



Transforming growth factor-beta1 and IL-13 response to allergen predict steroid needs in asthmatic children

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ARTICLE INFO

Article history:

Received 27 September 2012

Received in revised form

15 December 2012

Accepted 19 December 2012

Keywords:

Inhaled glucocorticosteroids

Immunotherapy

Asthma children

ABSTRACT

Background: The remission of asthma, which is induced during specific immunotherapy (SIT) or appears spontaneously in children is not completely understood and predictors of this phenomenon are still undefined.

Objective: To assess CD4⁺CD25⁺Foxp3⁺ Treg cells and cytokine/proliferation response to allergen-specific stimulation of PBMC as predictors of steroid sparing effect of SIT and steroid dosage needs without SIT during 5 years of follow-up in asthmatic children.

Methods: This is a 5-year long study of 32 asthmatic children, sensitive only to house dust mite (HDM). Eighteen children who had completed 5 years of HDM SIT – SIT group, and 14 children without SIT as a control group were studied. All patients had baseline clinical/immunological assessment; before and after observation the minimum effective ICS dose was defined and lung function was measured.

Results: In children from SIT group minimum effective ICS dose was reduced more than in children from control group (median reduction 65% vs. 0%; $p < 0.001$). Among patients in control group asthma severity was reduced after 5 years of observation in those who had at baseline higher TGF-beta1 and lower IL-13 answer to allergen stimulation of PBMC. Better response to 5 years immunotherapy was observed in those who had at baseline higher TGF-beta1 and lower proliferation answer to allergen stimulation of PBMC.

Conclusion: Similar processes may decide on both, SIT-induced and spontaneously appearing, reduction in asthma severity. Immunotherapy was much more effective than pharmacotherapy in our study. IL-13 overproduction may impede reduction of disease severity in asthmatic children independently from TGF-beta pathway.

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1. Introduction

Many different phenotypes of asthma have been defined based on different sets of parameters. Phenotypes may represent different pathophysiologic mechanisms and responses to medications [1]. Various regulatory and effector cells are involved in asthma development [2]. Different risk factors of more severe and persistent symptoms in asthmatic children have been defined [3,4]. On another hand, remission of asthma induced during SIT or appeared spontaneously in children is not completely understood and possible predictors of this phenomenon are still undefined. Asthma reduction during SIT, as defined in terms of reduction of inhaled

steroid use, has been studied in a few studies [5–7]. Steroid sparing effect of SIT, after 3 and 5 years course of Phostal (Stallergenens) has been observed [8].

It has been shown that mechanisms of SIT involve: (1) shifts the ratio of T-helper 2 (Th2) and T-helper 1 (Th1) cytokines in blood or in target organs, (2) increases dendritic cells toll like receptor 9 mediated innate immune function, which was previously impaired at baseline in allergic subjects, (3) induces allergen-specific regulatory T cells (Treg cell; IL-10-producing T cells and CD4⁺CD25⁺ T cells) and production of IL-10 and TGF- β by CD4⁺CD25⁺ cells, and (4) increases allergen-specific non-inflammatory blocking IgG1 and IgG4 antibody concentrations in the serum and in the local target organs [9].

TGF- β 1 displays pro-inflammatory properties by being a potent chemotactic factor and activator for inflammatory cells [10–12]. On the other hand, TGF- β 1 displays also anti-inflammatory action by regulating lymphocyte homeostasis, inhibiting predominantly Th2 cell response and promoting the differentiation of Tregulatory cells [13,14]. What is more, TGF- β 1 has been found as pivotal

Abbreviations: SIT, allergen-specific immunotherapy; ICS, inhaled glucocorticosteroids; FEV₁, forced expiratory volume in one second; PBMC, peripheral blood mononuclear cell; HDM, house dust mite.

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mediator of early SLIT-induced suppression of the T-cell responses to allergen and consequently predictor of SLIT, possibly due to TGF- β action: (1) maintenance of immunological self-tolerance in the CD4 T cell compartment, and (2) differentiation of Th1 and Th2 cells by inhibiting the expression of transcription factors T-BET and GATA-3 [15].

Immunological background describing spontaneously appearing remission of IgE-dependent allergy includes mainly the IL-10 and/or CD4(+)CD25(+) Treg cell pathways [16,17]. Early study showed that mucosal induction of tolerance against dietary antigens in children who have outgrown cow's milk allergy is associated with the development of CD4(+)CD25(+) Treg cells [16]. It has been suggested that *Dermatophagoides farinae*-specific anergy of T cells is likely to be induced by increased levels of IL-10, initially produced by specific T cells after exposure to relevant mite allergen in patients during remission [17].

We have previously analyzed relationship between changes in different immunoregulatory markers observed during SIT and steroid sparing effect of SIT [18]. In this study, we aim to define markers which could predict reduction in asthma severity in children treated with or without immunotherapy.

Our objective was to assess CD4⁺CD25⁺Foxp3⁺ Treg cells and cytokine/proliferation response to allergen-specific stimulation of PBMC as predictors of steroid sparing effect of 5 years SIT. Concomitantly we aim to evaluate steroid needs in asthmatic children without SIT during 5 years of follow-up.

2. Methods

2.1. Patients

Thirty-two boys and girls who participated in our two previous trials involving asthmatic children undergoing allergen immunotherapy [19,20] were included in the present study. Both trials were conducted at the same time, in the same Allergy Center and according to the same studies' protocols. Children treated with immunotherapy in the above trials were included in the present analysis as the SIT group ($n = 18$); screen failures (patients with indication for SIT who did not receive immunotherapy because of lack of parental/patients' consent for SIT) were included as the control group ($n = 14$).

All children had doctors' diagnosed allergic asthma and were sensitized only to house dust mites (HDM; *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), as evidenced by a positive skin prick test/presence of serum-specific IgE. Skin prick testing was performed with the inhalant allergens: *D. farinae*, *D. pteronyssinus*, Alternaria, Cladosporium, cat dander, dog dander, mixed grass pollen, rye, birch, hazel, ribwort, alder, *Artemisia vulgaris* (extracts from Allergopharma-Nexter, Reinbek, Germany). Six months before SIT qualification patients were treated with inhaled corticosteroids in a daily dose between 200 and 800 mcg. Asthma in all patients was controlled; there had been no exacerbations of a disease, nor any need for other treatment for a minimum of 6 months. Patients were required to be able to perform reproducible spirometry, exhibiting the resting forced expiratory value in 1 s (FEV₁) of at least 70%, and to have no contraindications for SIT. All patients suffered from rhinoconjunctivitis due to allergy of HDM. Since the effect of SIT on rhinoconjunctivitis symptoms was not the aim of this study we did not evaluate rhinoconjunctivitis questionnaire.

2.2. Study design

After 5 years of SIT (for SIT group) or after 5 years of observation (for control group) all patients from both groups were invited to the allergy clinic center to define clinical data: the minimum effective ICS dose and lung function measurement. Clinical data obtained

from above visit was analyzed in the context of baseline assessment from pre-study and the first visit of original trials [19,20]. Here we briefly describe procedures from visits of previous trials. During pre-study visit (at the beginning of a run-in period from September, 2005 till the end of March, 2006), all participants were informed about the purpose of the study, underwent skin testing. During this period, ICS dose was adjusted stepwise to define the lowest dose necessary to control asthma symptoms. During the first visit blood samples were obtained for immunological assessment, as well as clinical symptoms, and lung function was measured; patients from SIT group received the first subcutaneous dose of commercial *D. pteronyssinus* (50%) and *D. farinae* (50%) extracts (Phostal, Stalergenes, France). For patients from SIT group immunotherapy was continued throughout the study period according to a standard dose schedule. Increasing doses of HDM extract were administered up to a maintenance dose of 0.8 ml (1000 STU), which corresponds to 5.9 mcg of major allergens. This dose was continued monthly for 5 years. All children remained in the same environment during the study. Every 3 months visits were scheduled to adjust the corticosteroid doses to the current control of asthma symptoms, according to guidelines.

2.3. Principles for reducing the ICS dose

The minimal daily dose of ICS capable of maintaining asthma control was calculated according to guidelines [21]. The daily dose of ICS was reduced stepwise every 3 months until asthma was no longer controlled. The treatment was then continued with a dose one step higher. When the patient was asymptomatic for 3 months with the minimal dose, ICS treatment was discontinued. Uncontrolled asthma was defined according to guidelines [21] based on daytime/nighttime asthma symptoms, lung function, and the number of shortacting β_2 -agonist inhalations. Reduction in the minimal daily dose of ICS was calculated according to the following rule: (ICS dose after SIT – ICS dose before SIT)/ICS dose before SIT $\times$ 100% only September–March (the season with the maximum levels of natural exposure to HDM periods were included [22]). To make data comparable, ICS doses were converted into the budesonide doses when needed. In all patients the same inhaled steroid was maintained throughout the study.

2.4. Assessment of the immunological parameters

Peripheral Blood Mononuclear Cells (PBMCs) isolated from whole blood by density gradient centrifugation on Histopaque®-1077 were cultured for 72 h in standard mammalian cell culture conditions (37 °C, 5% CO₂, 80% relative humidity) in the presence of Derp2 allergen at a concentration of 10 μ g/ml. CD4, CD25 and Foxp3 expression on cultured PBMCs was estimated by flow cytometry (LSRII instrument, BD Biosciences) using appropriate fluorophore-conjugated monoclonal antibodies. The interleukin (IL)-10, IL-13 and transforming growth factor- β 1 (TGF- β 1) levels in cell culture supernatants were determined using ELISA-based specific tests (R&D) according to the manufacturer's instructions. Foxp3 cDNA quantitation was performed by a real-time PCR run on a LightCycler 480 thermocycler (Roche Applied Science) using LightCycler 480 SYBR Green I Master (Roche Applied Science) according to the manufacturer's protocol. The number of FOXP3 cDNA molecules in a sample was expressed in relation to the number of hypoxanthine phosphoribosyltransferase (HPRT1) cDNA molecules, HPRT1 being the reference gene of choice for lymphoid cells (PMID 16771605), using the relative quantitation feature of LightCycler 480 software. The HPRT1-specific primers used were 5'-TGACACTGGCAAAACAATGCA-3' and 5'-GGTCCTTTTCAC CAGCAAGCT-3' (sequence taken from ID no. 5 in the real-time

primer database RTPrimerDB, PMID 12519963). The *FOXP3*-specific primers used were 5'- AACAGCACATTCACAGAGTTCCT-3' and 5'- CATTGAGTGTCCGCTGCTTCT-3' (sequence taken from ID no. 3792 in the real-time primer database RTPrimerDB, PMID 12519963).

Lymphocyte proliferation was assayed by BrdU incorporation (BrdU Cell Proliferation Assay, Calbiochem) according to the manufacturer's instructions. Briefly, two parallel sets of duplicated samples for each patient containing 2×10^4 of pre-cultured mononuclear cells in 100 μ l final volume were transferred to the wells of 96-well plate. Twenty microliters of 2000 $\times$ diluted BrdU solutions were added to the first set, while 20 μ l of culture medium was added to the matching control set. Medium-only containing wells were used for blank corrections. The cells were then allowed to grow for 24 h in the standard culture conditions (37 °C, 5% CO₂, 80% relative humidity). Then the plate was centrifuged at 3000 RPM at 4 °C for 15' to settle down the cells and the supernatant was removed by suction. The 200 μ l of fixative/denaturing solution was then added to the cells and the plate was incubated in dark at RT for 30'. The contents of the wells were removed by inverting over sink and tapping on paper towel. The plate was then stored in dark at 4 °C overnight. The 100 μ l of 100 $\times$ diluted anti-BrdU antibody solution was then added to the wells and the plate was incubated at RT for 60' in dark. The antibody solution was then removed by inversion and the wells were rinsed with Washing Solution for three times. Then 100 μ l of reconstituted Peroxidase Goat Anti-Mouse IgG HRP Conjugate was added and the plate was incubated at RT in dark for further 30'. Again, the antibody solution was removed by inversion and the wells were rinsed with Washing Solution for three times. Finally, the whole plate was fled with H₂O and then tapped on the paper towel. The 100 μ l of substrate solution was added to each well. The plate was incubated at RT in dark for 15' and then 100 μ l of the Stopping Solution was added to each well. The absorbance was immediately measured in the microplate reader at 450 nm analytical wavelength and 550 nm reference wavelength. The result is given as the difference between averaged absorbances at A450 for BrdU samples and control samples calculated after blank and background (absorbance 550 nm) correction.

2.5. Statistical methods

We compared the baseline characteristics of the patients using Fischer exact two-tailed test for discrete variables and the Mann–Whitney test for continuous variables. To determine differences between groups, Mann–Whitney test was used. Correlations between study variables were assessed using Spearman's test. *P*-value <0.05 (two-tailed) was regarded as statistically significant. Power and sample size was calculated in original manuscripts [19,20].

2.6. Ethics

This study was approved by the Medical Ethics Committee of the Medical University. All parents or guardians and children provided written consent for participation in the study.

3. Results

Baseline characteristic of the patients is shown in Table 1; the minimum effective ICS dose at the beginning of the study was lower in the control group compared to SIT group. There were no other differences between the study groups (Tables 2 and 3).

Analysis of variance did not show any significant changes in FEV1 within both study groups after the study; there were no differences between the study groups.

Table 1
Baseline characteristics.

	Control group (n = 14)	SIT group (n = 18)	<i>P</i> -level
Age [years], mean $\pm$ SD	9.0 $\pm$ 1.89	9.5 $\pm$ 1.76	0.446
Male gender, n (%)	8(57)	11(61)	0.821
Duration of asthma [years], median (quartile range)	2.5(2–5)	3(2–4)	0.877
FEV1 [% pred.], mean $\pm$ SD	98 $\pm$ 8.9	93 $\pm$ 5.8	0.064
Specific IgE for <i>Dermatophagoides pteronyssinus</i> , median	72.1	68.2	0.53
Specific IgE for <i>Dermatophagoides farinae</i> , median	83.4	91.4	0.64
Minimum effective ICS dose [mcg/day], median (quartile range)	300(200–400)	400(400–600)	0.010

3.1. ICS dose reduction

After 5 years of observation the minimum effective ICS dose was reduced more in SIT group (median 62.5%) compared to control group (median 0%) (see Fig. 1).

In both groups reduction in minimum effective ICS dose was dichotomized into the subgroups according to the following rule: (i) less than median value defined as “worse responders to SIT” (for SIT group) or “maintained asthma severity” (for control group) and (ii) more than median value defined as “better responders to SIT” (for SIT group) or “asthma severity reduction” (for control group). In the next step, all immunological outcomes obtained at baseline (during the first visit) were compared between subgroups defined as above. Such approach allowed us to point out the best immunological predictors of reduced asthma severity, induced by SIT or naturally appearing. Since we studied asthmatic children who were controlled by inhaled corticosteroids, asthma severity was assessed by changes in minimal effective dose of ICS (Fig. 2).

3.2. Immunological predictors of steroid needs

In control group higher TGF- β 1 and lower IL-13 concentrations in supernatant of allergen-stimulated PBMC culture (at the beginning of 5 years of observation) have been shown in children who obtained reduced asthma severity after 5 years of observation.

In SIT group higher TGF- β 1 and lower proliferation answer to allergen stimulation of PBMC before SIT have been observed in children with better response to SIT compared to those with worse response to SIT.

3.3. Correlations

We showed that proliferation index negatively correlated with TGF- β 1 supernatant concentration of allergen-stimulated PBMC; relative expression of *Foxp3* positively correlated with IL-10 supernatant concentration of allergen-stimulated PBMC.

We also showed that minimum effective ICS dose was higher at baseline among those with higher proliferation answer to allergen stimulation of PBMC what was visible in changing trend of proliferation index (BrdU incorporation) across three category (200, 400, 600–800 mcg/day) of minimum effective ICS dose at baseline in whole study group (*p* = 0.05) (see Fig. 1).

4. Discussion

To our knowledge, this is the first trial that explores the importance of immunological parameters in the prediction of

Table 2
Immunological parameters by study groups.

	Control group (n = 14)			SIT group (n = 18)			<i>p</i> ^a
	Median	Percentile 25	Percentile 75	Median	Percentile 25	Percentile 75	
IL-10 (in supernatant) [pg/ml]	26.6	10.9	64.5	18.9	7.5	39.9	0.3744
IL-13 (in supernatant) [pg/ml]	20.6	13.5	30.9	21.6	16.7	28.1	0.9016
TGF-beta1 (in supernatant) [pg/ml]	1082.0	956.5	1439.6	1461.6	1085.9	2058.9	0.1134
%CD4 ⁺ CD25 ⁺ Foxp3 ⁺ cell	17.7	9.9	48.9	16.0	9.6	38.4	0.6395
Relative expression of Foxp3	49.5	35.1	64.3	36.5	26.9	55.1	0.3229
BrdU incorporation [arbitrary unit]	0.3	0.1	0.4	0.5	0.3	0.7	0.1071

^a Mann–Whitney test.**Table 3**
Correlations between all studied immunological parameters. Data are presented with Spearman's coefficient.

	IL-10 (in supernatant)	IL-13 (in supernatant)	TGF-beta1 (in supernatant)	%CD4 ⁺ CD25 ⁺ Foxp3 ⁺ cell	Relative expression of Foxp3	BrdU incorporation
IL-13 (in supernatant)	0.17	1.00	−0.15	0.01	−0.26	0.03
TGF-beta1 (in supernatant)	0.25	−0.15	1.00	−0.28	0.14	−0.61 ^a
%CD4 ⁺ CD25 ⁺ Foxp3 ⁺ cell	0.01	0.01	−0.28	1.00	0.05	0.38 ^b
Relative expression of Foxp3	0.60 ^a	−0.26	0.14	0.05	1.00	−0.21
BrdU incorporation	−0.46 ^a	0.03	−0.61 ^a	0.38 ^b	−0.21	1.00

^a Correlation is significant at the 0.01 level (two-tailed).^b Correlation is significant at the 0.05 level (two-tailed).

steroid needs in asthmatic children with or without allergen immunotherapy. Here we showed that in children treated with immunotherapy minimum effective ICS dose was reduced more than in children who were not treated with immunotherapy.

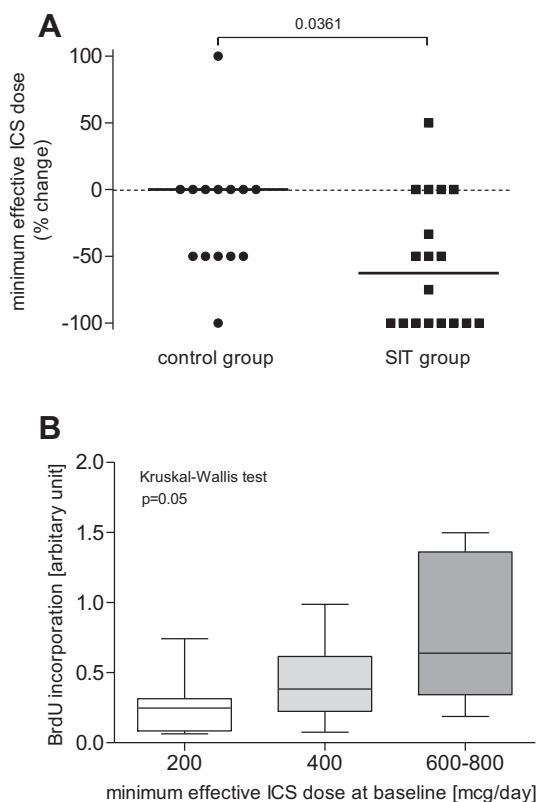


Fig. 1. (A) The reduction of the minimal daily dose of inhaled corticosteroid necessary to control the symptoms of asthma [% change] in each participant from control and SIT groups. (B) Association between minimum effective ICS dose at baseline and proliferation response of PBMC to allergen stimulation (BrdU incorporation). Reduction in ICS dose was calculated according to the following rule: (ICS dose after the first year of SIT – ICS dose at baseline)/ICS dose at baseline $\times$ 100%.

Among patients from control group asthma severity was reduced after 5 years of observation in those who had at baseline higher TGF-beta1 and lower IL-13 answer to allergen stimulation of PBMC. Among patients from SIT group better response to 5 years immunotherapy was observed in those who had at baseline higher TGF-beta1 and lower proliferation answer to allergen stimulation of PBMC. We showed that proliferation answer to allergen stimulation of PBMC correlates negatively with TGF-beta1, and expression of Foxp3 correlates with IL-10 production. Interestingly, minimum effective ICS dose was higher at baseline among those with higher proliferation answer to allergen stimulation of PBMC.

TGF-beta1 is one of the mediators involved in many aspects of persistent inflammation and tissue remodeling in asthmatic lung. Recently two-step model has been proposed [10]. In the first step, low expression in TGF-beta1 leads to deficient natural immunoregulatory processes allowing the induction of airway inflammation orchestrated by Th2 cells. In the second step, Th2 inflammation increases TGF-beta1 in the tissue, predominantly derived from migrating eosinophils, which activate the epithelial–mesenchymal transition and myofibroblast, and finally induces fibrosis. We studied children with relatively short lasting allergy disease. Therefore, we suspect that TGF-beta expression at initial stage of allergy process decided on the natural history of allergic asthma in our patients. Lower TGF-beta production in the answer to allergic stimulation caused worse response to SIT and impeded spontaneously appearing processes leading to allergy down-regulation, and consequently asthma remission. Unfortunately, our study does not allow to verify the second step of above hypothesis describing the role of TGF-beta1 in airway remodeling. We believe that our findings should be explained by TGF-beta1 mediated immunoregulation since significant negative correlation between TGF-beta1 production and proliferation answer to allergen has been found.

It has been shown that IL-13 is a key cytokine associated with asthma severity, lung function and airways hyper-responsiveness in asthmatic patients [23]. IL-13 production in the airway promotes the survival and migration of many pro-inflammatory cells such as eosinophils and macrophages [24]. Importantly, it has been shown that IL-13 together with IL-4 is able to induce an

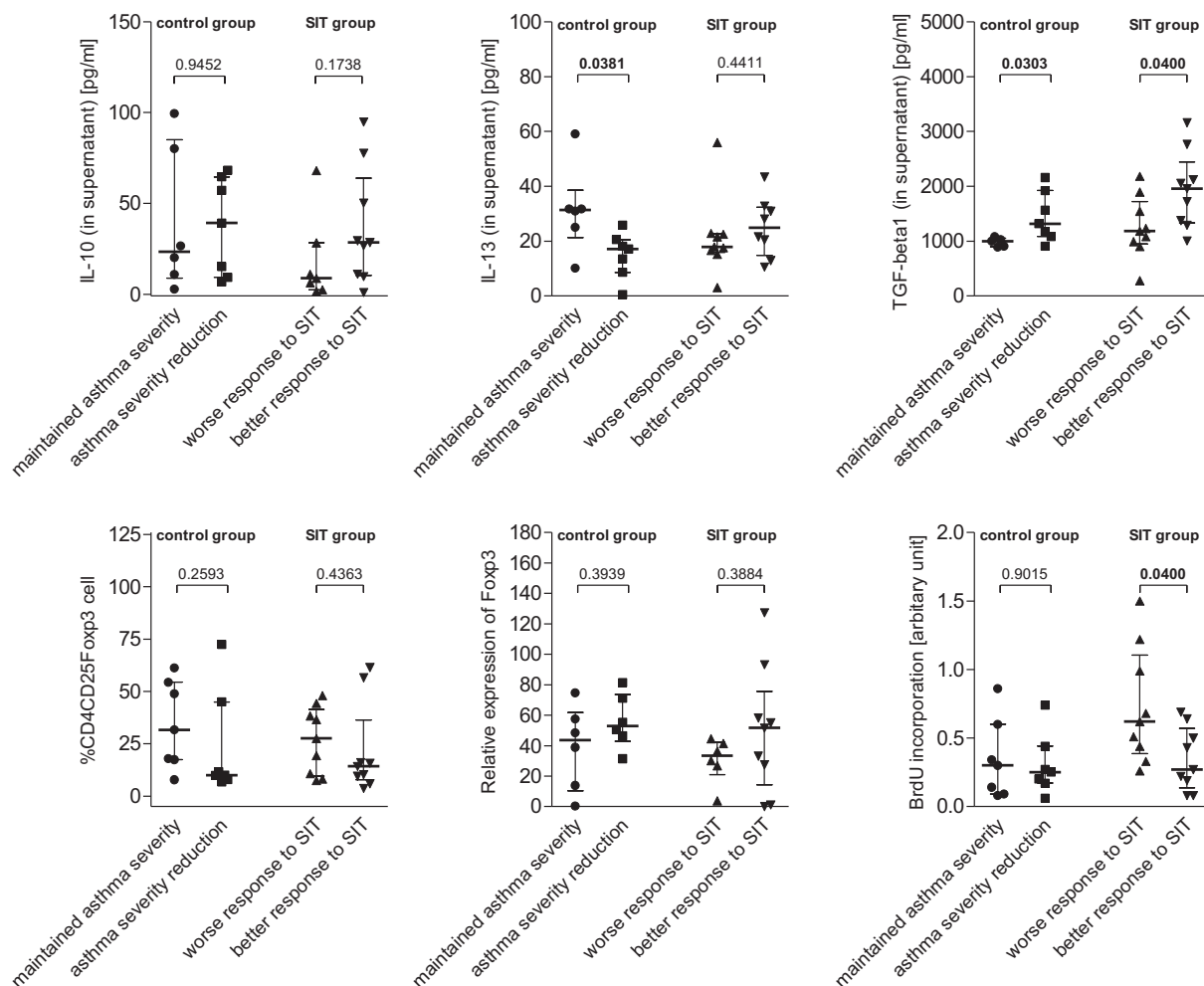


Fig. 2. The comparisons of immunological parameters at baseline between patients from SIT group with worse (less than median reduction in minimum effective ICS dose) vs. better (more than median reduction in minimum effective ICS dose) response to SIT and between patients from control group with maintained (less than median reduction in minimum effective ICS dose) vs. reduced (more than median reduction in minimum effective ICS dose) asthma severity. Data are presented as a value for each patient. For between-group comparisons Mann–Whitney tests were used. Reduction in ICS dose was calculated according to the following rule: (ICS dose after the first year of SIT – ICS dose at baseline)/ICS dose at baseline $\times$ 100%.

extracellular matrix protein-periostin which plays a crucial role in cell adhesion and subepithelial fibrosis [25]. Collectively, all above findings suggest that IL-13 is a relevant target for asthma therapy. Our results suggest that IL-13 overproduction on the response to allergen stimulation may affect the natural history of asthma independently from immunoregulatory processes such as the first step of TGF-beta pathway.

It has been previously shown that outgrown early childhood allergy is associated with the development of CD4(+)CD25(+) Treg. Therefore, we could suspect similar findings in our patients who experienced reduction in severity of allergic asthma. However, our children were allergic to house dust mite and we did not studied allergen-specific T regulatory cells what may explained the lack of relationship between T regulatory cells and asthma severity reduction.

During data analysis, we observed that the minimum effective ICS dose was lower among the control group. This difference may be attributed to the fact that the control group consisted of children who did not agree to SIT probably because of milder asthma symptoms. However, this fact did not influence comparability observed between the SIT and the control groups. What is more, conclusions from subgroup analysis could be underpowered because of relatively small sample size.

Our TGF-beta findings suggest that similar processes may decide on both, SIT-induced and spontaneously appearing, reduction in asthma severity. However, immunotherapy was much more effective than pharmacotherapy in our study. What's more, independently from TGF-beta pathway, IL-13 overproduction may impede reduction of disease severity in asthmatic children.

Funding

This study was funded by grant 503-2056-1 from the Medical University of Lodz, Poland.

Author contribution

Paweł Majak, Joanna Jerzyńska, Włodzimierz Stelmach, Iwona Stelmach: concept of the study, analysis and interpretation of data, manuscript preparation.

Katarzyna Smejda: acquisition of data, analysis and interpretation of data, manuscript preparation.

Conflict of interest

The authors declare that there are no conflicts of interest.

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