

Traitements immunomodulateurs actuels et futurs

Specific peptide immunotherapy

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Allergic responses are generally recognized as immunologic disorders characterised by the hyperproduction of IgE antibodies as well as tissue infiltration and activation of eosinophils and mast cells, releasing a variety of inflammatory and pro-inflammatory mediators. In contrast, a protective humoral immune response to allergens is characterized by specific IgG antibody production, in particular of the IgG4 class. This difference in allergen-specific antibody response enables the elucidation of the regulatory mechanisms in allergic responses in comparison to normal immunity. Specific antibodies to antigens develop after differentiation and clonal expansion of B lymphocytes. The single steps in development of distinct responses are under the control of specific T lymphocytes and depend on the recognition of antigenic peptide fragments and costimulatory interactions on the one hand, and the production of cytokines that are released upon T cell activation [1]. The qualitative and quantitative cytokine composition, mainly reflected by the formation of interleukin-4 (IL-4), IL-13 and gamma-interferon (IFN- γ) regulates the specific IgE and IgG4 antibody synthesis by B cells [1, 2]. In particular, the presence of IL-4 during initial antigen priming of naive T cells results in generation of Th2 cells, inhibiting Th1 cytokine development [3, 4]. IFN- γ can promote its own production [5] and IL-12 is a dominant factor inducing Th1 cell differentiation [4-8]. In contrast, cytokine secretion results from activation of specific

T cells by antigenic peptides, which are processed by antigen-presenting cells (APC) and presented in the context of MHC-II molecules to specific T cell receptors (TcR) [9]. Therefore, the nature of the three components in the ternary MHC-II/peptide/TcR complex is crucial for the development of IgE and/or IgG4 antibody responses and IgE-mediated allergic diseases or IgG4-related immunity. The recognition sites for T cell receptor (TcR) are linear peptide structures with a core region of nine-12 residues. They are different from those recognized by antibodies and B cells, which mostly recognize conformational structures on the antigen surface. Thus, the major investigations in specific allergy treatment are aimed at skewing distinct IL-4-, IL-5- and IL-13-dominated cytokine profiles by T cells into normal IFN- γ /IL-2-dependent immunity, most likely by differential T cell activation and changes in properties of antigen-specific recognition.

The symptoms of immediate and late-type allergic reactions can be controlled by various pharmacologic treatments. In contrast, the allergen-specific immunotherapy (SIT) represents the only curative approach for specific Type I allergy. SIT is most efficient in allergies to insect venoms and allergic rhinitis [10-16]. However, despite frequent usage for a long time, the mechanism behind SIT remained unclear. A rise in allergen-blocking IgG antibodies, particularly of the IgG4 class [17-18], the generation of IgE-modulating CD8⁺ T cells and a reduction in

the number of mast cells and eosinophils and release of mediators [19, 20], were found to be associated with successful SIT. Furthermore, SIT was found to be associated with a decrease in IL-4 and IL-5 production by CD4⁺ T cells, and in some cases with a shift towards increased IFN- γ production [21-26]. Recently, however, it appeared that the induction of an unresponsive or anergic state in peripheral T cells and the reactivation of the response by cytokines from the tissue microenvironment represent intermediate key steps in the basic mechanism of SIT [21-25]. Thus, conditions of the immunologic microenvironment and production of cytokines by tissue cells may finally determine whether a SIT develops towards a successful or an unsuccessful treatment. Therefore, for successful and safe SIT, allergen variants should be used, which contain intact recognition sites for T cells, whereas binding sites for required antibodies should be removed. Intact T-cell epitopes are required in order to enable the induction of specific T cell tolerance or anergy against the antigen/allergen. The antibody or B-cell epitopes are not only prerequisites for elicitation of adverse reactions; IgE antibodies also focus the allergen efficiently onto antigen-presenting B cells, which present it to T cells in a way that favors development of a Th2-dominated cytokine pattern and IgE production.

A specially suited model to study specific IgE and IgG regulatory mechanisms in humans is provided by the immune response to bee venom (BV), mainly because BV allergy in most cases results from dysregulated IgE/IgG antibody responses and does not reflect a true atopic disease. The BV phospholipase A2 (PLA) represents the major antigen in both normal immunity and bee sting allergy [27]. Of BV allergic patients, 99% react against native (n) PLA and 96% against recombinant (r) PLA produced in *Escherichia Coli* [28]. PLA is a glycoprotein of 14 to 16 kD consisting of 134 amino acid residues with a single carbohydrate side chain at position Asn¹³ [29]. The three-dimensional structure and biochemical function of PLA are well-defined [30]. Normally, most individuals initially elicit low-affinity IgG1 anti-PLA antibodies and develop high-affinity IgG4 antibodies to PLA after repeated exposure [31]. In contrast, some individuals generate PLA-specific IgE antibodies and develop bee sting allergy [27, 28].

ISOTYPE SWITCH OF BOTH IgE AND IgG DEPEND ON IL-4

Isotype switch and development of IgE antibodies by B cells, but also the switch to IgG4, requires T cell contact and IL-4 [32, 33]. Differential isotype production appears at the stage of isotype-specific memory B cell activation by antigens. At this level, production and secretion of IgE still depends on IL-4 or IL-13, whereas IgG secretion is associated with IFN- γ secretion by T cells [34]. Indeed, in the human in vitro response to PLA, increases in IL-4 correlated positively with IgE and negatively with specific IgG4 antibody generation by memory B cells [34, 35]. In contrast, increasing IFN- γ and decreasing IL-4:IFN- γ ratios proportionally correlated with a change in specific IgE:IgG4 ratio [34, 35]. The involvement of enzymatic activity in the immunologic response to PLA could be excluded by the usage of inactive rPLA that was mutated at His³⁴ by Asn or As [36].

ALLERGIC AND NON-ALLERGIC INDIVIDUALS RECOGNIZE THE SAME T CELL EPITOPES

Upon specific stimulation with PLA, the differences in cytokine secretion between T cells derived from IgE-producing allergic and IgG4-producing non-allergic subjects were mostly quantitative. Therefore, other individual features may be of more importance for inducing different cytokine profiles in T cells than recognition of particular antigenic sites. By screening the epitope specificity of T cell clones and PBMC of sensitized individuals with 62 overlapping dodeca- and 19 octadeca-peptides, three immunodominant sites in the PLA molecule could be mapped [37-39]. These T cell epitopes are represented by the peptides PLA40-63, PLA74-92 and PLA107-131, as indicated in *figure 1*.

Primary in vitro proliferative responses of unfractionated PBMC from allergic or healthy hyper-immune or SIT-treated individuals to the PLA peptides were restricted to the same immunogenic sites as found in T cell clones, and no other of the overlapping PLA peptides was active in sensitized subjects (*figure 2*). The same patterns of epitope reactivity

Major peptides

PLA 45-62	Glu-Ser-Lys-His-Gly-Leu-Thr-Asn-Thr-Ala-Ser-His-Thr-Arg-Leu-Ser-Cys-Asp
PLA 81-92	Tyr-Phe-Val-Gly-Lys-Met-Tyr-Phe-Asn-Leu-Ile-Asp
PLA113-124	Cys-Leu-His-Tyr-Thr-Val-Asp-Lys-Ser-Lys-Pro-Lys

Altered peptide ligands

⁸² PLA 81-92	Tyr- <i>Phe/Ala</i> -Val-Gly-Lys-Met-Tyr-Phe-Asn-Leu-Ile-Asp
¹¹⁸ PLA 113-124	Cys-Leu-His-Tyr-Thr- <i>Val/Ala</i> -Asp-Lys-Ser-Lys-Pro-Lys
¹²¹ PLA 113-124	Cys-Leu-His-Tyr-Thr-Val-Asp-Lys-Ser/ <i>Ala</i> -Lys-Pro-Lys

Figure 1. Amino acid sequences of minimal human T cell epitopes and altered peptide ligands from bee venom PLA.

were detected among all three groups of individuals. Thus, three dominant epitopes are involved in T cell recognition of PLA, independently of whether an individual was allergic or not and its levels of specific IgE and IgG4 antibodies. Among BV allergic patients > 90% responded to the peptide corresponding to PLA107-131, 70% of patients recognized PLA74-92 and 34% PLA40-63. Moreover, 70% reacted against the two epitopes PLA74-92 and PLA107-131. Only one out of 40 tested donors, a hyposensitized patient, did not respond significantly in the primary in vitro response to at least one of the overlapping peptides spanning the entire PLA molecule [37, 40]. Some proliferative response to PLA9-27 was observed in one out of 48 BV-sensitized individuals, and T cell reactivity was recently demonstrated against the entire C-terminal fragment of PLA spanning from amino acid 90 to 134 [38]. Probably the reactive site in the C-terminus may be shorter since we found a T cell clone reacting against PLA94-111 (unpublished). These findings may support the existence of minor or individual determinants within a larger cluster of antigenic sites in the PLA molecule. Generally, the lower proliferative responses were found among successfully hyposensitized patients, indicating that at least partial tolerance was induced during immunotherapy. None of the immunogenic peptides bound IgE antibodies or elicited skin reactions, indicating that they did not contain B cell epitopes.

Some of the T cell clones generated from peripheral blood of allergic subjects could not be mapped

to any of the linear peptide sequences in PLA, although they expressed clear reactivity to the native allergen. A few of them reacted to mellitin, which in traces was bound to nPLA. Using non-glycosylated nPLA and rPLA, as well as a number of different glycosylated and non-glycosylated PLA analogues, a glycosylation-dependent T cell epitope was found in PLA [41]. This carbohydrate-containing epitope was recognized by PBMC and T cell clones of some allergic individuals. The recognition of this epitope was HLA-DR-restricted and not inhibitable by the carbohydrate or peptide alone. Moreover, specific proliferation of T cell clones was abrogated after stepwise enzymatic deglycosylation of nPLA [18]. Based on the experimental design, superantigenic reactivity could be excluded. So far, such glycan-dependent T cell epitopes were only found in experimental animal models using synthetic antigens. Whether such epitopes play a role in development of allergy and elicitation of IgE antibodies remains to be elucidated.

THE ALLERGEN CONCENTRATION AND INDIVIDUAL AFFINITY OF ANTIGENIC PEPTIDE TO MHC CLASS II AND T CELL RECEPTORS ARE DRIVING FORCES IN GENERATION OF DISTINCT CYTOKINE PROFILES

A panel of CD4⁺ T cell clones specific to PLA was established from PBMC of human subjects allergic, hyposensitized or hyperimmune to bee sting [42].

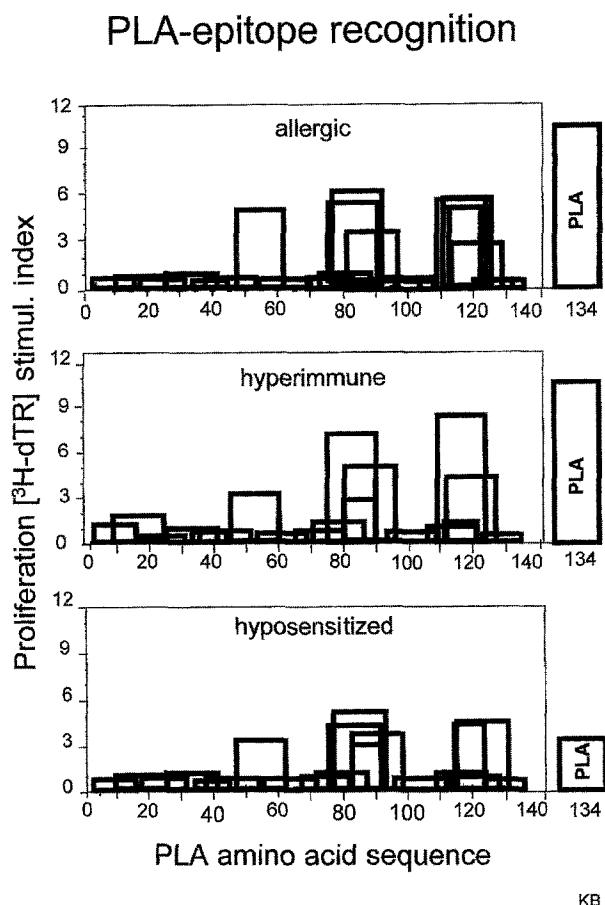


Figure 2. Allergics and nonallergics recognize the same and limited number of T cell epitopes within the PLA sequence of 134 amino acid residues. The stimulation index of proliferative responses of PBMC after stimulation with peptides covering the entire sequence of PLA is shown from 12-26 individuals in each group.

Depending on the T cell clone, optimal stimulation required 1 to 100 $\mu\text{g/mL}$ of PLA [42]. Upon high-dose antigen stimulation, all clones produced IL-4, IFN- γ and granulocyte-macrophage-colony-stimulating factor (GM-CSF), beside other cytokines. However, both absolute and relative amounts of secreted cytokines depended on the antigen concentration in the T cell culture [42, 43], and typically ten to 50 times lower threshold amounts were required for IL-4 induction than for IFN- γ .

The PLA-specific T cell clones could be classified according to the increasing or decreasing ratio of IL-4: IFN- γ production in response to increasing allergen

doses [43, 44]. A typical Th0 clone clearly showed a Th2 cytokine pattern at low antigen concentrations [44]. Increasing antigen concentrations, however, favored IFN- γ production, while IL-4 decreased at high antigen contents. In principle, the same feature could be observed in a Th2 cell clone; however, cytokine production generally required higher antigen concentrations. Suboptimal T cell activation with low amounts of antigen [42] or anti-CD3 [43] induced significant IL-4 synthesis by allergen-specific Th cells, whereas IFN- γ production by the same cells required stronger stimulatory signals [42-44]. These results demonstrate that particular cytokine patterns can be modulated by the dose of antigen and do not represent stable phenotypes. Thus, the individual HLA class II expression, determining the binding of antigenic peptide, influences the intensity of TcR engagement and the associated cytokine pattern [34, 42-45]. Low antigen concentration and suboptimal antigenic peptide binding capacity of MHC class II will generate weak T cell activation, predominantly a Th2 cytokine pattern and therefore IgE antibodies [34]. Accordingly, at the same antigen concentration individuals with high MHC-II affinity to immunogenic peptides display a higher MHC-II/antigen complex density on the APC surface and induce stronger T cell activation with sufficient IFN- γ to suppress IgE. The cytokine-regulating forces might be driven by individual HLA-class II-type expression and the strength of epitope binding in an APC/peptide/T cell receptor complex [42-44]. Therefore, the modulation of a T cell cytokine pattern by the dose of antigen may be a driving force for differential IgE and IgG antibody formation resulting in either allergy or immunoprotection.

ALTERED PEPTIDE LIGANDS

Recently, it was demonstrated that altered peptide ligands (APL) can modify specific T cell activation and cytokine production by their diversity in binding the TcR or MHC class II-presenting molecules (*figure 1*). By alanin substitution of the immunodominant epitope PLA74-92, a substantial decrease in proliferation of specific T cells and proportional cytokine release was obtained [46]. Only the Phe⁸² substituted APL, altered in one HLA-DP binding

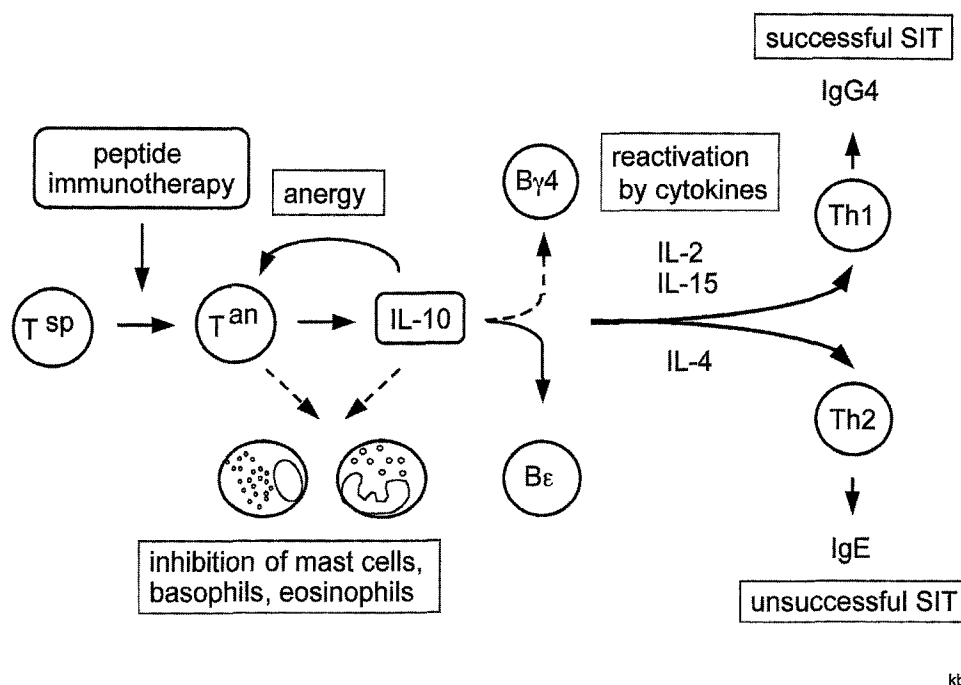


Figure 3. Mechanism of T cell peptide immunotherapy. Continuous treatment with high doses of allergen or a mixture of the peptides recognized by T cells establishes a specific anergy in peripheral T cells (T^{an}), which is characterized by suppressed proliferative and cytokine responses and simultaneous increase in IL-10 production. IL-10 suppresses specific T cells (T^{sp}) in an autocrine fashion. It also suppresses specific IgE and enhances IgG4 production. In consequence, activation, priming and survival of allergic inflammatory effector cells are downregulated. The anergic T cells can be reactivated by cytokines from the tissue microenvironment. In successful SIT, anergic T cells recover by the influence of microenvironmental IL-2 and/or IL-15 to produce Th0/Th1 cytokines. In an atopic individual IL-4 may reconstitute a Th2 cytokine pattern and reactivate an allergic response.

residue, however, inhibited IL-4 but had no effect on IFN- γ production. The location of the cytokine changing substitution is shown in *figure 1*. This effect of a particular amino acid substitution on cytokine secretion again appeared to result from changes in affinity to MHC class II of the APL compared to the native peptide. Most interestingly, stimulation with the Phe⁸²-mutated APL resulted in significant IgG4 increase and lower IgE production in vitro. Exposure of the T cells to high amounts of APL induced specific anergy with abrogated proliferation and cytokine synthesis on antigen rechallenge identical to the native peptide. The defective T cell activation was associated with alterations in transmembrane signaling, specifically with lack of tyrosine phosphorylation of the ZAP70 tyrosine kinase [46]. These results demonstrate that site-directed single amino acid mutations in T cell epitopes can modify specific T cell activation and cytokine production by their diversity in binding

TcR or MHC class II molecules and therefore change the type of immunologic response. Studies with APL of major allergens may enable ways of more controlled and strengthened PIT in the future.

SPECIFIC IMMUNOTHERAPY (PIT) WITH ANTIGENIC T CELL EPITOPE- PEPTIDES INDUCES SPECIFIC ANERGY

Multiple sites of T cell epitopes with an active core region of nine to 12 amino acids could be identified in different allergens. In mice, several studies demonstrated the induction of specific T cell tolerance or anergy by administration of peptides representing dominant T cell epitopes. In clinical trials, relatively long peptides of 27 and 36 amino acids of the major cat allergen Fel d 1 were applied in cat allergen-specific therapy. In bee venom allergy PIT was performed with a mixture of short peptides representing minimal-sized T cell epitopes (11, 12

and 17 mers) of the major bee venom allergen phospholipase A2. It is an interesting observation that only a few distinct sites in the PLA antigen are antigenic, and that the same peptides which activate T cell responses can induce an anergic state in Th0 and Th2 clones [19, 20]. Accordingly, a mixture of PLA peptides, each containing a single T cell epitope of PLA, was used for specific immunotherapy of patients with IgE-mediated systemic allergic reactions to BV [25, 44]. Increasing doses of the peptide mixture were subcutaneously administered over a two-month period. After therapy, specific proliferative responses to PLA and the peptides were decreased in PBMC of successfully treated patients. Moreover, the production of IL-4, IL-5 and IL-13, but also of IFN- γ and IL-2 was inhibited, indicating that a state of T cell anergy was induced against the PLA epitopes. The three PLA peptides elicited neither immediate nor delayed skin reactions in allergic patients and did not bind IgE antibodies. The patients were challenged one week after therapy with PLA and later with a live bee sting without allergic symptoms. B cells were not affected in their capacity to produce specific antibodies and serum levels of specific antibodies increased in favor of IgG4 after PLA and BV challenge. These results indicate that successful peptide immunotherapy is effective without risk of anaphylaxis due to induction of epitope-specific T cell anergy to the major allergen.

A final proof for the decisive and central role of T cell recognition and activation was recently provided by Haselden et al. They showed that short T cell epitope peptides from Fel d1 alone, beside induction of tolerance, can also directly initiate T cell-dependent late asthmatic reactions in sensitized asthmatic subjects, without requiring an IgE and mast cell-dependent response. This demonstrates the pivotal role of specific T cells, responding to single epitope peptides and initiating allergic responses. Therefore, there is a considerable rationale for targeting T cells in SIT.

SPECIFIC IMMUNOTHERAPY WITH WHOLE BV

Similar studies were undertaken in bee sting allergic patients treated with whole BV for two months [21, 22]. After two months of whole BV rush immu-

notherapy, PLA and T cell epitope peptide-specific T cell proliferation were suppressed and the PLA and PLA-peptide induced secretion of type 2 (IL-4, IL-5 and IL-13), as well as type 1 (IL-2 and IFN- γ) cytokines abolished. The suppressive effect was specific as the tetanus toxoid- or PPD-induced cytokine production and proliferation remained unchanged. In spite of the allergen-specific anergy in T cells, both anti-PLA IgE and IgG4 levels slightly increased during immunotherapy, while the PLA-specific IgE:IgG4 ratio changed in favor of IgG4. These findings indicated that BV immunotherapy, like peptide therapy, induces peripheral anergy in allergen-specific T cells. Again, B cells remained unaffected [21, 22].

The molecular mechanism of anergy induction in allergen-specific T cells was elucidated in PLA specific T cells [42, 43]. We demonstrated that incubation of human CD4⁺ PLA-specific Th2 cells with antigenic peptide, in the absence of professional APC or a second signal, results in a state of non-peripheral responsiveness or anergy. This appeared to be related to alterations in the signaling pathways mediated through the TcR. Anergic Th2 cells failed to respond. They showed increased tyrosine phosphorylation and suppressed activation of the p56^{lck} or ZAP70 tyrosine kinases. Therefore, the induction of anergy in Th2 cells represents a biochemically active process, associated with increased levels of basal tyrosine kinase activity, and CD25 upregulation and therefore with defective transmembrane signaling through the TcR [42, 43].

T CELL NON-RESPONSIVENESS IN SPECIFIC IMMUNOTHERAPY IS ASSOCIATED WITH INCREASED IL-10 PRODUCTION

By treatment with whole BV, the secretion of Th2 type (IL-4, IL-5 and IL-13), as well as Th1 type (IFN- γ) cytokines, was significantly inhibited already after seven days of immunotherapy [22, 23]. Simultaneously, the IL-10 production increased continuously. Intracytoplasmic staining of peripheral blood lymphocytes, combined with surface marker expression as analyzed by multicolor FACS analysis, revealed that IL-10 was first produced by allergen-

specific CD4⁺CD25⁺T cells and appeared later in B cells and monocytes [22, 23]. Neutralization of IL-10 in PBMC cultures reconstituted the abolished proliferative and cytokine responses of T cells. Moreover, successful immunotherapy led to a decrease in the allergen-specific IgE:IgG4 ratio. In vitro, IL-10 inhibited both allergen-specific and total IgE synthesis and enhanced IgG4 antibody formation by directly affecting memory B cells. These results indicate that increased IL-10 production during immunotherapy is responsible for peripheral anergy in specific T cells and therefore plays a decisive role in skewing the antigen-specific IgE:IgG4 ratio in favor of IgG4.

IL-2 AND IL-15 RECOVER ANERGIC T CELLS

By culturing PBMC from BV-treated patients in the presence of the antigen and IL-2 or IL-15, the specifically anergized T cell response could be restored with respect to specific proliferation and secretion of IL-2 and IFN- γ [21, 23]. In contrast, IL-4, IL-5 and IL-13 remained suppressed. Treatment of anergic T cells with IL-4 only partially restored the proliferation of T cells and induced a type 2 cytokine pattern at low levels. Reactivation of anergic T cells and modulation of cytokine patterns suggests an important role of microenvironmental cytokines resulting in success or failure of specific immunotherapy. In particular IL-15, being released by professional APC and various tissue cells in normal immune defence, may be of importance for establishing normal immunity. In contrast, the long-lasting success of allergen-specific immunotherapy may be difficult to achieve in an established atopic and polyallergic environment.

SPECIFIC IgE AND IgG4 ANTIBODY REGULATION BY PIT

The serum levels of specific IgE and IgG4 antibodies delineate allergic and normal immunity to allergens. Whereas peripheral anergy was demonstrated in specific T cells, the capacity by B cells to produce specific IgE and IgG4 antibodies was not abolished. In fact, specific serum levels of both isotypes

increased during the early phase of treatment. However, the increase in specific IgG4 was more pronounced and the ratio of specific IgE to IgG4 decreased by ten-fold within a few weeks [21, 22, 25]. Also, the in vitro production of PLA-specific IgE and IgG4 antibodies by PBMC changed in parallel to the serum levels of specific isotypes. A similar change in the specific isotype ratio was observed in SIT of various allergies. Moreover, IL-10 that was induced and increasingly secreted during SIT appears to counter-regulate antigen-specific IgE and IgG4 antibody synthesis. It is a potent suppressor of both total and PLA-specific IgE, while simultaneously increasing IgG4 formation [21, 22, 25]. Thus, IL-10 not only generates anergy in T cells, it also regulates specific isotype formation and skews the specific response from an IgE- to an IgG4-dominated phenotype.

CONCLUSIONS

Induction of specific anergy in peripheral T cells by IL-10 and subsequent reactivation of distinct cytokine patterns by cytokines from the tissue microenvironment are key events in the immunologic mechanisms of SIT. These mechanisms have implications, which may reach beyond specific allergy treatment, and induction of specific anergy may be of importance also in autoimmunity and GvH disease. It can facilitate tumor growth, parasite survival and AIDS development. The immunologic key steps of SIT and PIT are depicted in *figure 3*. Both SIT and PIT generate IL-10, which, in an autocrine way of action, induces specific anergy in peripheral T cells. Both types of treatment decrease the antigen-specific IgE:IgG4 ratio in peripheral blood. The reactivation and modulation of distinct cytokine patterns in anergic T cells suggest a pivotal role of microenvironmental cytokines in development of SIT. T-cell secreted cytokines are essential for the priming, survival and activity of inflammatory effector cells. Therefore, specific T cell reactivity is directly involved in the pathogenesis of allergic inflammation. IL-10 not only induces anergy in T cells, it also inhibits the activation of inflammatory reactions by mast cells and eosinophils. Finally, the generation of new Th2-type cells secreting allergic inflammatory

cytokine patterns can be suppressed by administration of high allergen doses inducing IFN- γ -dominated cytokine patterns.

Various attempts are currently being undertaken to increase the success of SIT and decrease its risk of side effects. The availability of recombinant allergen technology will permit excellent standardization of allergen preparations for clinical use. The aim of allergen modification is to decrease the allergenicity while retaining its immunogenicity. This could be achieved by destroying conformational B cell epitopes and simultaneously preserving linear T cell epitopes in allergens. Native allergens utilize IgE-facilitated antigen presentation, leading to increases in Th2 cytokine production. High amounts of IL-4 and IL-13 produced by classical allergens induce higher IgE production, whereas high IL-5 production lead to eosinophil activation and an increased eosinophil lifespan. Because of severe side effects, the high doses required for successful SIT may not be reached with native allergen extracts. In contrast, modified allergens or T cell epitope peptides lacking IgE binding sites or effector cell degranulation utilize phagocytic or pinocytic antigen uptake mechanisms, which induces a balanced Th0-/Th1-like cytokine pattern by T cells, resulting in lower IgE and higher IgG production by memory B cells. Targeting T cells by modified allergens enables the administration of higher doses, which induce tolerance in Th2 cells without risk of anaphylaxis. These properties are most consequently fulfilled by short peptides representing minimal required T cell epitopes. They are not recognized by IgE antibodies and B cells; therefore, they are not anaphylactogenic and do not induce antibody responses, but they express the full capacity of the antigen/allergen required for T cell activation and tolerance.

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