

Original article

The functional insufficiency of human CD4⁺CD25^{high} T-regulatory cells in allergic asthma is subjected to TNF- α modulation

Background: Natural CD4⁺CD25^{high}Foxp3⁺ regulatory T (nTreg) cells are important in maintaining immunologic tolerance, but their role in the pathogenesis of allergic asthma is unclear. We studied the function of nTreg cells in allergic asthmatic children and assessed the factors which may relate to the functional insufficiency of nTreg cells.

Methods: The percentage of CD4⁺CD25^{high} Treg cells, the expression of Foxp3, and the cell-induced suppressive activity of nTreg cells isolated from nonatopic controls, allergic asthmatics, and allergen-specific immunotherapy (AIT)-treated asthmatic patients were studied.

Results: Although the percentage of nTreg in peripheral blood mononuclear cells was increased, the expression of Foxp3 and its cell-induced suppressive activity were significantly lower in *Dermatophagoides pteronyssinus* (Der p)-sensitive asthmatic children when compared to nonatopic controls. In contrast, the expression of Foxp3 and the functional activity of nTreg cells were reversed in allergic asthmatics who received AIT. The addition of recombinant tumor necrosis factor (TNF)- α directly downregulated Foxp3 expression and abrogated the cell-induced suppressive function of Treg cells. The anti-TNF- α reagent, etanercept, restored the functional activity and Foxp3 expression of CD4⁺CD25^{high} Treg derived from allergic asthmatics.

Conclusions: The functional insufficiency of nTreg cells in patients with allergic asthma may be related to the enhanced production of TNF- α and its effect on the Foxp3 expression. These results may explain, in part, the effectiveness of anti-TNF- α therapy in the treatment of allergic asthma.

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Key words: allergen-specific immunotherapy; allergic asthma; Foxp3; natural regulatory T cells; tumor necrosis factor- α .

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Allergic asthma is characterized by a systemic exacerbated T helper type 2 (Th2) cytokine immune response to environmental allergens that leads to airflow obstruction, airway hyperresponsiveness (AHR), and ongoing active airway inflammation (1). Despite recent progress in the understanding of the role of Th2 and inflammatory cytokines, such as tumor necrosis factor (TNF)- α (2), in the pathogenesis of the disease, the mechanism underlying the development of allergen sensitization, and more importantly, the role of the regulatory pathway in the control of allergic diseases remains unknown. Although there is an extensive evidence that regulatory T cells (Treg) play a major role in controlling immune responses (3), studies exploring the role of CD4⁺ Treg cells in

allergic diseases have primarily focused on antigen-induced interleukin (IL)-10-producing Treg cells (i.e. inducible Treg; 4, 5).

Thymus-derived naturally occurring regulatory T cells (nTreg) are CD4⁺CD25⁺ T cells, functionally distinguished by their capacity to limit CD4⁺CD25⁺ T-cell proliferation and Th1/Th2 cytokines production (6, 7). Only few studies have focused on the role of nTreg in common allergic diseases. For example, children who have outgrown their cow's milk sensitivity have significantly higher levels of CD4⁺CD25⁺ T cells in their peripheral blood and are less responsive to stimulation with β -lactoglobulin *in vitro* than those who maintained a clinically active allergy (8). Natural Tregs have also been shown to suppress allergic responses to inhaled antigens such as grass or birch pollen (9–11). In contrast, animal studies on the role of nTregs in allergic asthma have yielded conflicting data. Although the development of AHR is unaffected by altering the number of

Abbreviations: AIT, allergen-specific immunotherapy; nTregs, naturally occurring regulatory T cells; Der p, *Dermatophagoides pteronyssinus*; HDM, house dust mite; PBMCs, peripheral blood mononuclear cells.

CD4⁺CD25⁺ Treg cells (12, 13), the development of eosinophilic airway inflammation is reported to be both increased (12), and paradoxically decreased (14), after depletion of naturally occurring CD4⁺ CD25⁺ Treg cells. This discrepancy may be due to the different genetic backgrounds of the mice studied (15). However, the factors that influence the functional capacity in Treg cell-mediated suppression of allergen-specific immune responses have not been delineated in allergic asthmatic patients.

Herein, we have demonstrated that the expression of Foxp3 and cell-induced suppressive activity in CD4⁺CD25^{high} Tregs were significantly decreased in allergic asthmatic patients. We also find that TNF- α , a pleiotropic cytokine, plays a critical role in bridging innate and adaptive immunity in chronic inflammatory disease (16), is increased in Der p-stimulated peripheral blood mononuclear cells (PBMCs) of allergic asthmatics, and impairs the regulatory activity of natural Treg cells via the TNF- α receptor 2 (TNFR2) signaling pathway to down-modulate Foxp3 expression. The addition of the soluble TNF- α receptor-IgG₁Fc fusion protein, etanercept, reversed the expression of Foxp3 and the loss of inhibitory activity of Treg cells in allergic asthmatics, which may explain, at least in part, the mechanism of functional insufficiency of natural Tregs in allergic asthma.

Materials and methods

Study subjects

The study population was recruited from the allergy and asthma specialty clinic at the National Cheng Kung University Hospital, and consisted newly diagnosed allergic asthmatic children (mean age, 11.2 years), allergic asthmatic children treated with allergen-specific immunotherapy (AIT) for 1 year (mean age, 10.8 years), and age-matched nonatopic controls. The definition of asthma was based on GINA guideline. All asthmatic children had a history of atopy and were sensitive to the house dust mite (HDM), *Dermaphagoides pteronyssinus* (Der p) as evaluated by prick skin test and specific immunoglobulin (Ig) E antibody to Der p using the Unicap system (Pharmacia Diagnostics, Uppsala, Sweden). Age-matched nonatopic controls did not have any of the above allergic asthmatic symptoms in their previous physical check-ups. The AIT protocol was initiated with a weekly injection of 10⁻⁶ g/ml of Der p (Allermed, San Diego, CA, USA). The dose was increased gradually until a maximum tolerated dose was reached (usually within 6 months) and was then maintained at 4-week intervals. The total dose of crude mite allergen administered ranged from 126.5 to 219.5 μ g. The effectiveness of AIT was evaluated by both the frequency of allergic asthmatic attacks and the amount of medication consumed and compared with those same parameters the year before treatment. In the current study, only good responders to AIT that demonstrated $\geq 75\%$ decrease in both parameters were recruited. In all study subjects, no inhaled or oral steroids or bronchodilators were taken for at least 1 week and 6 h, respectively, before blood sampling. This study was approved by the Human Research Committee of the National Cheng Kung University Hospital and informed consent was obtained from all subjects or their guardians.

Antibodies and reagents

Der p extract (1 g lyophilized whole body extract in ether; Allergon, Engelholm, Sweden) was dissolved in pyrogenic-free isotonic saline, filtered through a 0.22 μ m filter, and stored at -70°C before use. The lipopolysaccharide concentration of the preparations was <0.96 EU/mg of Der p (Limulus amoebocyte lysate test; E-Toxate; Sigma-Aldrich, St Louis, MO, USA), etanercept (Enbrel[®], Wyeth Pharmaceuticals Co., Philadelphia, PA, USA), which has been used clinically, was purchased and prepared according to the package insert.

Isolation of cells and flow cytometry

Peripheral blood mononuclear cells were separated by Ficoll-Paque Plus (Amersham Bioscience, Uppsala, Sweden) gradient centrifugation (850 g at room temperature for 20 min). Non-CD4⁺ cells in PBMCs were removed by LD column with biotin-antibody cocktail and antibiotin microbeads (Mileny Biotech, Bergisch Gladbach, Germany). CD4⁺ cells were incubated with anti-CD25 beads (3 μ l per 10⁷ cells), and CD25⁻ and CD25⁺ fractions were recovered using a MS-magnetic column according to the manufacturer's instructions (Mileny Biotech). The purity of the cell populations was determined by flow cytometry using the following monoclonal antibodies (mAb; eBioscience, San Diego, CA, USA): FITC-anti-CD4 (RPA-T4), PE-isotype control IgG1, and PE-anti-Foxp3 and PE-anti-CD25 (4E3) (Mileny Biotech). The percentage of CD25^{high} cells was determined according to a typical Treg cell phenotype and based on the mean fluorescent intensity of CD25 ≥ 100 (11).

Cellular proliferation and ELISA

The suppressive capacity was determined by culturing 1×10^4 CD4⁺CD25⁻ T cells in a 1 : 1 mixture of CD4⁺CD25^{high} cells in the presence of 2.5 μ g/ml of soluble antihuman CD28 (CD28.2; eBioscience) and 2.5 μ g/ml anti-CD3 mAb (OKT3; eBioscience) coated 96-well plates (Bio-Whittaker, Walkersville, MD, USA) for 3 days. The culture conditions were incubated without or with various dosages of Der p (Allergon). At the end of the culture periods, cell proliferation of CD4⁺CD25⁻ T cells was assessed using a WST-8 (modified tetrazolium salt) cell proliferation kit (Cell Counting Kit-8, Dojin, Japan) according to the manufacturers' protocols instead of a direct cell count. A human TNF- α ELISA kit (R&D System, Inc., Minneapolis, MN, USA) was used for the assay of TNF- α levels in the supernatants of different cellular conditions.

Preincubation experiments of TNF- α

Purified CD4⁺CD25⁻ and CD4⁺CD25^{high} T cells were incubated for 3 days with 100 U/ml IL-2 (eBioscience), or various dose of recombinant human TNF- α (eBioscience) started from 1 to 100 ng/ml in the medium supplemented with IL-2. Afterward, cells were washed extensively and used in the assays of Foxp3 expression as described above. We found the maximal inhibition effect was achieved at the concentration of 50 ng/ml of TNF- α , and was adopted for the subsequent experiments.

Statistics

Data were shown as mean $\pm$ SD and analyzed by a two-tailed Mann-Whitney U-test using GRAPHPAD PRISM 4.0 (GraphPad, San Diego, CA, USA). P-values of <0.05 were regarded as significant.

Results

Decreased expression of Foxp3 and impaired cell-induced suppressive activity of CD4⁺CD25^{high} T cells in allergic asthmatic patients

We obtained >90% purity of isolated CD4⁺CD25[−] T cells (range: 90–95%), in which Foxp3 expression was almost undetectable (range: 0.8–6.7%). The purity of isolated CD4⁺CD25^{high} T cells was over 95% and Foxp3 expression was 82.5% (range: 49–90%; Supplementary Fig. S1). As illustrated in Fig. 1, the percentage of CD4⁺CD25^{high} T cells was slightly increased in allergic asthmatics, but not to a significant level, when compared to nonatopic controls (controls: $0.20 \pm 0.18\%$, $n = 16$ vs allergic asthmatics: $0.54 \pm 0.18\%$, $n = 23$; Fig. 1A). The average Foxp3 expression of CD4⁺CD25^{high} T cells in allergic asthmatic individuals was significantly decreased when compared to those of age-matched nonatopic controls (controls: $94.29 \pm 6.60\%$, $n = 10$ vs allergic asthmatics: $91.05 \pm 3.01\%$, $n = 10$; $P < 0.05$; Fig. 1B). Moreover, the CD4⁺CD25^{high} T cell-induced suppression of effector CD4⁺CD25[−] T-cell proliferation was impaired in allergic asthmatics. The addition of CD4⁺CD25^{high}Foxp3⁺ Tregs suppressed the proliferation of CD4⁺CD25[−] T cells in nonatopic controls, but the suppressive function was defective in allergic asthmatic individuals (controls: $72.15 \pm 6.33\%$, $n = 10$ vs allergic asthmatics: $26.93 \pm 6.07\%$, $n = 10$; $P < 0.001$; Fig. 1C).

Comparison of Foxp3 expression and the cell-induced suppressive activity of CD4⁺CD25^{high} T cells during stimulation with Der p extract

To study the effect of HDM allergen on the function of natural Treg cells, PBMCs collected from study subjects were incubated with Der p extract ($5 \mu\text{g}$ per 10^6 PBMCs) for 6 days, then subjected to analysis for their respective cellular numbers of CD4⁺CD25^{high} T cells and Foxp3 expression using flow cytometry (Supplementary Fig. S2). After stimulation with Der p extract, the percentage of CD4⁺CD25^{high} T cells in nonatopic controls as well as in allergic asthmatics and AIT-treated patients did not change significantly when compared to cell preparations without Der p stimulation (Fig. 2A). In contrast, the expression of Foxp3 in CD4⁺CD25^{high} T cells was significantly reduced in allergic asthmatic individuals after Der p stimulation (medium: $76.71 \pm 23.13\%$ vs Der p: $50.61 \pm 37.0\%$, $n = 20$, $P < 0.01$; Fig. 2B), while in nonatopic controls and AIT-treated patients, the expression of Foxp3 did not exhibit a significant change after Der p stimulation (Fig. 2B). The cell-induced suppressive activity of CD4⁺CD25^{high} T cells in nonatopic controls was not influenced by Der p, but those from Der p-sensitive asthmatics were significantly impaired (Fig. 2C). The percentage inhibition in allergic asthmatic individuals was significantly decreased when compared to non-

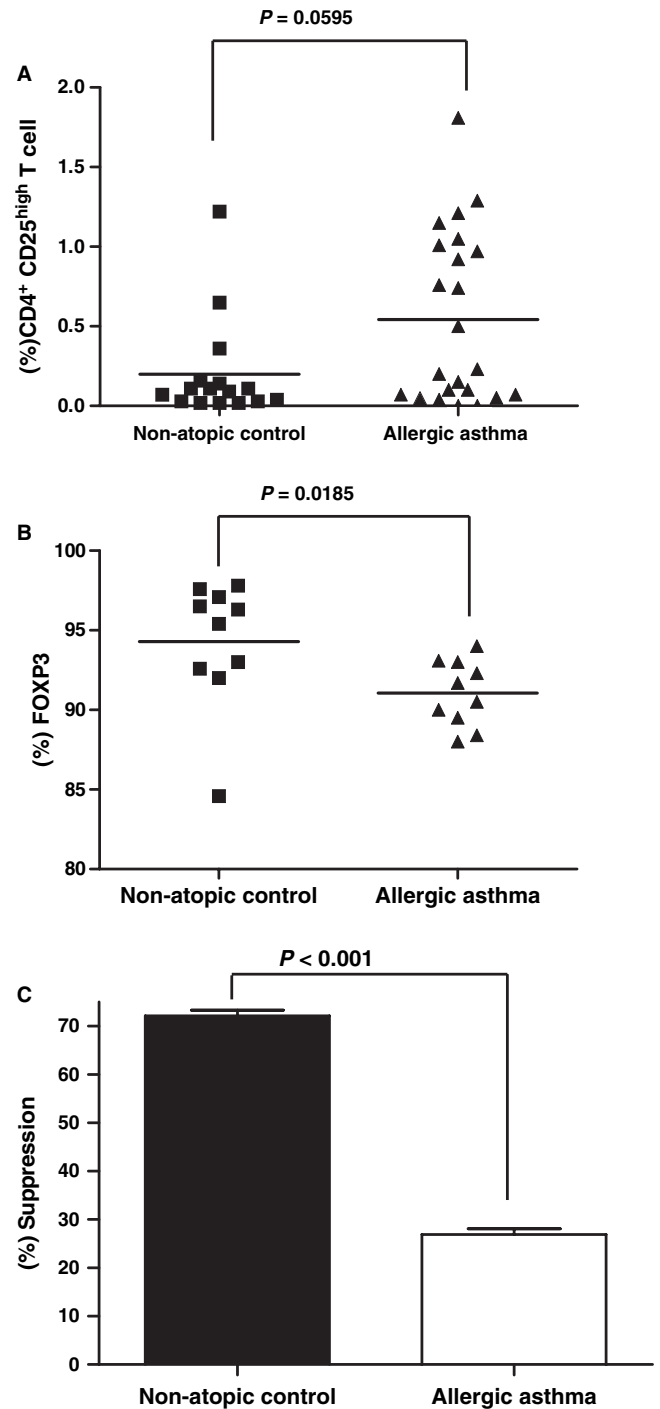


Figure 1. Comparison of percentages of CD4⁺CD25^{high} regulatory T cells in peripheral blood mononuclear cell (A), Foxp3 expression in CD4⁺CD25^{high} regulatory T cells (B), and the suppressive activities of CD4⁺CD25^{high} regulatory T cells in allergic asthmatics and nonatopic controls (C).

topic controls (controls: $68.52 \pm 9.87\%$, $n = 20$ vs allergic asthmatics: $27.72 \pm 14.9\%$, $n = 20$; $P < 0.001$; Fig. 2C). In contrast, the cell-induced suppressive activity of CD4⁺CD25^{high} T cells in AIT-treated asthmatics was

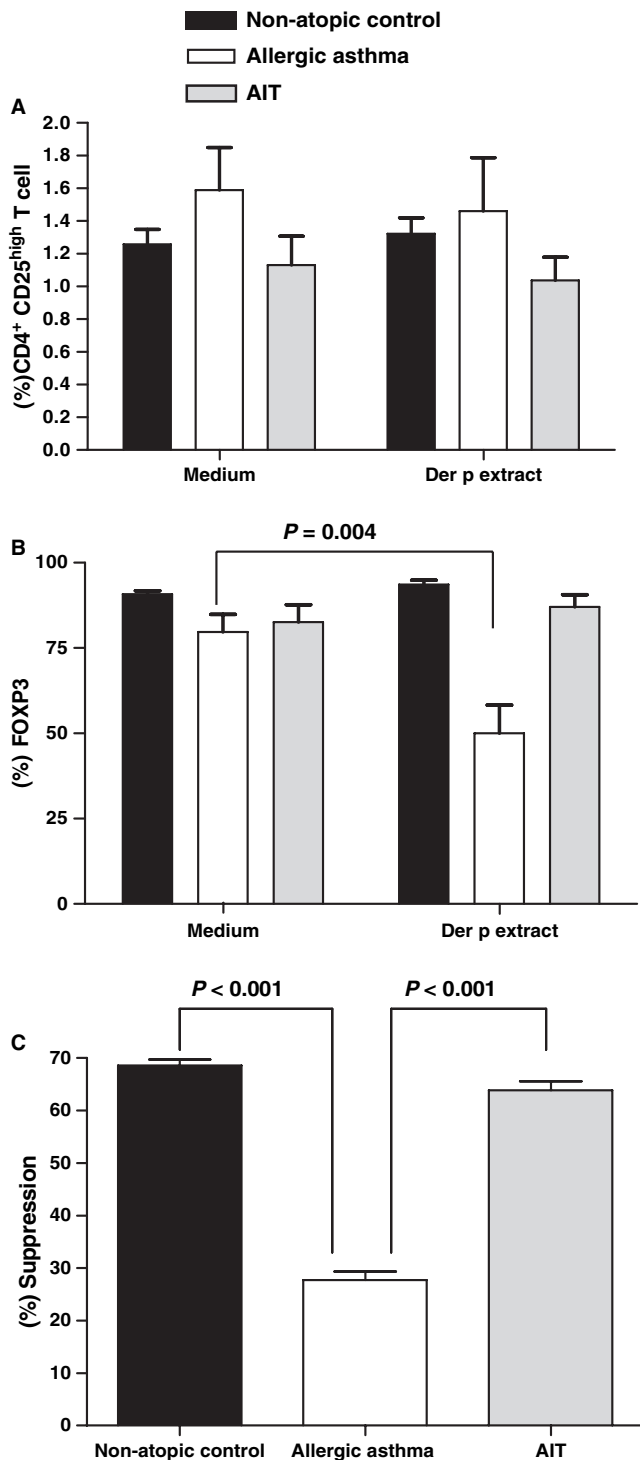


Figure 2. The percentages (A), Foxp3 expression (B), and suppressive activity (C) in CD4⁺CD25^{high} regulatory T cells, when cultured with medium, or stimulated with Der p for 6 days in nonatopic controls ($n = 20$), allergic asthmatics ($n = 20$), and allergen-specific immunotherapy-treated asthmatic children ($n = 20$).

reversed when compared to allergic asthmatics (allergic asthmatics: mean, $27.72 \pm 14.9\%$, $n = 20$ vs AIT: $63.87 \pm 11.51\%$, $n = 20$; $P < 0.001$; Fig. 2C).

The effect of TNF- α on Foxp3 expression in CD4⁺CD25^{high} T cells. Previously, we and others have found that there are increased amounts of TNF- α production in sera and in allergen-stimulated PBMCs from allergic asthmatic children (17, 18). The role of TNF- α in the pathogenesis of asthma is not limited to its biologic effects in airway inflammation and remodeling (19, 20); perhaps, the most prominent effect of TNF- α is that of induction and aggravation of AHR (21, 22). From the assay of TNF- α production in the culture supernatants of Der p-stimulated PBMCs ($5 \mu\text{g}$ per 10^6 PBMCs), there were significantly higher levels of TNF- α in allergic asthma ($1076.8 \pm 195.4 \text{ pg/ml}$, $n = 10$), when compared to AIT-treated allergic asthma ($334.3 \pm 109.9 \text{ pg/ml}$, $n = 10$) and nonatopic controls ($229.6 \pm 66.9 \text{ pg/ml}$, $n = 10$, $P < 0.001$; Fig. 3A). Moreover, we found the Foxp3 expression in recombinant TNF- α -stimulated CD4⁺CD25^{high} T cells was significantly decreased (IL-2 only: $80.5 \pm 8.9\%$ vs IL-2 + TNF- α : $46.0 \pm 28.3\%$, $n = 10$, $P < 0.001$; Fig. 3B,C).

A soluble TNF- α receptor fusion protein, etanercept, reversed Foxp3 expression and the cell-induced suppressive activity of CD4⁺CD25^{high} T cells in allergic asthmatics

To further examine the role of TNF- α in downregulating the function of human CD4⁺CD25^{high} Treg cells, etanercept ($1 \mu\text{g/ml}$), a soluble fusion protein combining two p75 TNF receptors (TNFR2), was added to the Der p-stimulated PBMCs ($5 \mu\text{g}$ per 10^6 PBMCs) culture system. We found that etanercept increased the Foxp3 expression (Der p only: $39.1 \pm 25.1\%$ vs Der p + etanercept: $73.4 \pm 36.2\%$, $n = 10$, $P < 0.01$; Fig. 4A) and enhanced the cell-induced suppressive activity (OD: 450 nm ; Der p: 1.52 ± 0.19 vs Der p + etanercept: 0.95 ± 0.25 , $n = 10$, $P < 0.05$; Fig. 4B) in CD4⁺CD25^{high} Treg cells from allergic asthmatic individuals.

Moreover, Fig. 5 showed that human CD4⁺CD25^{high} T cells basally expressed the TNF- α receptor, TNFR2 ($62.02 \pm 16.3\%$, $n = 10$), and TNFR2 expression was enhanced ($79.5 \pm 12.7\%$, $n = 10$) in CD4⁺CD25^{high} T cells isolated from the Der p-stimulated PBMCs, but not to a significant difference ($P = 0.056$). The expression of TNFR2 in CD4⁺CD25^{high} Treg cells was significantly reduced in etanercept-treated ($1 \mu\text{g/ml}$), Der p-stimulated PBMCs ($58.1 \pm 19.2\%$, $n = 10$) when compared to those of nontreated, Der p-stimulated PBMCs in allergic asthmatic patients ($P = 0.032$, Fig. 5).

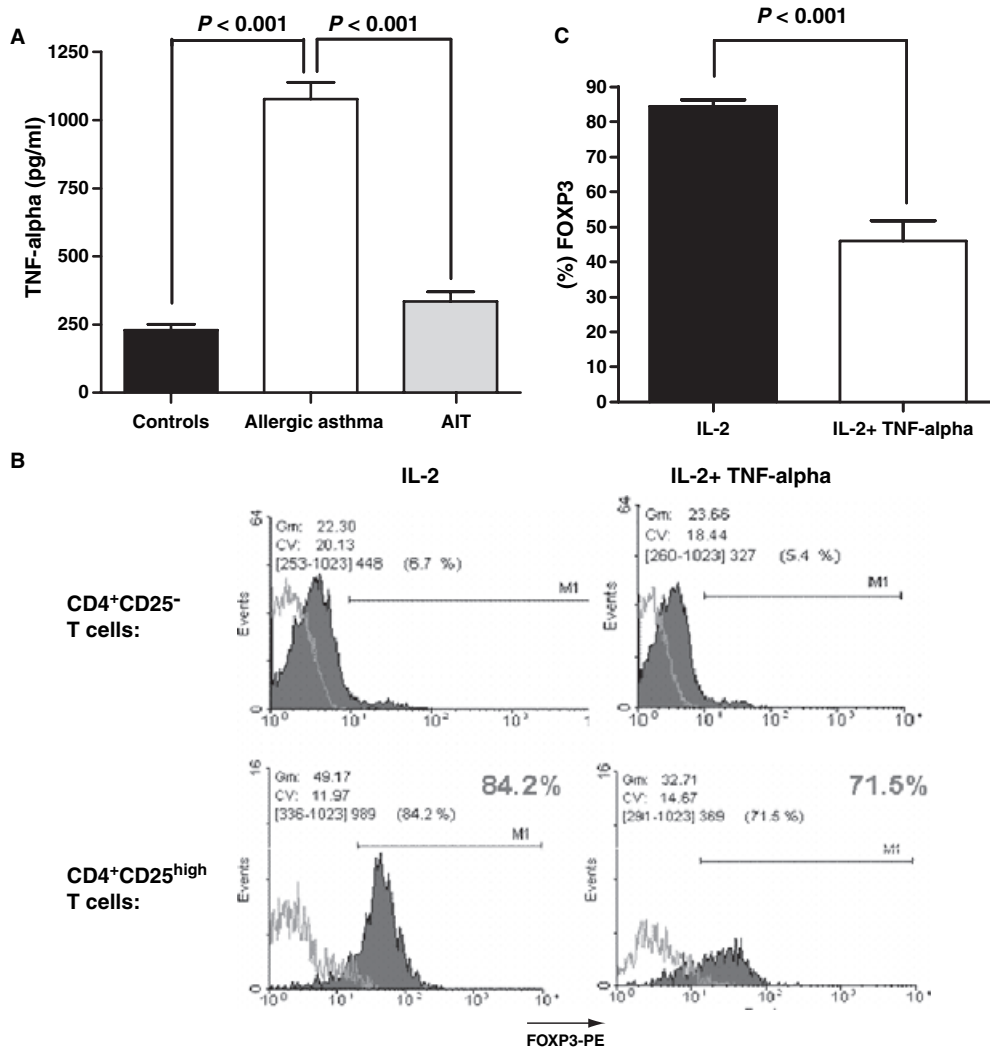


Figure 3. The levels of tumor necrosis factor (TNF)- α in the culture supernatants of Der p-stimulated peripheral blood mononuclear cells in nonatopic controls ($n = 10$), allergic asthmatics ($n = 10$), and allergen-specific immunotherapy-treated asthmatics ($n = 10$) (A). Flow cytometry analysis of Foxp3 expression in CD4⁺CD25⁻ T cells (upper panels) and CD4⁺CD25^{high} regulatory T cells (lower panels) when incubated with IL-2 or IL-2 plus recombinant TNF- α (B), and the effect of recombinant TNF- α on the expression of Foxp3 in CD4⁺CD25^{high} regulatory T cells from nonatopic controls ($n = 10$; C).

Discussion

Our results have shown that there is a functional insufficiency of natural Treg cells in allergic asthmatic children, as demonstrated by the decreased expression of Foxp3 and impaired cell-induced suppressive activity on effector T cells. We also found that TNF- α was able to down-modulate Foxp3 expression and led to functional insufficiency of natural Treg cells. Furthermore, this impaired activity was reversed in AIT-treated patients and by the addition of anti-TNF- α agent in the culture cells of allergic patients. Several studies (8–11) have examined the role of natural Tregs in common allergic diseases, and suggested that CD25⁺ Tregs in allergic patients may not be dysfunctional *per se*, but may rather

be affected by various genetic and environmental factors resulting in the dysregulation of Th2 response to allergen. In line with their findings, our results showed that the total number of CD4⁺CD25^{high} T cells was increased in allergic asthma, while the expression of Foxp3 levels was significantly lower when compared to normal controls, and reversed in AIT-treated asthmatics, indicating that the expression of Foxp3, as well as the function of natural Treg cells was influenced by the environmental signals that likely prevented them from suppressing allergen-induced Th2 cytokines responses. Further evidence of environmental influence on the function of Treg cells in allergic diseases comes from the report of Mamessiere et al. (23) that a Th2-to-Th1 switch occurs during ultra-rush venom immunotherapy (VIT), in parallel with

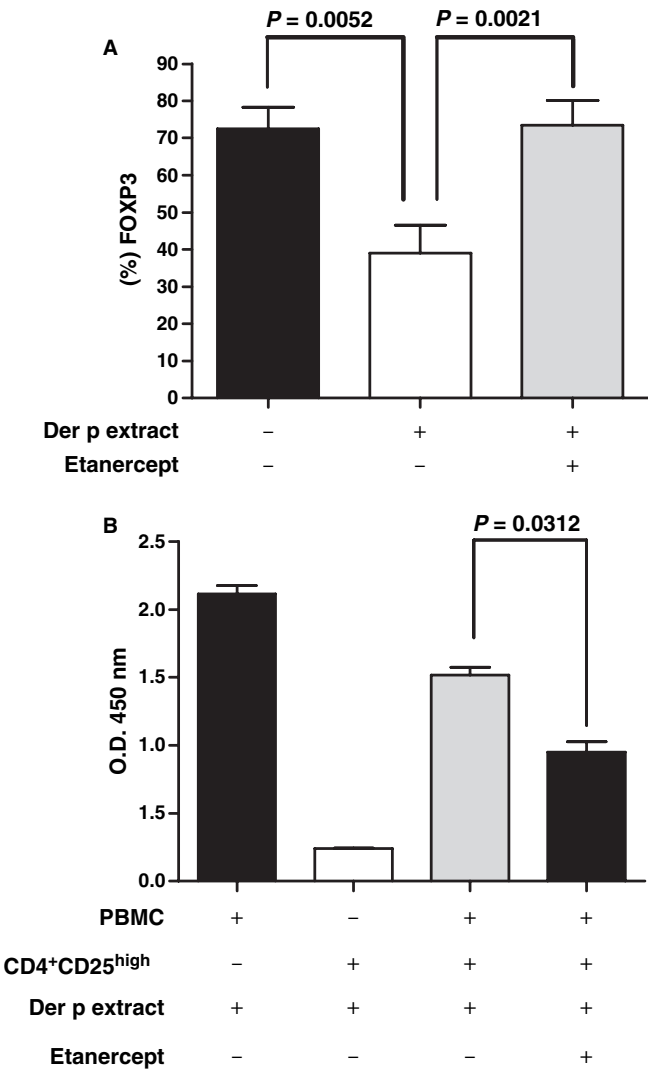


Figure 4. Effect of etanercept on the expression of Foxp3 in CD4⁺CD25^{high} regulatory T cells (A), suppressive activity of CD4⁺CD25^{high} regulatory T cells (B) in allergic asthmatic patients (n = 10).

natural and acquired Tregs increase. These events occur earlier and at a higher level in less severe subjects, suggesting that VIT tolerance induction is easier to achieve in these patients, which is in accordance with the inflammatory environment hypothesis.

The expression of Foxp3, which appears to serve as a master switch gene for Treg development, is closely correlated with the function of CD4⁺CD25⁺ T cells in mice and CD4⁺CD25^{high} T cells in humans (24). The spontaneous development of allergic airway inflammation, hyper-IgE, and eosinophilia in addition to various autoimmune syndromes in Foxp3 mutant mice, and the subsequent rescue of these animals if transferred with wild-type CD25⁺ Tregs, provide compelling evidence for the important role of CD25⁺ Treg and Foxp3 in allergic

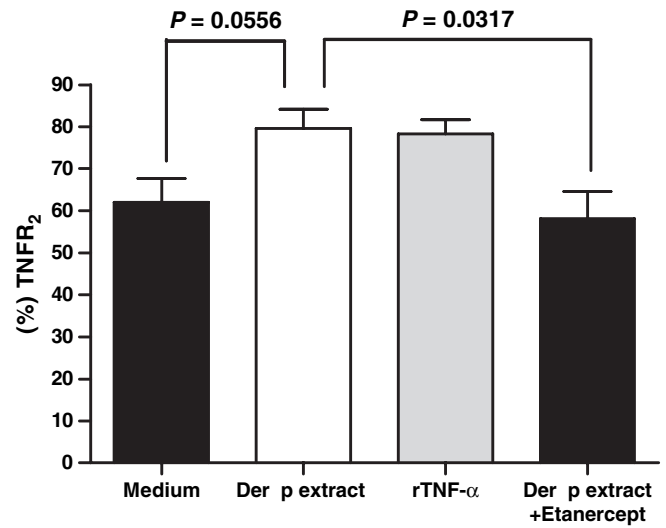


Figure 5. The expression of TNFR2 in CD4⁺CD25^{high} regulatory T cells when stimulated with Der p, recombinant tumor necrosis factor- α , and Der p plus etanercept in allergic asthmatic patients (n = 10).

diseases (25). But there is still limitation to rely on the expression of Foxp3 for the evaluation of the suppressive activity of natural Tregs. It is found that Foxp3⁺CD4⁺/CD25^{low} cells with suppressive activity also exist, and nonregulatory human CD4⁺ and CD8⁺ T cells can be induced to transiently express Foxp3, but without suppressive activity (26, 27). Although the augmentation or impairment of suppressive activity in naturally Tregs are not totally depended on Foxp3 expression, our results showed that Der p-induced TNFR2 expression in natural Tregs from Der p-sensitive allergic asthmatics (Fig. 5), and adding recombinant TNF- α significantly downregulated Foxp3 expression and inhibited suppressive activity of natural Tregs (Fig. 4), suggesting the important role of TNF- α on Foxp3 expression in the function of natural Tregs. In fact, several studies have indicated that TNF- α , IL-7, and IL-15 in the inflamed joints exerted a negative effect on tissue-derived CD4⁺CD25^{high} Treg cells *in vivo*, and this impaired cell-induced suppressive activity was restored by the addition of antagonists for these cytokines (28, 29). However, the role of these inflammatory cytokines in regulating the function of natural Treg cells or their impact on effector cells is presently unknown.

Tumor necrosis factor- α contributes to the dysregulated inflammatory response seen in the asthmatic airway; this has been demonstrated by the findings of increased TNF- α mRNA (18) and protein (17, 19) in the sera and airways of patients with asthma, as well as several properties of TNF- α that might be relevant to airway inflammation (19, 20) and AHR (20–22) of asthma in general, and airway remodeling in refractory asthma (30, 31). Our results are the first to show that allergen-induced TNF- α production *in vivo* (17–20) and *in vitro* (17, 21, 22)

in allergic asthmatic patients is the major cause for functional insufficiency of natural Treg cells via the down-modulation of Foxp3 expression. Two recently published studies (32, 33) have evaluated a possible therapeutic target for severe and refractory asthma (i.e. corticosteroid-dependent) using the TNF- α blocking agent, etanercept, which can improve asthmatic symptoms, pulmonary function, and bronchial hyper-responsiveness in chronic severe asthma. These clinical observations, accompanied with our results of the effect of etanercept on the restoration of suppressive activity of natural Treg cells *in vitro*, further confirm the important role of TNF- α and the functional insufficiency of natural CD4⁺CD25^{high} Foxp3⁺ Tregs cells in the pathogenesis of allergic asthma.

In conclusion, we have found that functional insufficiency of natural Tregs in allergic asthma may be related to the enhanced production of TNF- α and its effect on the Foxp3 expression in Treg cells. These results may provide a new therapeutic modality in the treatment of allergic asthma.

Supplementary Material

The following supplementary material is available for this article:

Figure S1. Representative flow cytometry results of Foxp3 expression in isolated CD4⁺CD25⁺ T cells and CD4⁺CD25^{high} regulatory T cells

Figure S2. Comparison of Foxp3 expression in CD4⁺CD25^{high} regulatory T cells isolated from allergen-stimulated PBMCs from representative subject of non-atopic control (A), allergic asthmatic (B), and AIT-treated asthmatic patient (C)

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Acknowledgment

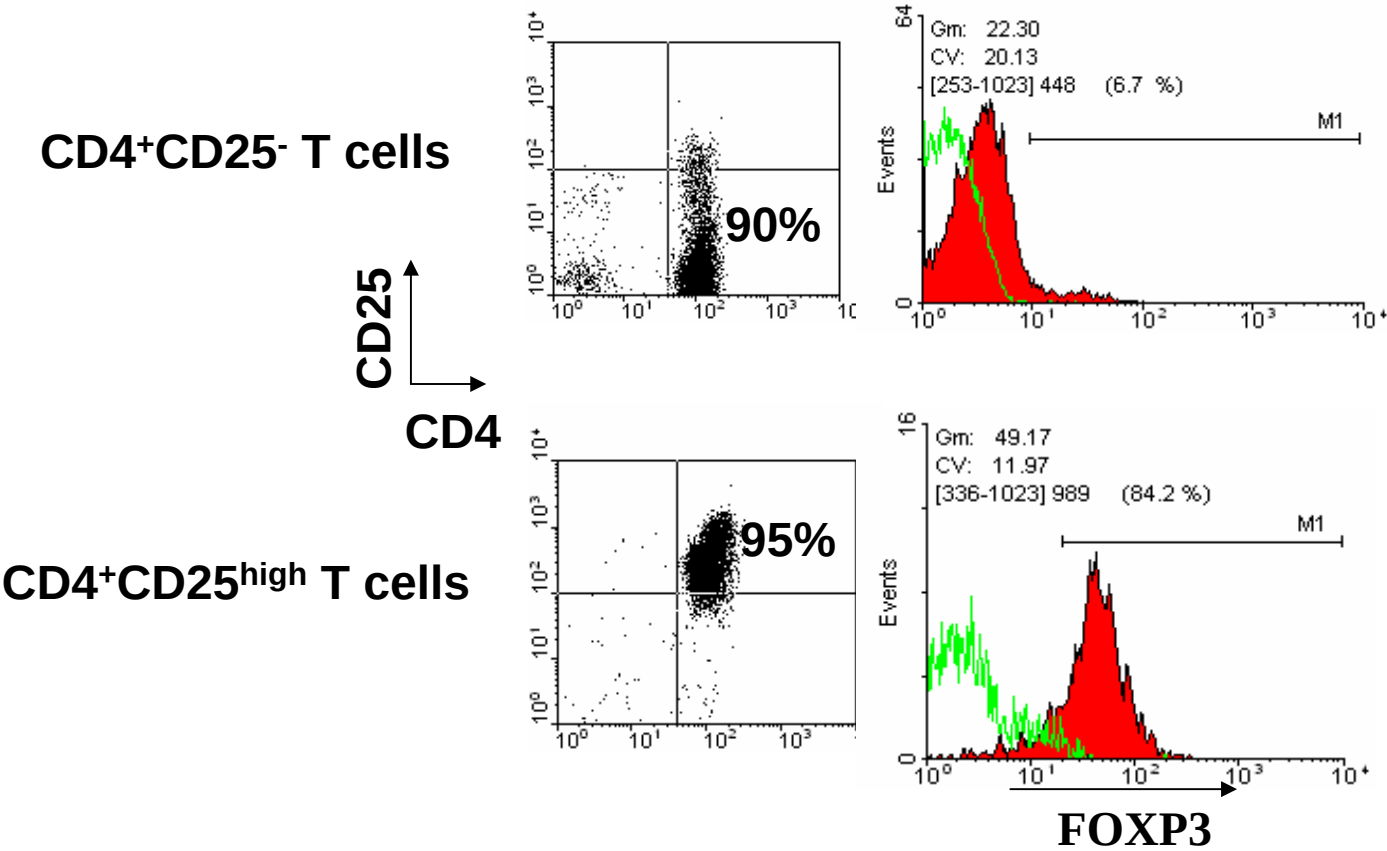
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References

- Wills-Karp M. Interleukin-13 in asthma pathogenesis. *Immunol Rev* 2004;**202**:175–190.
- Berry M, Brightling C, Pavord I, Wardlaw AJ. TNF- α in asthma. *Curr Opin Pharmacol* 2007;**7**:1–4.
- Akdis M, Blaser K, Akdis CA. T regulatory cells in allergy: novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. *J Allergy Clin Immunol* 2005;**116**:961–968.
- Francis JN, Till SJ, Durham SR. Induction of IL-10⁺CD4⁺CD25⁺ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 2003;**111**:1255–1261.
- Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszczyk M, Blaser K et al. IL-10 and TGF- β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 2003;**33**:1205–1214.
- Sakaguchi S. Naturally arising Foxp3-expressing CD25⁺CD4⁺ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005;**6**:345–352.
- Coomes JL, Robinson NJ, Maloy KJ, Uhlig HH, Powrie F. Regulatory T cells and intestinal homeostasis. *Immunol Rev* 2005;**204**:184–194.
- Karlsson MR, Rugtveit J, Brandtzaeg P. Allergen-responsive CD4⁺CD25⁺ regulatory T cells in children who have outgrown cow's milk allergy. *J Exp Med* 2004;**199**:1679–1688.
- Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J et al. Relation of CD4⁺CD25⁺ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004;**363**:608–615.
- Grindebacke H, Wing K, Andersson AC, Suri-Payer E, Rak S, Rudin A. Defective suppression of Th2 cytokines by CD4⁺CD25⁺ regulatory T cells in birch allergies during birch pollen season. *Clin Exp Allergy* 2004;**34**:1364–1372.
- Bellinghausen I, Klostermann B, Knop J, Saloga J. Human CD4⁺CD25⁺ T cells derived from the majority of atopic donors are able to suppress TH1 and TH2 cytokine production. *J Allergy Clin Immunol* 2003;**111**:862–868.
- Jaffar Z, Sivakuru T, Roberts K. CD25⁺CD4⁺ T cells regulate airway eosinophilic inflammation by modulating the Th2 cell phenotype. *J Immunol* 2004;**172**:3842–3849.
- Hadeiba H, Locksley RM. Lung CD25⁺CD4⁺ regulatory T cells suppress type 2 immune responses but not bronchial hyperreactivity. *J Immunol* 2003;**170**:5502–5510.
- Suto A, Nakajima H, Kagami SI, Suzuki K, Saito Y, Iwamoto I. Role of CD4(+) CD25(+) regulatory T cells in T helper 2 cell-mediated allergic inflammation in the airways. *Am J Respir Crit Care Med* 2001;**164**:680–687.
- Lewkowich IP, Herman NS, Schleifer KW, Dance MP, Chen BL, Sproles AA et al. CD4⁺CD25⁺ T cells protect against experimentally-induced asthma by altering the dendritic cell phenotype and activation state. *J Exp Med* 2005;**202**:1549–1561.

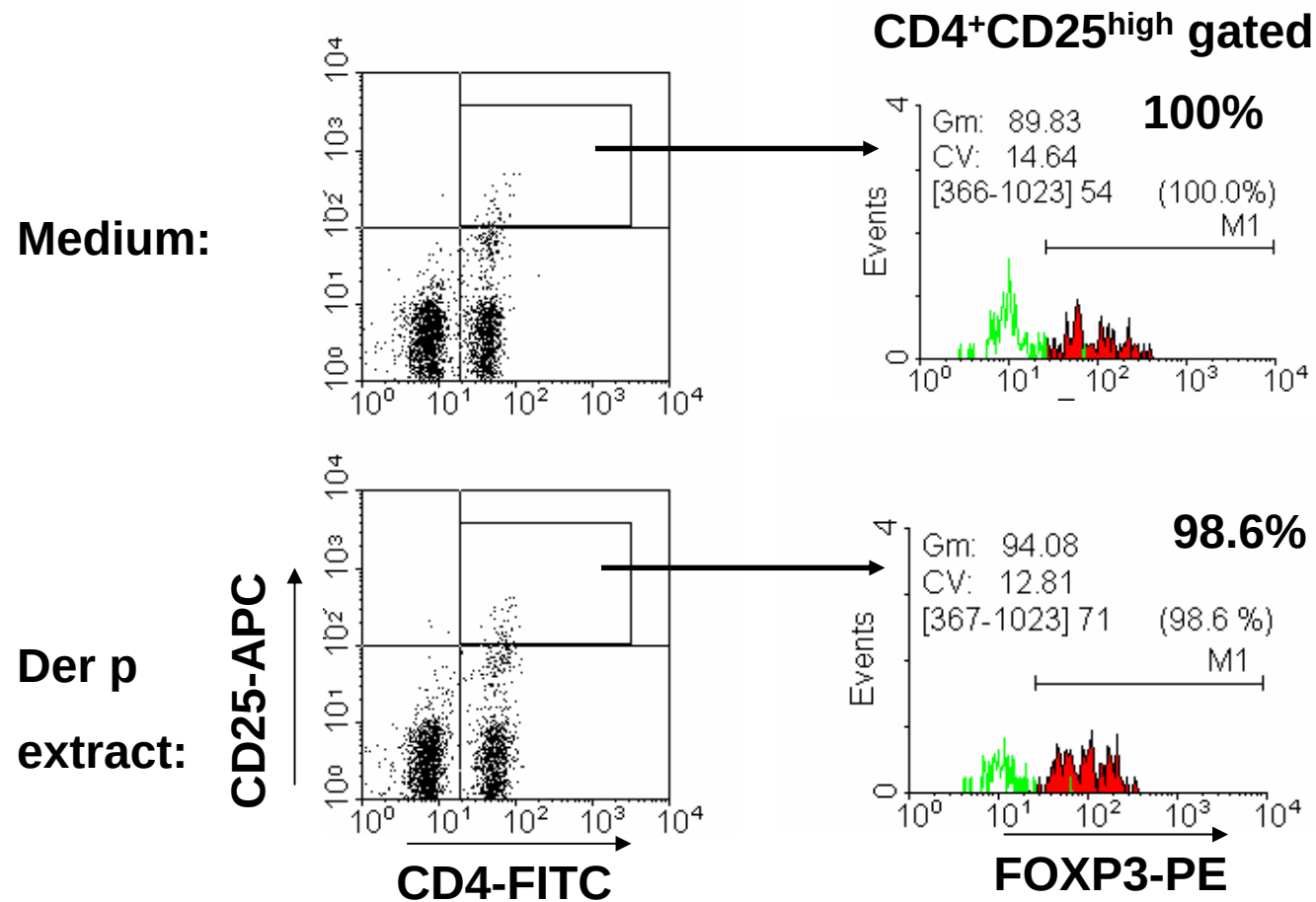
16. Pasparakis M, Alexopoulou L, Episkopou V, Kollias G. Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *J Exp Med* 1996;**184**:1397–1411.
17. Wang JY, Lei HY, Hsieh KH. The effect of immunotherapy on interleukin 1 and tumor necrosis factor production on monocytes in asthmatic children. *J Asthma* 1992;**29**:193–201.
18. Ying S, Robinson DS, Varney V, Meng Q, Tsicopoulos A, Moqbel R et al. TNF alpha mRNA expression in allergic inflammation. *Clin Exp Allergy* 1991;**21**:745–750.
19. Bradding P, Roberts JA, Britten KM, Montefort S, Djukanovic R, Mueller R et al. Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines. *Am J Respir Cell Mol Biol* 1994;**10**:471–480.
20. Thomas PS, Yates DH, Barnes PJ. Tumor necrosis factor alpha increases airway responsiveness and sputum neutrophilia in normal human subjects. *Am J Respir Crit Care Med* 1995;**152**:76–80.
21. Amrani Y, Chen H, Panettieri RA Jr. Activation of tumor necrosis factor receptor 1 in airway smooth muscle: a potential pathway that modulates bronchial hyper-responsiveness in asthma? *Respir Res* 2000;**1**:49–53.
22. Choi IW, Kim S, Kim YS, Ko HM, Im SY, Kim JH et al. TNF- α induces the late-phase airway hyperresponsiveness and airway inflammation through cytosolic phospholipase A2 activation. *J Allergy Clin Immunol* 2005;**116**:537–543.
23. Mamelessier E, Birnbaum J, Dupuy P, Vervloet D, Magnan A. Ultra-rush venom immunotherapy induces differential T cell activation and regulatory patterns according to the severity of allergy. *Clin Exp Allergy* 2006;**36**:704–713.
24. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 2005;**22**:329–341.
25. Lin W, Truong N, Grossman WJ, Haribhai D, Williams CB, Wang J et al. Allergic dysregulation and hyperimmunoglobulinemia E in Foxp3 mutant mice. *J Allergy Clin Immunol* 2005;**116**:1106–1115.
26. Ziegler SF. FOXP3: of mice and men. *Annu Rev Immunol* 2006;**24**:209–226.
27. Gavin MA, Torgerson TR, Houston E, deRoos P, Ho WY, Stray-Pedersen A et al. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci USA* 2006;**103**:6659–6664.
28. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4⁺CD25^{high} T-regulatory cells. *Blood* 2006;**108**:253–261.
29. Ruprecht CR, Gattorno M, Ferlito F, Gregorio A, Martini A, Lanzavecchia A et al. Coexpression of CD25 and CD27 identifies Foxp3 regulatory T cells in inflamed synovia. *J Exp Med* 2005;**201**:1793–1803.
30. Amrani Y, Panettieri RA Jr, Frossard N, Bronner C. Activation of the TNF alpha-p55 receptor induces myocyte proliferation and modulates agonist-evoked calcium transients in cultured human tracheal smooth muscle cells. *Am J Respir Cell Mol Biol* 1996;**15**:55–63.
31. Sullivan DE, Ferris M, Pociask D, Brody AR. Tumor necrosis factor-alpha induces transforming growth factor-beta1 expression in lung fibroblasts through the extracellular signal regulated kinase pathway. *Am J Respir Cell Mol Biol* 2005;**32**:342–349.
32. Howarth PH, Babu KS, Arshad HS et al. Tumour necrosis factor (TNF- α) as a novel therapeutic target in symptomatic corticosteroid dependent asthma. *Thorax* 2005;**60**:1012–1018.
33. Berry MA, Hargadon B, Shelley M, Parker D, Shaw DE, Green R et al. Evidence of a role of tumor necrosis factor α in refractory asthma. *N Engl J Med* 2006;**354**:697–708.

Supplement figure 1



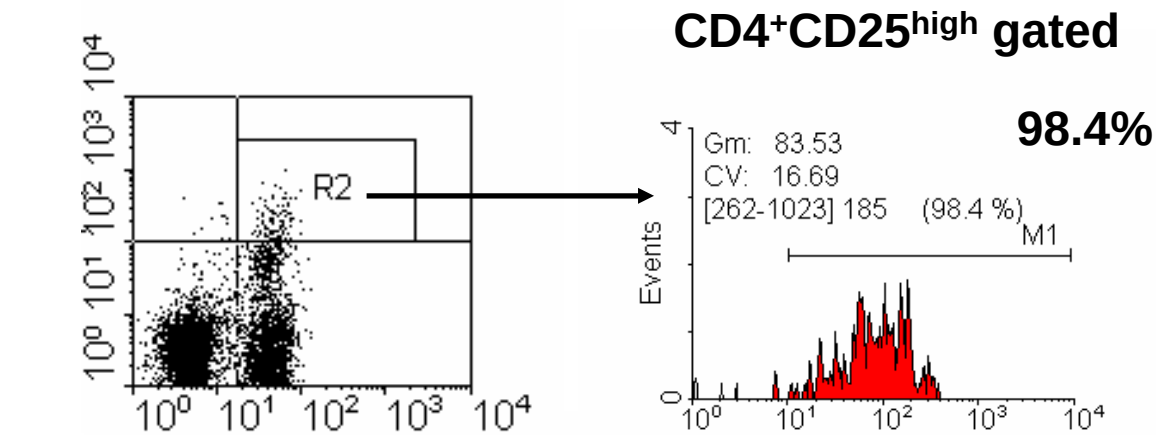
Supplement figure 2

(A). Non-atopic Controls:

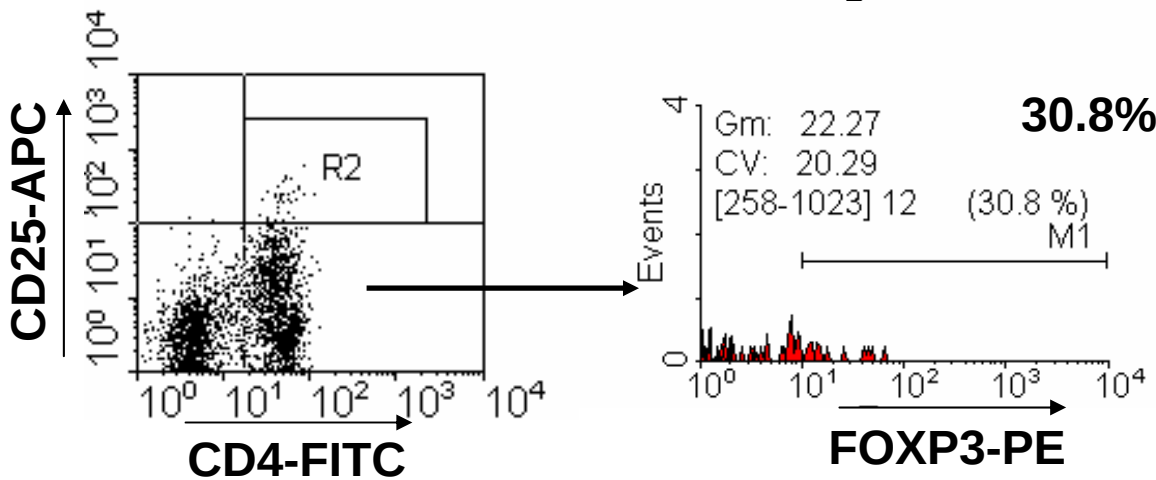


(B). Allergic asthmatics :

Medium:

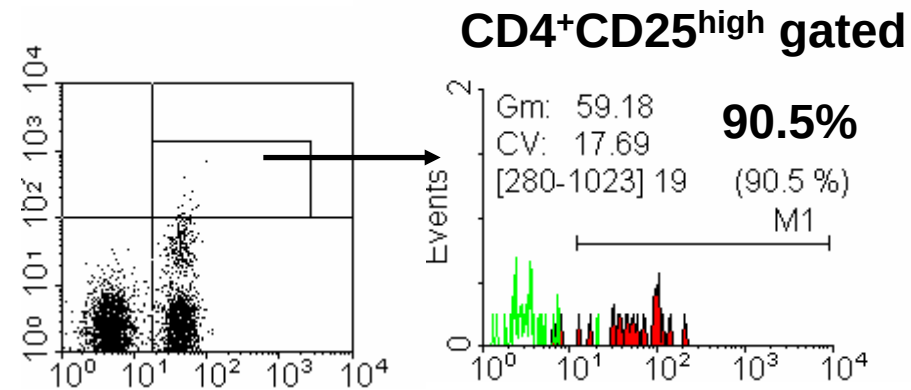


Der p
extract:



(C). AIT-treated allergic asthmatics:

Medium:



Der p
extract:

