	[116]
Safety of sublingual-swallow immunotherapy in children and adults	Modified	+	+		(+)		[117]
Long-term efficacy of preseasonal grass pollen immunotherapy in children	Modified	+	+	+			[118]
Efficacy of combination treatment with anti-IgE plus specific immunotherapy in polysensitized children and adolescents with seasonal allergic rhinitis	Modified	+	+				[104]
Safety and efficacy of specific immunotherapy with standardized allergenic extracts adsorbed on aluminium hydroxide	Modified	+		+			[119]
Single-course specific immunotherapy with mixed pollen allergoids: results of a multi-centre study	Modifies	+					[120]
A pre-seasonal birch/hazel sublingual immunotherapy can improve the outcome of grass pollen injective treatment in bisensitized individuals. A case-referent, two-year controlled study	Modified	+		+			[121]
A case control study of dermatophagoides immunotherapy in children below 5 years of age	SIT	+	+	+			[122]
Prevention of new sensitizations in asthmatic children monosensitized to house dust mite by specific immunotherapy. A six-year follow-up study	SIT	+	+	+			[123]
Three years of specific immunotherapy with house-dust-mite extracts in patients with rhinitis and asthma: significant improvement of allergen-specific parameters and of nonspecific bronchial hyperreactivity	SIT	+		+			[124]
Effects of specific immunotherapy in allergic rhinitic individuals with bronchial hyperresponsiveness	SIT	+		+			[125]
A double-blinded, comparative study of the effects of short preseason specific immunotherapy and topical steroids in patients with allergic rhinoconjunctivitis and asthma	SIT	+		+			[4]
Induction of allergy to new aeroallergens during specific immunotherapy (SIT) in grass pollen sensitive patients	SIT	+			+		[126]
How long does the effect of birch pollen injection SIT on apple allergy last?	SIT	+					[127]
Benefits of immunotherapy with a standardized *Dermatophagoides pteronyssinus* extract in asthmatic children: a three-year prospective study	SIT	+	+				[128]

that control peripheral tolerance to allergen and which create the basis for the adaptation of SIT to more complex, atopic diseases.

Cellular requirements for tolerance induction to allergen by SIT

A key feature in the pathogenesis of allergy lies in the generation of allergen-specific IgE instead of IgG and IgA antibodies, which discriminates allergic from non-allergic inflammations. The immediate hypersensitivity reaction is initiated by an allergenic compound, generally soluble proteins or glycoproteins, penetrating through epithelial surfaces and then binding to specific IgE antibodies that were generated before by a first allergen contact. To generate allergen-specific IgE, it is necessary that naïve B cells recognize the allergen and receive help from IL-4-producing T cells, which is an essential cytokine for switching from IgM to IgE. The IL-4 production by T cells is also dependent on the presence of antigen [7]. The crucial role of the T cell is further reflected by the fact that the degree of T cell activation strictly correlates with the number of low-affinity IgE receptor (CD23) -bearing B cells [8], eosinophil cationic protein (ECP) and other serum factors of allergic inflammation [9]. This clearly demonstrates that allergy is a T cell-dependent disease [9, 10]. Thus, it is not surprising that peptides [11, 12] or engineered allergen vaccines [13, 14] lacking B cell epitopes are as efficient in SIT as native allergens. Peptides or engineered allergens appear in fact to be of advantage for the reduction of side effects of SIT, which appear when the allergen cross-links specific IgE on mast cells or basophils, bound to high-affinity FcεRI, leading to activation and degranulation of the cells, and causing the allergic symptoms or even anaphylactic shock.

The SIT shows a reduction in allergen responsiveness in terms of skin prick test and immunological differences become apparent starting on day 7 [15, 16]. It was a key finding that SIT against bee venom allergies induces T cell unresponsiveness (anergy) in vitro, 28 days following initiation of SIT [16, 17]. These experiments support the concept that SIT restores the natural peripheral tolerance to allergens by re-introducing T cell tolerance. This concept of tolerance re-induction is supported by the molecular analysis of SIT and natural tolerance of allergens, as investigated in bee venom-induced immune responses of healthy and bee venom-allergic patients [15].

Molecular mechanisms of SIT

The ex vivo culture of PBMC isolated during the course of SIT showed increased IL-10 production by T cells along with the induction of T cell anergy. Later on, monocytes and B cells also produce IL-10 [15]. Similar observations were made with SIT directed against house dust mite or birch pollen, with the interesting difference that TGF-β was also increasingly produced by T cells [18, 19], suggesting that allergen entering tissues via mucosal surfaces triggers different, TGF-β-dependent tolerization pathways. The T cell anergy could be prevented by the addition of IL-10-neutralizing antibodies or soluble TGF-β receptors to the culture. The cytokines IL-10 and TGF-β are both known to suppress T cell activity [20, 21]. Thus, SIT is accompanied by increasing suppression of the allergen-specific response. The changes on the T cell levels are followed by changes of the B cell phenotype. The cytokines IL-10 and TGF-β also affect B cell activity towards IgG4 and IgA production. Both are suppressive for IgE. Accordingly, the allergen-specific isotype pro-

Table 2 Phenotype of T regs controlling allergen-specific T cells

Phenotype	Function	Reference
Antigen specific	Allergen-driven suppression	Akdis M, et al (submitted)[a]
Non-proliferating	Terminally differentiated?	Akdis M, et al (submitted)
CD4	T cell subset	[15], Akdis M, et al (submitted)
IL-10	Suppression of T cells and APCs	[15], Akdis M, et al (submitted)
IL-10R	Differentiation into T reg?	Akdis M, et al (submitted)
TGF-β	Suppression of T cells and APCs	[19]
CD25	IL-2 receptor, survival and gowth of the T cells	[18]
CTLA-4	Homolog of CD28, inhibits T cell activity	Akdis M, et al (submitted)
PD-1	Receptor of the PDL1, inhibits T cell activity	Akdis M, et al (submitted)
CD105	TGF-β co-receptor, surface binding of TGF-β	Schmidt-Weber CB,et al (submitted)[b]

[a] T regulatory 1 cells in allergic and healthy immune response
[b] Endoglin expression acts as a TGF-β mediator of CD25$^+$ regulatory T cells

file shifts during SIT towards normal IgG4/IgA levels, and the ratio of specific IgE to IgG4 or IgA changes about 100–1,000-fold [18].

It could be shown that suppression not only plays a role in SIT but also maintains allergen tolerance in healthy individuals, who are sensitized to allergen, but do not show allergic symptoms. It was demonstrated that neutralization of IL-10 or TGF-β reveals allergen responsiveness, particularly to allergens to which everyone is exposed, such as food allergens or house dust mites [18]. The T cells which produce these suppressive cytokines are low in IL-4 and IFN-γ, and thus are neither typical Th1 nor Th2 cells. They are called regulatory T cells (T reg) [22, 23]. T regs have been demonstrated to suppress immune reaction in several experimental systems in vitro and in vivo [24, 25, 26, 27, 28, 29, 30, 31]. Recent studies defined a CD25$^+$ subset of T cells, which has a suppressive capacity in vitro and in vivo [32, 33, 34, 35, 36]. However, it is currently not fully established whether CD25$^+$ T cells are identical to IL-10- and/or TGF-β-producing T regs [35], since the suppression was in some cases contact dependent [34]. However, suppressive cytokines may be secreted into an immunological synapse [37] or may act in a surface-bound form on the target cell [38]. This CD25$^+$ T reg subset also plays an important role in allergen-specific immunotherapy (SIT), since SIT-induced unresponsiveness could be prevented by depletion of CD25$^+$ T cells in vitro [18], indicating that allergen reactivity is controlled by a CD25$^+$ and IL-10/TGF-β-producing T reg population. The T reg phenotype controlling allergen-specific effector cells (Table 2), particularly of mucosal origin, may have a different phenotype from those T regs controlling autoantigens or tumor antigens. It should be considered that CD25 is not a satisfactory marker for T regs, since recently activated T cells also express CD25. Other activation markers, such as the cutaneous lymphocyte-activation antigen (CLA), are known to be on one hand an activation marker for recently activated T cells, but on the other hand are also indicative for terminal differentiated cells, which have already synthesized cytokines and secrete them without de novo synthesis following TCR engagement [39]. In analogy, it might be that T regs represent terminal differentiated cells derived from Th1 or Th2 memory cells. This hypothesis is in fact supported by the observation that T regs can be generated by CD46 stimulation of both memory and naïve T cells [40]. The combination of CD25 with different activation markers, such as HLA II or CD69, may improve the identification of T regs [41]. An interesting development is the discovery of the FOXP3 transcription factor, which appears to be essential for the generation of CD25$^+$ T regs [42, 43, 44]. In contrast to the CD25 gene, FOXP3 is not

up-regulated following T cell activation; however, its nuclear/intracellular localization does not allow the use of this gene for diagnostic purposes. It is particularly interesting that FOXP3 is operatively involved in silencing the IL-2 gene expression, as demonstrated by FOXP3 overexpression in the Jurkat cell line [44]. Current studies are now focused on the effect of FOXP3 on T reg genes such as IL-10 and TGF-β.

Additional genes of diagnostic and pharmacological interest might become apparent when the molecular mechanisms underlying T cell suppression are fully understood. Initial studies on these mechanisms revealed that T cell suppression is linked to costimulation of T cells [45, 46, 47, 48]. Biochemical analysis of costimulation revealed that the phosphatidyl inositol 3-kinase (PI3K) plays a key role in this process [49, 50]. IL-10 suppresses the association of the PI3K with CD28 [51] as well as other costimulatory molecules, and thereby directly prevents costimulatory signals. The PI3K phosphorylates phosphatidyl inositol 2-phosphates (PIP2) into phosphatidyl 3-phosphates (PIP3). Interestingly, the immune inhibitory receptor of B cells or NK cells also addresses PIP3 lipids by activating PIP-phosphatases (e.g., SHIP; [52]), suggesting that prevention of PIP3 increases represents a general principle of immune suppression. The PIP3 lipids recruit molecules to the membrane, which contain pleckstrin or FYVE domains. One of the best known downstream targets of PI3K is the PKB or Akt kinase, which regulates diverse cellular processes such as proliferation and apoptosis [53].

The PI3K is, however, not only stimulated by costimulatory molecules. It is also a target for growth factor-mediated signals, including IL-2 and IL-4. Therefore, PIP3 levels can reflect multiple signals from the current environment of T cells, which will influence suppressive mechanisms. The PKB/Akt kinase collectively converts these signals in proliferation and/or survival signals. Recent investigations in our laboratory showed that IL-10 and TGF-β induce a negative feedback on PIP3 mobilization by inducing the phosphatidylinositol phosphatase PTEN (C.B. Schmidt-Weber, unpublished results), which is otherwise induced by CD28 engagement as a natural recovery mechanism of PIP3-mediated activation [54]. In contrast to IL-10, TGF-β mobilizes the SMAD2/3-4 complex, which interacts with other transcription factors to bind DNA elements that will down-regulate expression of the particular gene [55]. Alternative means of TGF-β suppression would include the transforming growth factor-activated kinase-1 (TAK1) or the activation of phosphatases [56], which could immediately block or reduce signal transduction of TCR-mediated signals [57]. The suppression of $CD25^+$ T cells is contact mediated, but the molecular details are currently unknown [58]. Further research will be necessary to define tissue conditions where suppression operates to control peripheral tolerance and affect SIT.

The T reg concept

It was originally believed that Th2 cells can be inhibited, if T cell differentiation is skewed towards Th1 cells, lacking IL-4 or IL-13 for IgE isotype switching. The current understanding of T cell regulation favors a relationship of T cell populations where Th0, Th1 or Th2 cells are in a balance with a regulatory T cell population (T effector/T reg). The allergy relevant Th2 cells, producing typically IL-4, IL-5 and IL-13, are generated by differentiation of naïve T cells recognizing processed and MHC class II-presented allergen by dendritic cells in the presence of IL-4. This cytokine is also needed for differentiation of the Th2 pathway, whereas IL-12 is required for Th1 differentiation [59]. The two pathways

inhibit one other, which leads to the hypothesis that allergy emerges in consequence of reduced Th1 cell frequency (two population model). Since Th1 cells are generated by infections and other inflammatory reactions, it was hypothesized that the pathogen-low environment in industrialized countries allows a shift of the Th1-Th2 balance towards the proallergenic Th2 cells.

One obvious argument against the concept of Th1/Th2 balance, is that allergen-specific T cells express a Th2-like cytokine pattern not only in allergic, but also in healthy individuals [60]. Furthermore, it is important to notice that, although Th2 cells are important for IgE switching, IFN-γ typically expressed by Th1 cells also plays an important role in tissue destruction in chronic disease, particularly in keratinocyte/epithelial cell death in atopic dermatitis and asthma [61, 62]. In severe asthma, IL-12 serum levels are in fact known to be elevated [63], and alveolar monocytes are also primed to produce more IL-12 compared to healthy individuals [64]. The cell death-inducing IFN-γ in peripheral tissues can also be provided by Th2 cells, if they have been exposed to IL-12 [65], despite lower expression of the IL-12R [66]. Interestingly, the IL-12-primed Th2 cells keep their ability to produce IL-5 [67], which may be particularly relevant for asthma. Taken together, these studies show that, although T cell differentiation towards Th1 and Th2 cells is important, the balance will not determine whether T cells become pathogenic or not. The fact that it is not Th1/Th2 balance, but rather the T effector/T reg balance that determines the general outcome of an immune response to given antigen [21, 68, 69] represents the current concept. Although a shift in T cell populations lies beneath this concept, it does not necessarily depend on a distinct Th2 cell subset as a cause of allergy pathogenesis. This concept is likely to change the strategy in hygiene hypothesis-motivated studies in a sense that infections are important for the generation, maintenance and survival of T regs [70]. In fact, danger signals provided by viral or microbial infections are assumed to play an important role in the regulation of peripheral tolerance [71], which should be considered as contraindicative for SIT.

On the basis of the T reg concept, current research is focused on obvious questions of how T regs are generated and how the target cells are suppressed by the T regs. The answer to these questions will allow SIT strategies to be improved and provide keys for improving induction and maintenance of peripheral T cell tolerance to allergen.

New therapeutic approaches

The change in immunological concepts is likely to affect the therapeutic rationale for the design of SIT. Adjuvant therapies are likely to improve the treatment efficacy if applied in a regimen supporting tolerance re-induction.

Vaccine optimization

The most obvious improvement of SIT is the generation of optimized allergens, reducing unwanted IgE-mediated side effects. Successful allergen engineering included fragmentation of the allergen [72], destruction of three-dimensional structure by di-/trimerization [13], mutagenesis of B cell epitopes by site-directed mutagenesis [73], dissection of the allergen in peptides [74, 75] or by fusion of several different major allergens into a single

molecule (Kussebi et al., unpublished results). Besides the increased safety of these vaccines, it may be an advantage that the vaccine is not subjected to IgE-facilitated antigen presentation [76], which is supposed to further enhance IgE production in secondary responses. Is efficient antigen presentation also required for the activation and/or generation of T regs? This question remains open, since the origin of T regs and the mechanism of T reg-mediated suppression is not yet completely understood.

A broader range of indications of SIT for multiple forms of allergy requires the design of vaccines covering several allergen specificities. Ubiquitous allergens such as Bet V1, which cross-react with multiple allergens of other allergenic sources, may be engineered by T cell epitope shuffling to generate super-vaccines covering multiple specificities.

Adjuvants in SIT: cyclosporine A and rapamycin

The activity of T regs is at least limited in vitro by IL-2 [77, 78, 79, 80] and IL-15 [81, 82]. These cytokines can resolve T cell unresponsiveness (anergy) and prohibit anergy induction by IL-10 [77, 78, 79, 80]. Since both cytokines are secreted in conditions of acute inflammation, it appears that a balance of T regs and T effectors can only be established if acute inflammation is controlled. Thus, it would be favorable to use anti-inflammatory drugs to facilitate SIT against multiple allergenic responses and if unknown allergens contribute to perpetuation of the allergic inflammation. Cyclosporine A (CsA) and rapamycin efficiently block IL-2 expression and may help generate a pro-tolerogenic microenvironment. However, CsA [83] and rapamycin [84] are also known to inhibit the IL-10 gene expression and may, therefore, delay or block T reg activity, possibly preventing re-induction of peripheral tolerance.

Adjuvants in SIT: anti-histamines and other non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in anti-inflammatory therapy and generally address G protein-coupled receptors (GPCR; [77, 78, 79, 80]). These GPCR are expressed on smooth muscle, epithelia and endothelial cells and induce contraction, vasodilatation and various other inflammatory reactions. For example, GPCR agonists or antagonists are used as therapies for asthma either by promoting airway smooth muscle relaxation (beta2 adrenergic receptor agonists) or by inhibiting inflammation in the nasal mucosa and airways (cysteinyl leukotriene receptor antagonists; [85]). Recent studies showed that GPCR can negatively regulate T cell activation [86, 87, 88, 89, 90, 91], mediated by the activation of the adenylate cyclase (AC) and subsequently increased intracellular cAMP [92, 93, 94]. Thus, IgE-triggered mast cell-released mediators negatively feedback on the T cell population, which is responsible for the initiation of IgE antibodies by B cells. Histamine, prostaglandins (PG) and leukotrienes (LT) are recognized by GPCR, of which four are known for PGE_2 (EP1-4), two for PGD_2 (DP, CRTH2), four for histamine (HR1-4), two for LTB_4 and two for cysteinyl LT. All of these GPCRs can either signal via the phospholipase C pathway to mobilize Ca^{2+} transients or via activation of the AC, increasing intracellular cAMP. For T cells expressing H2R, it was shown that the histamine-induced cAMP pathway mobilizes protein kinase A (PKA), which negatively regulates T cell proliferation [87] by down-regulation of IL-2 and IFN-γ [95, 96] and by increased IL-10 production [97]. This could also be shown for T cells isolated from asthmatic patients [98]. We recently demonstrated that histamine enhances the responsiveness of

Th2 cells to TGF-β in an H2R-dependent fashion, resulting in more efficient IL-4 suppression by TGF-β [99]. The latter findings indicate that GPCR signaling is tied into the regulatory network of suppressive cytokines, but it is not clear under which circumstances GPCR ligands enhance or reduce T reg-mediated suppression. Although the effect of anti-GPCR supplemented SITs were not studied in view of T cell immunology, anti-H1R antagonists were successfully used to reduce side effects of the treatment [100, 101, 102]. Since the antagonists are specific, histamine can still act on H2R to suppress T cells under these treatment regimens. Interestingly a recent study demonstrated that anti-H1R therapy during the initial rush protocol improved treatment in the sense of reduced skin sensitivity and allergen-specific IgE [103], suggesting improved tolerance induction.

Adjuvants in SIT: anti-IgE

Anti-IgE treatment covers the advantages of reduced IgE binding to the vaccine, reducing the risk of side effects on one hand and limiting the release of mast cell mediators, which are the targets of NSAIDs, on the other. Initial studies demonstrate significantly decreased allergic symptoms in patients receiving SIT and anti-IgE compared to SIT alone; however, anti-IgE was given 12 weeks after the first vaccine [104]. Further studies are now required to verify an immunological improvement in terms of T cell tolerance and long-term efficacy of anti-IgE supplement of SIT.

Adjuvants in SIT: steroids

Although steroids are potent immunosuppressors, it is currently not clear whether and how steroids affect the regulation of peripheral tolerance. Steroids are assumed to suppress the immune system by blocking cytokine production by blocking a crucial transcription factor (NF-κB) by transcriptional up-regulation of its natural, cellular inhibitor IκB [105]. However, steroids show more immediate effects and also induce long-lasting changes in the differentiation of T cells. Very soon (3–4 h) after systemic steroid administration, glucocorticoids induce a redistribution of cells, which can be measured as reduced T cell counts and increased NK cell numbers [106]. The disappearing T cells were shown to migrate into the bone marrow [106], but remaining T cells are functionally intact [107]. The redistribution is possibly mediated by the steroid-mediated suppression of the adhesion molecules CD62L and CD11a [108]. This redistribution also affects T regs, which are found more frequently in the peripheral blood following steroid treatment (Karagiannidis et al., unpublished results). The origin and the immunological consequence of this steroid effect is still unclear.

Steroids promote chromatin remodeling [109, 110], which is known to be an important event in the differentiation of T effector cells [111] and, therefore, affects long-lasting immunological memory. Although this effect of steroids is problematic because of its antigen unspecificity, it is interesting to note that in vitro differentiation of T cells in the presence of steroids promotes the generation of IL-10-expressing T cells [112]. Further studies are necessary to understand the relationship of steroids, T regs and SIT. However, in vitro studies suggest that steroids do not necessarily inhibit T regs, and thus may go well along with SIT.

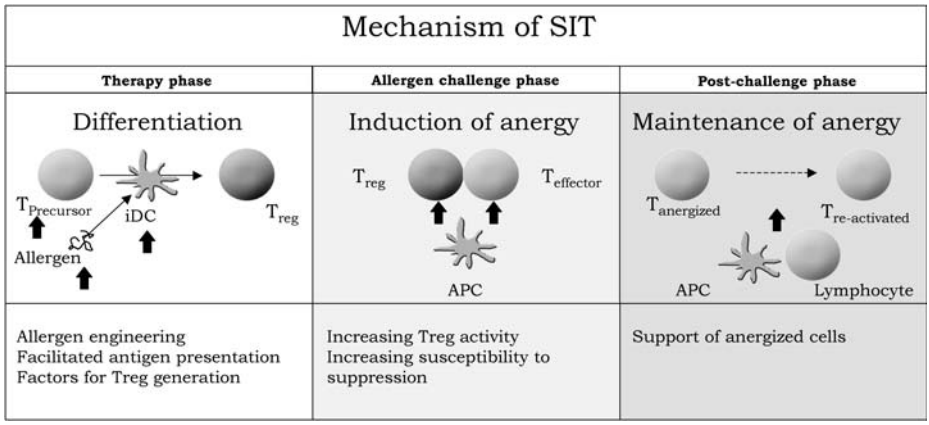

Fig. 1 The cartoon categorizes three different phases in the induction of T cell tolerance by SIT. *Black arrows* indicate therapeutic targets. Targets of therapy improvement may evolve not only from the therapy phase, which is critical for the differentiation of the T cells into T regs in the presence of antigen and T reg-promoting APC such as iDC, but also in the allergen and post-allergen challenge phase. The allergen-challenge phase is characterized by the confrontation of the immune system with the allergen following immunotherapy, and T reg activity should induce anergy in upcoming T effector cells. Following this anergy induction phase, anergized cells are subjected to the microenvironment, which may influence the anergized cells. In particular, inflammatory conditions providing IL-2 and IL-15 may be counter-productive and may break anergy, representing molecular targets for maintenance of anergized cells (*SIT* specific immunotherapy, *T reg* regulatory T cell, *APC* antigen-presenting cells, *iDC* immature dendritic cells)

Conclusion

Although the potential of SIT is currently not fully used for the treatment of allergies, it has been proven as the only curative and non-symptomatic treatment of allergy. The analysis of underlying mechanisms of SIT indicate that generation of T regs, which control peripheral tolerance, is a key to treatment success. Available drugs and novel molecular targets are currently being tested to interfere with the differentiation of T regs, induction and maintenance of T cell suppression (Fig. 1). Of great importance for SIT is the immunological monitoring of SIT and its progress into the post-challenge phase, which is important for the evaluation of treatment success, which in turn determines the treatment-duration. The understanding of T reg-mediated suppression is likely to facilitate the identification of specific, molecular targets and will also improve SIT strategies and monitoring.

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