IL-10 and TGF-β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy

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The regulation of normal and allergic immune responses to airborne allergens in the mucosa is still poorly understood, and the mechanism of specific immunotherapy (SIT) in normalizing the allergic response to such allergens is currently not clear. Accordingly, we have investigated the immunoregulatory mechanism of both normal and allergic responses to the major house-dust mite (HDM) and birch pollen allergens — *Dermatophagoides pteroynyssinus* (Der p)1 and Bet v 1, respectively — as well as the immunologic basis of SIT to HDM in rhinitis and asthma patients. In normal immunity to HDM and birch pollen, an allergen-specific peripheral T cell suppression to Der p 1 and Bet v 1 was observed. The deviated immune response was characterized by suppressed proliferative T cell and Th1 (IFN-γ) and Th2 (IL-5, IL-13) cytokine responses, and increased IL-10 and TGF-β secretion by allergen-specific T cells. Neutralization of cytokine activity showed that T cell suppression was induced by IL-10 and TGF-β during SIT and in normal immunity to the mucosal allergens. In addition, SIT induced an antigen-specific suppressive activity in $CD4^+$ $CD25^+$ T cells of allergic individuals. Together, these results demonstrate a deviation towards a regulatory/suppressor T cell response during SIT and in normal immunity as a key event for the healthy immune response to mucosal antigens.

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1 Introduction

Cytokines, which stimulate or inhibit effector functions of distinct T cell subsets, are critical in the control of immune responses [1–4]. Th1 cells, predominantly secreting IL-2, IFN-γ and TNF-β, are implicated in cell mediated immunity, including inflammation, cytotoxicity and delayed-type hypersensitivity reactions. Th2 cells, producing IL-4, IL- 5 and IL-13, are associated with strong antibody responses and eosinophilia. The dysregulation of these two subtypes of responses results in various immunopathologic conditions [5–7].

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The first two authors contributed equally to this study.

Abbreviations: Der p: *Dermatophagoides pteroynyssinus* **HDM:** House-dust mite **SIT:** Specific immunotherapy **sTGF-βR:** Soluble TGF-βRII–Fc chimeric protein **TT:** Tetanus toxin

Allergy is associated with high serum levels of allergen-specific IgE and with eosinophilia. in particular, the generation of IL-4 and IL-13 is associated with IgE production and IL-5 with increased eosinophil survival [8–10]. Normal immunity to allergens is characterized by predominant IgG antibody formation, especially of the IgG4 class [11]. IgG4, like IgE, requires IL-4 for isotype switch. The production of IgG4 antibodies by memory B cells depends on the presence of IFN-γ, whereas IgE remains IL-4 dependent and is suppressed by IFN-γ [12, 13].

IL-10 inhibits IgE and enhances IgG4 production [14, 15]. IL-10 is a general inhibitor of proliferative and cytokine responses in T cells and is produced by mononuclear phagocytes [16, 17], natural killer cells [18] and by both Th1 and Th2 type lymphocytes [19]. It can suppress cytokine synthesis in T cells by inhibiting accessory CD28–B7.1 receptor interaction [20]. TGF-β is a pleiotropic cytokine known to affect T cell proliferation [21, 22], differentiation [23–25], apoptosis [26], antigen presentation, effector functions of macrophages, the expression of MHC class I and II [27] and CD40 and IL-12 [28].

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TGF- β also induces B cell activation and Ig isotype switch to IgA [29, 30].

Clinical allergen tolerance induced by specific immunotherapy (SIT) is associated with changes in cytokine production by $CD4^+$ T cells [31–36]. In this study, we demonstrate that IL-10 and TGF-β cooperate in inducing peripheral T cell suppression both during natural exposure to aeroallergens in healthy individuals and during SIT in allergic rhinitis and asthma patients. It appears that both of these suppressive cytokines and $CD4^+$ $CD25^+$ T cells are pivotal in the control of specific immune responses to high doses of aeroallergens that are being encountered via mucosal tissues.

2 Results

2.1 The regulatory T cell response in HDM-SIT is mediated by IL-10 and TGF-β

We investigated changes in T cell responses to *Dermatophagoides pteroynyssinus* (Der p)1, the major house-dust mite (HDM) allergen, during the first 70 days of HDM-SIT. Der p 1-induced specific T cell proliferation was measured in 6-day cultures of PBMC obtained before treatment. After 70 days of SIT, specific proliferation was significantly suppressed in all 10 patients ($p<0.001$), whereas the proliferation induced by control antigen [tetanus toxin (TT)] did not change (Fig. 1A). In experiments investigating the mechanisms of decreased specific peripheral T cell proliferation, IL-10 and TGF-β were demonstrated as the responsible suppressive cytokines. We used an IL-10Rα-chain blocking antibody and a soluble TGF-βRII–Fc chimeric protein (sTGF-βR) to block the effects of endogenous IL-10 and TGF-β in cultures. Suppressed specific T cell responses in HDM-SIT can be re-established by blocking of endogenous IL-10 (Fig. 1B). Blocking of the IL-10R resulted in increased specific T cell proliferation before treatment, which was even more enhanced during HDM-SIT. A similar increase in specific T cell proliferation was observed by neutralization of TGF-β in parallel cultures. These findings were confirmed by measurements of IL-10 and TGF-β in parallel cultures.

Fig. 2 shows changes in cytokine production during 70 days of treatment. SIT resulted in significantly increased production of both IL-10 and TGF-β after 28 days of treatment ($p<0.01$). A further increase was observed after 70 days of SIT ($p<0.005$). Patients who showed high increases in IL-10 also showed high TGF-β increase with a significant correlation ($p<0.001$) (Fig. 2B). IL-5, IL-13 and IFN-γ secretion was significantly decreased after 28 ($p<0.05$) and 70 days ($p<0.01$) of SIT.

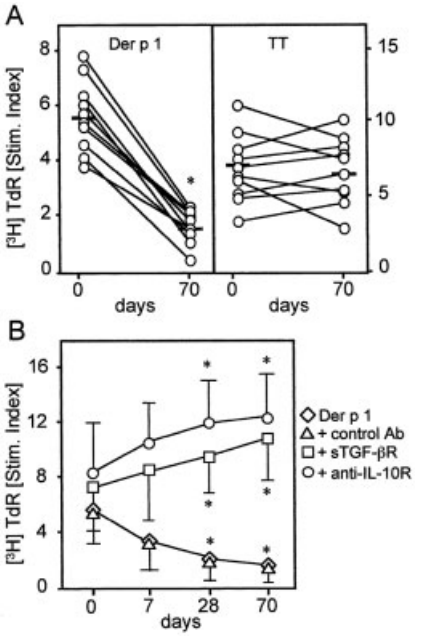

Fig. 1. IL-10- and TGF-β-mediated T cell suppression during HDM-SIT. (A) Suppression of T cell proliferation after stimulation with specific allergen (Der p 1) after 70 days of SIT. PBMC obtained before and 70 days after HDM-SIT from 10 mite-allergic subjects were stimulated with Der p 1, and [^{3}H]thymidine (TdR) incorporation was measured. Results shown are the mean of triplicate cultures. Horizontal lines show the overall means. T cell proliferation did not change after stimulation with control antigen (TT) after 70 days of HDM-SIT. (B) The suppressed specific T cell response in HDM-SIT can be re-established by neutralization of endogenous IL-10 and TGF-β. PBMC obtained at different time points of HDM-SIT were stimulated with Der p 1 alone or together with blocking anti-IL-10R mAb or neutralizing sTGF-βR. Rat IgG was used as control Ab. Blocking of IL-10 or TGF-β resulted in reconstitution and an additional increase of T cell proliferation during SIT. The results shown are the mean ± SD values obtained in 10 subjects. *$p<0.01$.

To determine the cellular source of IL-10, intracytoplasmic IL-10 and co-expression of surface markers were analyzed in fresh PBMC and specific T cells after 10 days of culture with Der p 1. $CD4^+$ $CD25^+$ T cells were gated for IL-10 content immediately after isolation of the PBMC. Increased intracellular IL-10 production in these activated $CD4^+$ $CD25^+$ T lymphocytes was observed on day 70 (Fig. 2C). The percentage of IL-10-containing cells increased from 2.5 % before SIT to 34.2% at day 70 ($p<0.01$). In T cell cultures specifically stimulated with Der p 1, the percentage of IL-10-containing cells increased from 3.6% before SIT, to 38.5% at day 70

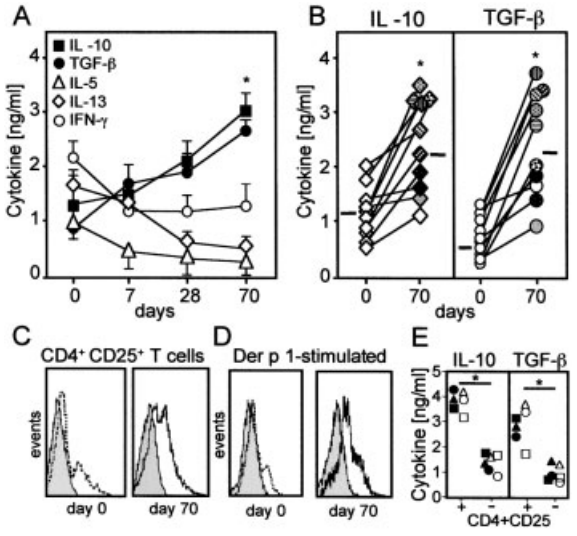

Fig. 2. Mite-SIT induces increased IL-10 and TGF-β production. Changes in cytokine production during HDM-SIT were determined in cultures of PBMC stimulated with Der p 1. (A) IL-10 and TGF-β increased, whereas IL-5, IL-13 and IFN-γ decreased, during SIT. Results shown are representative for 9 other patients. (B) Increased secretion of both IL-10 and TGF-β after 70 days of SIT in 10 subjects. Horizontal lines show the overall means. Cytokine production of unstimulated PBMC was less than 15% and is omitted in the figure. (C) Intracytoplasmic IL-10 content of peripheral blood $CD4^+$ $CD25^+$ T cells during HDM-SIT. (D) Intracytoplasmic IL-10 content of cultured Der p 1-specific T cells during HDM-SIT. The filled curve represents the isotype control. A similar intracytoplasmic IL-10 pattern was obtained in 7 other HDM-SIT patients (C, D). (E) Purified $CD4^+$ $CD25^+$ (indicated by + on the × axis) and $CD4^+$ $CD25^-$ (–) T cells from three patients before (open symbols) and after (filled symbols) SIT were stimulated by immobilized anti-CD3, and supernatants were taken after 72 h. IL-10 and TGF-β were measured by ELISA. *$p<0.001$.

($p<0.01$) (Fig. 2D). In repeated measurements of intracytoplasmic IL-10 content in HDM-allergic individuals who did not receive HDM-SIT, no significant changes were observed in the respective cells. Currently available anti-TGF-β antibodies were not suitable for intracytoplasmic staining of TGF-β.

To further support the finding that allergen-specific T cells that express IL-10 and TGF-β are a subset of $CD4^+$ $CD25^+$ T cells, we purified $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells before and after SIT and analyzed their cytokine profile by anti-CD3 stimulation. IL-10 and TGF-β secretion was significantly higher in $CD4^+$ $CD25^+$ T cells compared with $CD4^+$ $CD25^-$ T cells of HDM-allergic individuals (Fig. 2E) ($p<0.001$). There was no difference in IL-10 and TGF-β production of $CD4^+$ $CD25^+$ or $CD4^+$ $CD25^-$ T cells before and after SIT, probably due to the low frequency of Der p 1-specific T cells.

The involvement of $CD4^+$ $CD25^+$ T cells in the suppression of T cell response induced by SIT was further investigated. $CD4^+$ $CD25^+$ T cells purified after 70 days of SIT significantly suppressed Der p 1-specific proliferation of PBMC (Fig. 3A). In contrast, $CD4^+$ $CD25^-$ T cells did not show any suppressive effect. Interestingly, the enrichment of PBMC with $CD4^+$ $CD25^-$ T cells enhanced Der p 1-induced proliferation both before and after SIT. Simi-

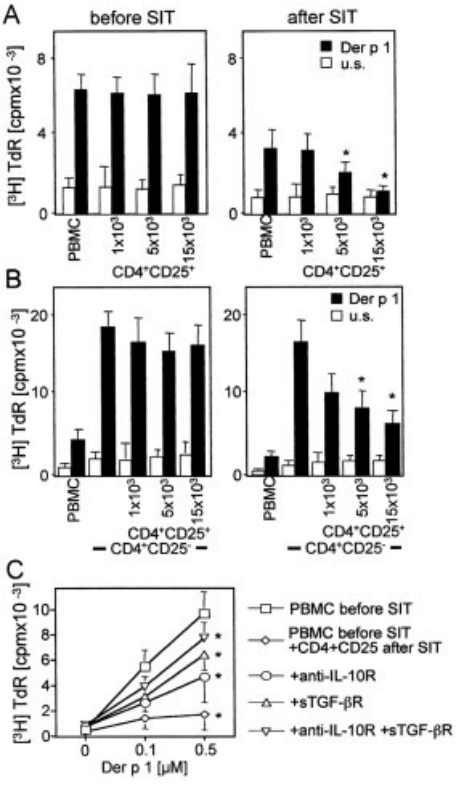

Fig. 3. Antigen-specific proliferation of PBMC is suppressed by $CD4^+$ $CD25^+$ T cells after SIT. (A) PBMC (10^5) enriched with different numbers of autologous $CD4^+$ $CD25^+$ cells obtained before and after 70 days of SIT were stimulated with 0.3 μM Der p 1 or left unstimulated (u.s.). (B) Der p 1-specific PBMC proliferation enhanced by $CD4^+$ $CD25^-$ cells is again suppressed by $CD4^+$ $CD25^+$ generated after SIT. PBMC (10^5) enriched with both 15×10^3 $CD4^+$ $CD25^-$ cells and different numbers of $CD4^+$ $CD25^+$ cells were stimulated with Der p 1. Incorporation of [^{3}H]thymidine (TdR) was measured after 6 days (cpm, counts per minute). (C) Der p 1-specific proliferation of PBMC (10^5) obtained before SIT, which were enriched with $CD4^+$ $CD25^+$ T cells (15×10^3) obtained after SIT. The responses to different concentrations of Der p 1 are significantly suppressed. Blocking of IL-10 or TGF-β partially reconstituted antigen-specific proliferation (C). Similar results were obtained in five different patients before and after HDM-SIT. *$p<0.001$.

larly, the addition of $CD4^+$ $CD25^+$ cells to these cultures resulted in suppression of Der p 1-specific T cell proliferation only after SIT (Fig. 3B). Before SIT the $CD4^+$ $CD25^+$ T cells did not show any antigen-specific suppressor activity. The enrichment of PBMC obtained before SIT with $CD4^+$ $CD25^+$ T cells that were obtained after SIT induced Der p 1-specific suppression.

Blocking of either IL-10 or TGF-β by anti-IL-10R mAb or sTGF-βR reconstituted the Der p 1-specific T cell suppression and increased antigen-specific proliferation. Neutralization of both IL-10 and TGF-β showed an additive effect in blocking the $CD4^+$ $CD25^+$ T cell induced suppression after SIT (Fig. 3C). These results suggest that Der p 1-specific T cells exist in both the $CD4^+$ $CD25^+$ and the $CD4^+$ $CD25^-$ T cell subsets. The antigen-specific suppressor activity achieved after SIT is confined to the $CD4^+$ $CD25^+$ T cell subset and their IL-10 and TGF-β production.

During the first 70 days of HDM-SIT, both the Th1 cytokine IFN-γ and the Th2 cytokines IL-5 and IL-13 decreased significantly. Accordingly, the effect of neutralization of IL-10 and TGFβ was investigated in Der p 1-stimulated PBMC cultures. The blocking of IL-10R and neutralization of TGF-β resulted in full recovery of IFN-γ, IL-13 and IL-5 production (Fig. 4).

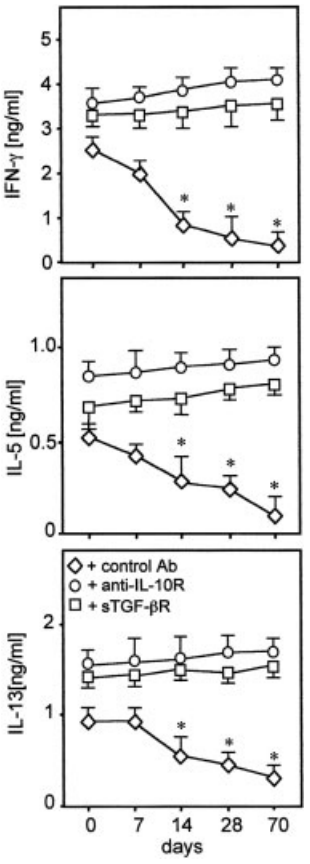

Fig. 4. Neutralization of IL-10 and TGF-β restores suppressed cytokine production in HDM-SIT. PBMC from different time points of HDM-SIT were stimulated with Der p 1 alone or together with neutralizing anti-IL-10R mAb or sTGF-βR. The levels of IFN-γ, IL-5 and IL-13 in cell supernatants were measured by ELISA after 5 days of culture. Similar results were obtained in all of the six other HDM-SIT patients. $^*p<0.05$.

2.2 IL-10 and TGF-β mediate peripheral suppression in T cells of healthy subjects

The mechanisms of normal immune response to natural allergen exposure in healthy individuals was investigated in Der p 1-specific and Bet v 1-specific T cell stimulations (Fig. 5A). A tolerant state was observed in specific T cells, and neutralization of endogenous IL-10 and TGF-β resulted in enhanced T cell responses to aeroallergens. Neutralization of both IL-10 and TGF-β in Der p 1-stimulated PBMC cultures of normal donors induced significantly increased proliferative responses.

To support these findings, the specific T cell response to the birch pollen allergen Bet v 1 was investigated during the pollen season. Consistently, we found increased T cell proliferation after neutralization of IL-10 and TGF-β; PBMC of healthy individuals secreted relatively higher levels of IL-10 and TGF-β in comparison with IL-4, IL-5 and IL-13 after Bet v 1 stimulation. In addition, Bet v 1-induced production of IFN-γ, IL-4, IL-5 and IL-13 was significantly enhanced in parallel cultures after neutralization of IL-10 and TGFβ (Fig. 5B).

If both cytokine neutralization approaches were used together, antigen-induced T cell proliferation as well as antigen-induced Th2 cytokine production showed a tendency to increase. Although these changes were not statistically significant in comparison with blocking of IL-10 or TGF-β alone, they imply an additive effect and cooperation between both cytokines. These results demonstrate that T cell response to allergens is actively suppressed by IL-10 and TGF-β in healthy subjects.

2.3 Specific IgA and IgG4 characterize the healthy antibody response

The healthy immune response to Der p 1 demonstrated specific IgA and IgG4 antibodies in serum (Fig. 6). Healthy individuals did not show IgE or IgG1 antibodies to Der p 1. The increases in serum specific IgA and IgG4 concentrations coincide with increased TGF-β and

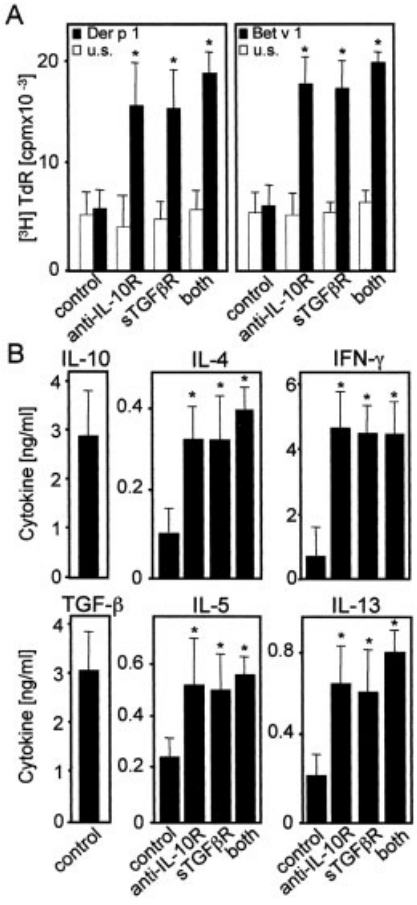

Fig. 5. IL-10 and TGF-β-mediated immune suppression to Der p 1 and Bet v 1 in nonallergic individuals. PBMC from healthy subjects were stimulated either with Der p 1 and Bet v 1 alone or together with neutralizing anti-IL-10R Ab or sTGF-βR. Rat IgG was used as a control. Incorporation of [^{3}H]thymidine (TdR; cpm, counts per minute; u.s., unstimulated) (A) and Betv 1-induced IL-4, IL-5, IL-13, IFN-γ, IL-10 and TGF-β (B) were determined after 5 days. In both Der p 1- and Bet v 1-stimulated cultures neutralization of IL-10 and TGF-β enhanced T cell proliferation and cytokine production. Data shown are the mean ± SD of three different healthy individuals. *$p<0.01$.

IL- 10, respectively. This may account for the role of IgA and TGF-β as well as IgG4 and IL-10 in peripheral mucosal immune response to allergens in healthy individuals. HDM-SIT did not change specific IgE after 70 days of treatment. However, a significant increase in specific IgA ($p<0.01$), IgG1 ($p<0.05$) and IgG4 ($p<0.01$) was observed.

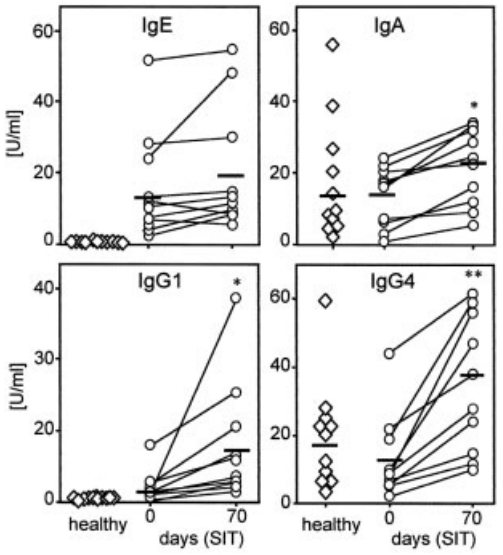

Fig. 6. Characterization of the healthy antibody response and changes during HDM-SIT. Serum Der p 1-specific IgE, IgA, IgG1 and IgG4 Ab were measured in 10 patients before and after 70 days of HDM-SIT, and in 11 healthy controls. Horizontal lines show the overall means. *$p<0.01$, **$p<0.001$.

2.4 Distinct suppressive effects of IL-10 and TGF-β on T cells

IL-10 directly acts on T cells to induce suppression by altering the co-stimulatory CD28 signaling pathway [37]. Blocking of endogenous IL-10 renders the T cells responsive to costimulation on its own. The underlying mechanisms of T cell suppression by IL-10 and TGF-β were compared. In freshly purified CD45RO$^+$ T cells, anti-CD28-mAb-induced T cell proliferation was significantly suppressed by both IL-10 and TGF-β (Fig. 7A). TGF-β but not IL-10 inhibited the anti-CD3-induced T cell proliferation. IL-15 reconstituted the suppressive effect of both IL-10 and TGF-β. These data demonstrate a direct effect on CD28 costimulation by both IL-10 and TGF-β since these experiments were performed in pure T cells. IL-10 and TGF-β differed in their suppressive capacity for anti-CD3-induced T cell proliferation, demonstrating a difference in signaling pathways and suppressive mechanisms. To analyze whether IL-10- and TGF-β-mediated T cell suppression leads to non-responsiveness or induces cell death, long-term viability of the cells was determined after 12 days (Fig. 7B). Although T cells did not proliferate in IL-10- or TGF-β-suppressed conditions, they showed long-term survival in the same range as the highly proliferating cells.

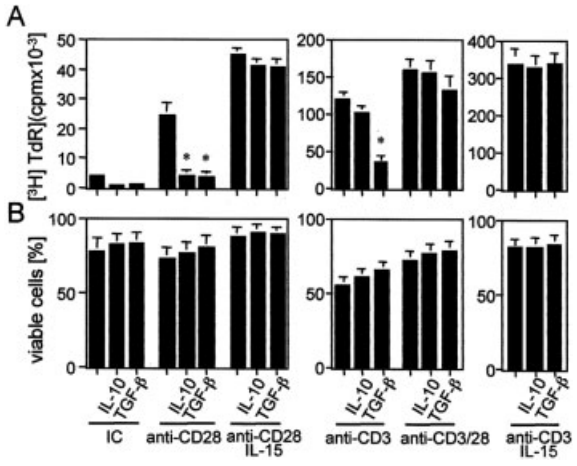

Fig. 7. IL-10 and TGF-β have distinct suppressive effects on T cells. Purified CD45RO$^+$ T cells were stimulated with anti-CD28 and anti-CD3 mAb, or both, in the presence or absence of IL-15. IL-10 or TGF-β were used from the start. (A) T cell proliferation was measured after 5 days (TdR, thymidine; cpm, counts per minute). (B) Determination of cell viability after 12 days showed no significant difference between the IL-10- or TGF-β-suppressed and non-suppressed conditions. The results are representative of three experiments in triplicates. IC, isotype control. *$p<0.001$.

3 Discussion

The present study demonstrates IL-10- and TGF-β-mediated active suppression of specific T cell responses as an essential mechanism in immune response to mucosal allergens. A distinct immune response pattern is associated with modified antibody production. IL-10-mediated distinct immune response represents a key step in natural exposure to bee venom of healthy individuals and in bee venom SIT [15, 37, 38].

Successful SIT with aeroallergens reduces allergen-specific responses assessed by skin and provocation tests [39–41]. This results from the modulation of T cell functions [31, 33–38]. CD4$^+$ T regulatory cells that specialize in the suppression of immune response are pivotal in maintenance of peripheral tolerance [42–45]. T regulatory cells include Tr1 cells, which produce high levels of IL-10 and are generated by chronic activation of CD4$^+$ T cells in the presence of IL-10, as well as Th3 cells, which are induced following oral administration of antigen and secrete predominantly TGF-β. T regulatory cells are enriched within CD4$^+$ CD25$^+$ cells [46–48].

In the present study, we demonstrate that after SIT, enrichment of the cultures with CD4$^+$ CD25$^+$ cells enhances the SIT-induced T cell suppression and that this effect is allergen-specific. Additionally, we demonstrate that allergen-specific T cells that express IL-10 and TGF-β are contained mostly within the fraction of CD4$^+$ CD25$^+$ T cells. The suppressive effect of CD4$^+$ CD25$^+$ cells is partially blocked by neutralization of either secreted or membrane-bound IL-10 and TGF-β by using anti-IL-10R mAb and sTGF-βR.

Evidence from animal models accounts for an additional active mechanism of immune suppression whereby a distinct subset of T cells inhibits the activation of conventional T cells in the periphery [42–45]. These T cells can prevent the development of autoimmunity, indicating that the normal immune system contains a population of professional regulatory T cells. Membrane CTLA-4 expression, IL-10 production, TGF-β production and membrane TGF-β expression were suggested to play a role [15, 46–48]. The phenotypic features of CD4$^+$ CD25$^+$ human *** The regulatory T cells so far described additionally include CCR8, CCR4, and TNFRII [49, 50]. Changes in these receptors and others on CD4$^+$ CD25$^+$ T cells after SIT remain to be elucidated.

TGF-β is able to down-regulate both Th1 and Th2 responses in the control of specific immune responses to high antigen doses in mucosal tissues. In human lungs, TGF-β is produced by various cell types and in very high amounts by bronchial epithelial cells [51, 52]. It also plays a crucial role in the induction of oral tolerance through activation of antigen-specific TGF-β-producing T cells [29]. Oral administration of antigens generates antigen-specific TGF-β-secreting Th3 regulatory T cells [53, 54]. In this study, if both cytokine neutralization approaches were used together, antigen-induced T cell responses showed a tendency to increase, demonstrating a cooperative effect between the two cytokines. IL-10 was reported to suppress the CD28 signaling pathway [37]. Here, we demonstrate that TGF-β directly affects T cell functions by inhibiting both the TCR/CD3 and the CD28 costimulatory pathways. IL-15 as a T cell survival and growth factor reconstituted the suppression mediated both by IL-10 and TGF-β.

The serum levels of specific IgE and IgG4 antibodies delineate allergic and normal immunity to allergen. In this study, the increase in TGF-β observed during HDM-SIT was associated with a significant increase in specific IgA to Der p 1 and a decrease in the ratio of specific IgE to IgA. Moreover, high levels of serum specific IgA and IgG4 against Der p 1 were observed in healthy individuals, while Der p 1-specific IgE and IgG1 were not detectable. It is important to note that IgA and IgG4 represent non-inflammatory isotypes, whereas IgG1 and IgE binding to allergens can initiate an inflammatory response, via com-

plement activation and mast cell degranulation, respectively.

The principal finding of this study is that IL-10 and TGF-β cooperate in suppression of the immune response to aeroallergens and control allergic inflammation due to mucosal allergen exposure in healthy individuals as well as in SIT of HDM allergic patients. The antigen-specific suppressive activity achieved after SIT is confined to the $CD4^+$ $CD25^+$ regulatory T cells. In particular, a distinct regulatory/suppressor immune response profile occurs, which promotes IgA and IgG4 antibody formation against allergen along with suppressed T cell proliferation and decreased Th2 cytokines. Thus, a modified allergen-specific T cell response may contribute to the regulation of Ig synthesis and suppression of effector cell recruitment and activation, which leads to inhibition of allergic inflammation, and clinical improvement in desensitized patients.

4 Materials and methods

4.1 Study population

Fifteen individuals (mean age 29 years) who were allergic to the HDM, Der p, who had perennial allergic rhinitis or both allergic rhinitis and asthma, and who fulfilled inclusion and exclusion criteria with positive skin test responses to Der p [positive test with specific IgE to Der p (>0.70 kU/l), as well as positive conjunctival provocation tests with Der p extract] were enrolled in this study. The first 10 patients were studied for T cell proliferation, cytokine responses and serum antibodies. An additional 5 patients were studied for the analysis of the role of $CD4^+$ $CD25^+$ T cells, because of limitation in cell numbers. The patients were not sensitized to other common allergens as assessed by disease history, SPT and serum specific IgE measurements. The patients received SIT with HDM extract (NovoHelisen Depot, Allergopharma, Reinbek, Germany) according to a clustered semi-rush protocol. In this regimen, two or three injections of increasing vaccine doses [initial dose 5 therapeutic units (TU)] at 30-min intervals were applied weekly. The maintenance dose of 5000 TU was administered monthly after 6 weeks. None of the patients received oral corticosteroids. Patients had not taken any long-acting antihistamine for at least 6 months before initiation of SIT. Short-acting antihistamines were stopped at least 14 days before skin testing. Institutional review board approval and informed consent from all patients were obtained. After 3 months of treatment, an improvement in rhinitis score (25±10 before HDM-SIT *vs* 19±8 after treatment) and asthma score (15±8 before treatment *vs* 12±6 after treatment) was observed. Blood samples were taken before HDM-SIT and 7, 14, 28 and 70 days after starting HDM-SIT. Eleven healthy non-allergic (aged 25–70 years) individuals were studied as controls.

4.2 T cell cultures

PBMC were isolated from peripheral venous blood as described previously [36]. PBMC (10^6/ml) were stimulated in a 48-well plate with 0.3 μM of Der p 1 (Allergopharma). Supernatants were harvested at day 5 for cytokine detection [36]. T cell proliferative responses were determined by stimulation of 2×10^5 PBMC for 6 days with titrated doses of Der p 1 or Bet v 1 (kindly provided by Dr R. Valenta, Vienna, Austria) allergens or 0.1 U/ml TT (Serum Institute of Berne, Switzerland) in 200 μl medium in 96-well flat-bottom tissue culture plates in triplicates [36]. They were pulsed with 1 μCi/well [^{3}H]thymidine (Du Pont / New England Nuclear, Boston, MA, USA) and incorporation of labeled nucleotide was determined, after 20 h, in an LKB beta plate reader (Wallax, Pharmacia, Turku, Finland). IL-10 was neutralized in cultures with 4 μg/ml anti-IL-10R mAb (DNAX Research Institute, Palo Alto, CA, USA) [55]. TGF-β was neutralized in cultures with 100 ng/ml recombinant human sTGF-βR (R&D Systems Europe Ltd., Basel, Switzerland). The neutralizing activity of the two approaches was controlled in titrated doses. The IL-10R-blocking mAb was compared with the isotype control and sTGF-βR was compared with bovine serum albumin. Rabbit IgG, rat IgG or mouse IgG1 (Coulter Corp., Nyon, Switzerland) served as control antibodies.

$CD4^+$ $CD25^+$ cells were isolated as described previously [56, 57]. $CD4^+$ T cells were negatively selected from PBMC using AutoMACS. $CD4^+$ $CD25^+$ T cells were positively selected by using anti-CD25 microbeads. The purities of $CD4^+$ $CD25^+$ T cells and $CD4^+$ $CD25^-$ T cells were more than 97% as assessed by flow cytometry. Autologous PBMC (10^5) obtained before or after SIT were reconstituted with different numbers of $CD4^+$ $CD25^+$ and/or $CD4^+$ $CD25^-$ T cells in 96-well plates in 200 μl supplemented RPMI 1640 medium. Incorporation of [^{3}H]thymidine was detected after 5 days. To analyze their IL-10 and TGF-β secretion profiles before and after SIT, $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells (10^5) were stimulated in 96-well plates in 200 μl medium. Supernatants were harvested after 72 h for cytokine detection. To analyze the effect of IL-10 and TGF-β on CD28 costimulation, $CD45RO^+$ T cells were negatively selected by using microbead-labeled anti-CD14, anti-CD16, anti-CD19 and anti-CD45RA mAb (the isolated cells were more than 98% pure) and stimulated with 10 μg/ml plate-bound anti-CD3 and anti-CD28 mAb [37] in triplicates. Flat-bottom 96-well plates (Costar, Corning, NY, USA) were coated with the mAb for 2 h at 37°C in PBS pH 7.4. Fifty ng/ml IL-10 (Schering AG, Keniworth, NJ, USA), 50 ng/ml IL-15 (PharMingen, St. Louis, MO, USA) or 25 ng/ml TGF-β (R&D Systems) were added from the start. Incorporation of [^{3}H]thymidine was determined after 5 days. Cell death was analyzed by the uptake of 50 μM ethidium bromide and following flow cytometry in parallel cultures after 12 days.

4.3 Quantification of cytokines

The solid-phase sandwich ELISA procedures for IFN-γ, IL-4, IL-5, IL-10 and IL-13 have been described previously [15, 36]. The sensitivity of the IFN-γ ELISA was <10 pg/ml and the sensitivity of IL-4 ELISA was <50 pg/ml (kindly provided by Dr C. H. Heusser, Novartis, Basel, Switzerland). The sensitivity of the IL-5 ELISA was <50 pg/ml, the sensitivity of the IL-10 ELISA was < 50 pg/ml and the detection limit for IL-13 was 100 pg/ml (PharMingen). TGF-β was measured using the solid-phase sandwich ELISA with a sensitivity of <10 pg/ml (R&D Systems). Supernatants (100 μl) were acidified with 4 μl of 1 N HCl, incubated for 1 h at 37°C and neutralized with 4 μl of 1 N NaOH prior to TGF-β analysis.

Immediately after isolation, PBMC were stained for the surface markers CD4 and CD25 with appropriate mAb then intracellular IL-10 was stained after fixing and permeabilizing the cells with a paraformaldehyde and saponin solution (PermeaFix™, Ortho Diagnostic Systems Inc., Raritan, NJ, USA) [15]. All antibodies and isotype controls were from PharMingen. Intracytoplasmic IL-10 content was also measured in cultured specific T cells during HDM-SIT. PBMC were stimulated with 0.3 μM Der p 1 and cultured for 10 d. Thereafter the cells were stimulated for 5 h with a mixture of 4 μg/ml anti-CD3 (Ortho Diagnostic Systems Inc.) and anti-CD28 mAb (CLB, Amsterdam, The Netherlands), in the presence of 2 μM monensin (Sigma Chem. Co., Buchs, Switzerland). The multicolor fluorescence analysis was performed on an Epics Profile flow cytometer (Coulter Corp.).

4.4 Quantification of allergen-specific antibodies

The IgE, IgA, IgG1 and IgG4 anti-Der p 1 specific antibody contents in serum were measured in duplicates by ELISA [15, 36]. ELISA plates (Maxisorb, Nunc GmBH & Co KG, Wiesbaden, Germany) were coated with 10 μg/ml Der p 1 and incubated with serum at different dilutions. Biotinylated anti-IgE mAb 6–7 were kindly provided by Dr C. H. Heusser (Novartis) and peroxidase-labeled ExtrAvidine (Sigma Chem. Co.) was used to develop IgE anti-Der p 1. Anti-IgG4 mAb RJ4 (Oxoid Ltd., Basingstoke, UK) and peroxidase-labeled anti-mouse Ig antibodies (Tago AG, Burlingame, USA) were used in IgG4 anti-Der p 1 ELISA. Mouse anti-human IgG1 mAb (Zymed, South San Francisco, CA, USA) and goat anti-human-IgA (Chemicon, Hofheim, Germany) and appropriate peroxidase-labeled secondary antibodies were used to detect IgG1 and IgA anti-Der p1 antibodies. Specific antibody binding to coated plates was controlled with hydrolyzed milk powder and human serum albumin (Sigma Chem. Co.). A pooled serum from Der p 1 allergic patients was used as arbitrary standard (10 U/ml).

4.5 Statistical analysis

Student's *t* test, Pearson's correlation analysis and the Mann-Whitney U test were used for statistical analysis to compare results at different time points of immunotherapy.

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