

The effect of specific immunotherapy on the expression of costimulatory molecules in late phase reaction of the skin in allergic patients

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Summary

Background Specific immunotherapy (SIT) modulates immune responses to allergens resulting in improvement of allergic symptoms. However, the mechanisms behind the clinical changes are not clear. Participation of costimulatory molecules on antigen-presenting cells and T cells in the process of antigen recognition is suggested to be of essential importance. The SIT effect on expression of costimulatory molecules has not been earlier examined.

Methods Forty-one birch-allergic patients were treated with SIT or placebo. After 1 year of treatment skin biopsies were obtained 24 h following allergen challenge. Sections were stained with antibodies against: EG2 (eosinophils), CD4 (T cells), CD68 (macrophages), CD1a (Langerhans cells), CD28 (on T cells) and costimulatory molecules (CD80, CD86).

Results Following allergen challenge number of the CD4⁺ and CD68⁺ cells increased significantly ($P = 0.002$, 0.0001 , respectively) in the placebo, but not in the SIT-treated patients. The difference between groups was significant ($P = 0.003$, 0.01 , respectively). The numbers of EG2⁺ cells increased significantly in both groups. CD80⁺ cell numbers increased in the placebo ($P = 0.01$) but not in the SIT group. The number of CD86⁺ cells increased in both groups (placebo, $P = 0.001$; SIT, $P = 0.01$) but significantly less in the SIT group ($P = 0.05$). The numbers of CD28⁺ cells increased in the placebo ($P = 0.001$) but remained unchanged in the SIT group. The difference between the groups was significant ($P = 0.05$).

Conclusion There were lower numbers of cells expressing costimulatory molecules in SIT-treated than in placebo-treated patients. Decreased costimulation may lead to diminished immune response following allergen exposure. This could be an important factor contributing to the clinical improvement after SIT.

Keywords costimulatory molecules, immunotherapy, late phase reaction, skin biopsies

Submitted 17 December 2003; revised 17 May 2004; accepted 02 August 2004

Introduction

Specific immunotherapy (SIT) is considered to be an effective treatment of allergic rhinitis (AR). Moreover, this treatment is the only intervention that offers long-lasting amelioration of clinical symptoms [1] and prevents the onset of new sensitizations [2] as well as development of asthma in patients with AR [3]. Although the clinical improvement during SIT has been well documented [1, 4] the underlying cellular mechanisms are still not clear.

During antigen presentation, antigen-presenting cells (APCs) take up proteins, process them and present peptides, in the context of major histocompatibility complex (MHC) class II molecules, to CD4⁺ T cells with the corresponding T cell receptors (TCR). Simultaneously with this first signal, the

second signal is passed on by interaction between costimulatory molecules such as CD80 and CD86 (B7 family molecules) on the surface of APCs and CD28 on the T cells [5]. Incomplete costimulation may lead to T cell unresponsiveness [6].

We have employed the model of late phase reaction (LPR) in the skin in order to examine the effect of SIT on costimulatory molecules expression. To our knowledge there is no earlier study investigating the expression of CD80, CD86 and CD28 molecules on cells in skin biopsies from SIT- and placebo-treated patients.

Materials and methods

Patients

Forty-one birch-allergic patients (25 females, 16 males), mean age 33 years (range 19–46 years), were included in the study. All patients had a clinical history of birch pollen-induced

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Table 1. Clinical data of the patients

	Placebo	SIT
No. of patients	22	19
Sex: M/F	8/14	8/11
Age (years): mean (range)	32 (28–35)	33 (30–37)
SPT (mm): median (range)	5.75 (4.5–7)	5.5 (4.5–8)
Specific birch IgE (CAP system)	4 (3–5)	3 (2–5)

SPT, skin prick test; SIT, specific immunotherapy.

rhinoconjunctivitis. The birch allergy was confirmed by positive skin prick test: ≥ 3 mm and serum-specific IgE antibodies to birch: class ≥ 2 (CAP system, Pharmacia Diagnostics, Uppsala, Sweden). Twenty-two patients received placebo and 19 SIT, with Alutard[®] (Alutard, ALK-A/S, Hørsholm, Denmark). All patients provided written informed consent. The Ethics Committee of Sahlgrenska University Hospital approved the study (Table 1).

Clinical symptoms

Patients recorded symptoms of running and blocked nose, and eye symptoms on a four-point scale (0, no symptoms; 1, mild; 2, moderate; 3, severe). Symptoms were recorded every day for 2 weeks before season (February) and for 6 weeks during the season (later half of April and May). Total rhinoconjunctivitis symptoms score was counted as daily average of nasal and ocular symptoms scores for 2 weeks in February and 18 days covering the peak of the pollen season (28 April–15 May).

Biopsy sampling

Allergen challenge was performed and biopsies were collected after 1 year of SIT treatment, outside birch pollen season.

To evoke the LPR, 50 SQ-U birch pollen allergen extracts (Aquagen[®] SQ *Betula verrucosa*; ALK-Abello, Hørsholm, Denmark) was injected intracutaneously in forearm. Albumin diluent (Aquagen[®] SQ) were injected in the opposite forearm and served as a negative control.

The size of the LPR was measured and skin biopsies were obtained 24 h following challenge with allergen or diluent. The contours of the LPR area was marked with a pen and transferred to a transparent sheet by pressing it against the forearm. The size of the area was determined by using a computerized scanner and expressed in mm².

Biopsies were taken from both allergen and diluent provoked arm, using a 3 mm disposable punch, under local anaesthesia with Xylocain[®] (Astra AB, Södertälje, Sweden). Samples were snap frozen, embedded immediately in TissueTek (Zoeterwoude, Netherlands) OCT medium and stored at -70°C . For staining 6 μm thick sections were cut by cryostat (Leica CM1800, Nusstoch bei Heidelberg, Germany), placed on slide and air-dried at room temperature.

Immunohistology

Monoclonal mouse anti-human antibodies were used to stain T cells (anti-CD4, 1:50, Dakopatts, Glostrup, Denmark); macrophages (anti-CD68, 1:400, Serotec Ltd, Oxford, UK); eosinophils (anti-EG2 (eosinophil cationic protein), 1:50,

gift from Dr Christer Pettersson, Pharmacia Diagnostics); Langerhans cells (anti-CD1a, Dakopatts). For detection of costimulatory molecules the following monoclonal antibodies (MoAbs) were used: anti-CD80 (1:100, Biodesign International Industrial Park Road, Saco, ME, USA); anti-CD86 (1:2000) and anti-CD28 (1:10), both purchased from Pharmingen (San Diego, CA, USA).

The alkaline phosphatase anti-alkaline phosphatase (APAAP) method was used as previously described [7] to stain sections with anti-CD4 and -EG2 MoAbs. Briefly acetone fixed, for anti-CD4, and formalin fixed, for anti-EG2 sections, were incubated with: (1) primary antibody for 1 h, (2) rabbit anti-mouse (1:50) for 30 min, and (3) rabbit APAAP for 30 min (1:50). The Fast Red substrate was used to develop the staining. Staining with anti-EG2 was performed in a semi-automated immunostainer (Shandon Cadenza, Astmoor, UK).

The avidine-biotin method (ABC method) was used to stain macrophages [8]. Sections were incubated with: (1) endogenous peroxidase blocking solution (10 mM glucose, 1 U/mL glucose oxidase, 1 mM sodium azide) [9] for 20 min, (2) normal rabbit serum (1:50) for 20 min, (3) primary antibody for 1 h in 37°C , (4) rabbit anti-mouse IgG biotinylated F(ab')₂ fragments for 30 min (1:400, Dakopatts), and (5) biotinylated peroxidase conjugated to avidine (ABC/HRP complex) for 30 min (Vector Laboratories, Burlingame, CA, USA). The reaction was visualized with 3-amino-9-ethylcarbazole (AEC) substrate (Dako Corporation, Carpinteria, CA, USA). The EnVision[™] (Dako Corporation) system was used to enhance the staining of the costimulatory molecules. Sections were incubated with: (1) endogenous peroxidase blocking solution, (2) Tris-buffered saline with 0.05% Tween for 1 h, (3) normal goat serum (1:50) for 20 min (4) primary antibody overnight at 4°C , and with (5) EnVision[™] for 30 min in RT. The staining was visualized with AEC (Dako Corporation).

The sections were counterstained with haematoxylin and mounted in water-based mounting medium.

Quantification

Cells were counted, in a blinded manner, using a Nikon Eclipse E600 microscope, at 400 times magnification. Easy Image 2000 Measuring System (Bergström Inst AB, Solna, Sweden) was used as software. Cells in the epithelium and in all accessible fields under the basal membrane were counted. The average values for epithelial and subepithelial readings were added and positively stained cells were expressed as number of cells per 1 mm².

Statistical methods

The difference between total symptom score before and during the season was evaluated with Wilcoxon's (signed-rank) test and the difference between groups Mann-Whitney U-test.

The differences in the number of positively stained cells between the diluent and the allergen challenged arm were evaluated by Wilcoxon's (signed-rank) test for matched pairs.

Comparisons between treatment groups for each marker were done with the Mann-Whitney U-test.

Correlations were tested with Spearman's rank coefficient test. A $P \leq 0.05$ was considered statistically significant. All tests were performed using a software package (StatSoft, Tulsa, OK, USA).

Results

Clinical results

Symptoms from 2 weeks around the peak are presented in results.

The increase in total symptom score during the pollen season was significant for both placebo ($P = 0.0001$) and treated group ($P = 0.0002$). The difference between the groups was not significant before the season; however, during the season SIT-treated patients had significantly less symptoms ($P = 0.003$) than placebo-treated patients (Fig. 1). During the season median values for the total symptom score were 0.8 (quartile range = 0.78) for the SIT treated and 1.27 (quartile range = 0.78) for the placebo-treated patients, respectively.

Late phase reaction in the skin

The size of LPR after 1 year of treatment was significantly smaller in the immunotherapy-treated group compared with the placebo-treated group ($P = 0.00001$). Median LPR size for SIT group was 132 mm² and for placebo 2242 mm² (quartile range values were 372 and 2984 mm², respectively).

Skin biopsy specimens

The number of EG2⁺ cells increased significantly following challenge in both the placebo and the SIT group. There was no significant difference in increase between the groups. The numbers of CD4⁺ (Fig. 2a) and CD68⁺ (Fig. 2b) cells increased more after provocation in the placebo group ($P = 0.002$ and 0.0001 , respectively) than in the SIT-treated patients ($P = 0.003$ and 0.01 respectively). We did not find any changes in the number of CD1a⁺ cells.

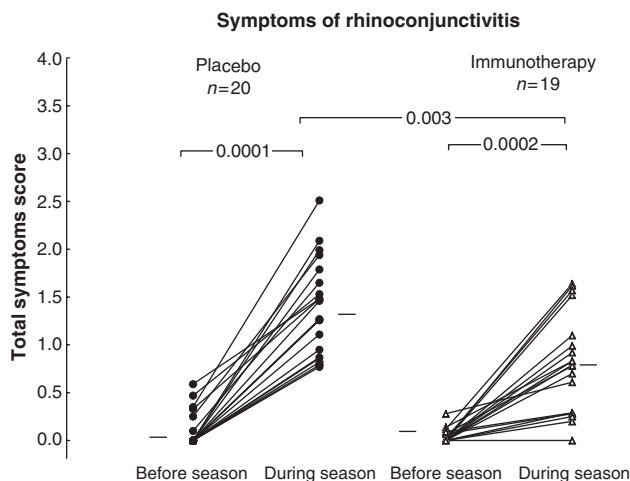


Fig. 1. Rhinoconjunctivitis symptoms score, during 18 days covering the peak of birch pollen season and 2 weeks before the start of season, for immunotherapy- and placebo-treated patients after 1 year of treatment.

CD80⁺ (Fig. 2c) cells increased significantly in the placebo group ($P = 0.01$) but remained unchanged in the SIT-treated patients. There was no difference between the groups.

We found a significant increase in the number of CD86⁺ (Fig. 2d) cells in both treatment groups (placebo, $P = 0.001$; and SIT, $P = 0.01$) but the increase in the SIT patients was significantly lower ($P = 0.05$). The number of CD28⁺ (Fig. 2e) cells increased significantly in the placebo group ($P = 0.001$), but not in the SIT group, and the difference between the groups was significant ($P = 0.05$). Relationships were also found between LPR size and numbers of infiltrating CD4⁺ T cells ($P = 0.001$; $R = 0.5$) and CD68⁺ macrophages ($P = 0.001$; $R = 0.5$) in skin biopsies.

Discussion

In our study biopsies obtained from the sites of the LPR were used to investigate the effects of SIT treatment compared with placebo treatment on numbers of inflammatory cells and expression of costimulatory molecules.

The numbers of CD80⁺ cells increased in the placebo but not in the SIT-treated patients. Higher numbers of CD86 expressing cells were found following allergen challenge in both groups, but the increase in the SIT group was significantly lower. The numbers of CD28⁺ cells increased in the placebo group but remained unchanged in the SIT group. Higher numbers of CD4⁺ and CD68⁺ cells were found in the placebo-treated patients compared with the SIT group. Prominent influx of EG2⁺ cells was found in both treatment groups.

The concentration of birch allergen extract used in skin challenges was relatively high and chosen based on a previous study by Frew [10], showing that allergic patients develop a LPR in the skin providing a sufficient dose of allergen is injected. We have confirmed earlier findings that successful immunotherapy decreases the size of LPR in skin after intracutaneous allergen injection [11, 12].

The CD80 (B7-1) and CD86 (B7-2) costimulatory molecules are expressed on professional APCs including activated B cells, monocytes [13], macrophages, dendritic cells, Langerhans cells, but also keratinocytes [14] and eosinophils [15]. CD28 is expressed on T cells [14] and has also been reported on blood eosinophils [16].

APCs, such as macrophages and Langerhans cells express MHC class II molecules and deliver costimulatory signals via, e.g. CD80 and CD86, thus enabling a complete T cells activation [17].

There are a limited number of studies examining the expression of costimulatory molecules in tissue sections taken from allergic sites. In SIT-treated patients the numbers of cells expressing all of the investigated costimulatory molecules in skin sections was lower than in skin sections from placebo-treated patients. Explanations for this could be a lower number of inflammatory cells expressing the molecules of interest or lower density of these molecules on the surface of the cells. Using flow cytometry we have found that 3 days following rush immunotherapy the median fluorescence intensity significantly decreased for CD86 on monocytes and CD28 on T cells [18].

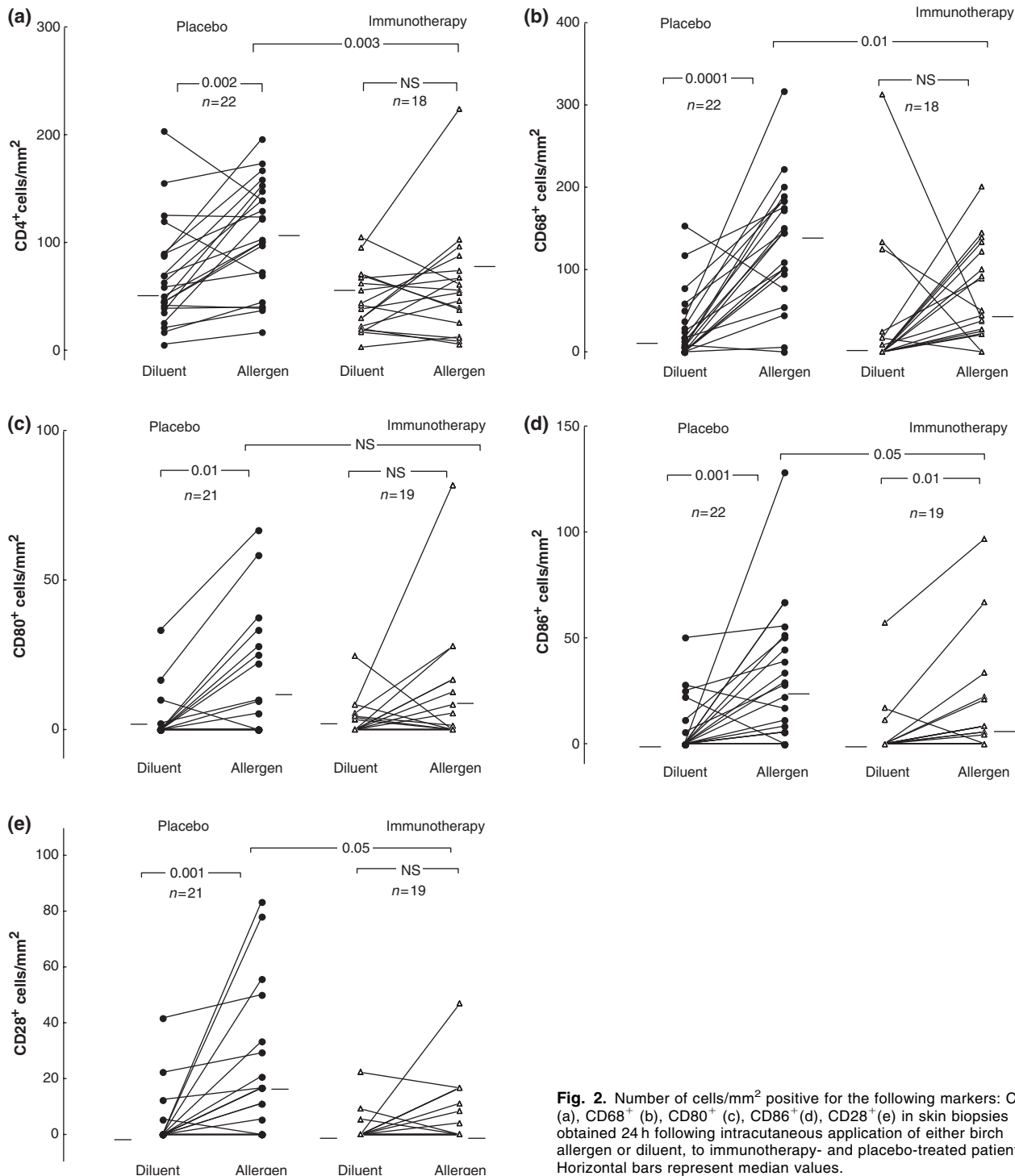


Fig. 2. Number of cells/mm² positive for the following markers: CD4⁺ (a), CD68⁺ (b), CD80⁺ (c), CD86⁺ (d), CD28⁺ (e) in skin biopsies obtained 24 h following intracutaneous application of either birch allergen or diluent, to immunotherapy- and placebo-treated patients. Horizontal bars represent median values.

The detection of CD28 in skin biopsies seems to depend on severity of inflammation. Investigation of allergic contact dermatitis biopsies revealed that the majority of T cells were positive for CD28, while T cells in normal skin was negative for CD28 [14]. In a study of Hattori et al. [19] performed on nasal tissue no expression of CD28⁺ cells was found in allergic patients without provocation or in non-AR patients. However, following allergen challenge significant increase in the number of CD28⁺ cells was found, although the numbers were low.

In our study the number of CD28⁺ cells expressed in the skin LPR site of patients treated with immunotherapy was significantly lower compared with the placebo-treated group. However the numbers of CD28⁺ cells were lower than the number of CD4⁺ cells. Consistent with previous findings we observed a significant influx of CD4⁺ cells into the LPR site in the placebo group [20] but not in the SIT-treated group [11]. The correlation between the numbers of infiltrating CD4⁺ T cells and the size of the LPR, after 1 year of SIT, indicates the important role

of this cell population in the inflammatory allergic reaction.

Inhibition of CD28 tyrosine phosphorylation (associated with IL-10 receptor ligation) by IL-10 affects CD28 mediated T cell response and activation [21] resulting in a state of unresponsiveness [22]. A number of studies have suggested the increase in IL-10 as a key step in induction of allergen specific anergy and central mechanism in the action of immunotherapy. Akdis et al. [23] showed that immunotherapy decreased production of IL-5 and IL-13 and increased production of IL-10 by peripheral blood mononuclear cells from bee venom-treated patients. Addition of IL-10 to cultures of murine macrophages reduced expression of costimulatory molecules [24]. IL-10 has also been shown to inhibit expression of B7 molecules on human monocytes [25] and suppress B7/CD28 interaction dependent production of IL-5 from human T cells [26].

It has recently been shown that IL-10 originates from peripheral blood CD4⁺CD25⁺ cells and increases significantly following 1 year of grass SIT [27]. Increased expression of IL-10 has also been shown with *in situ* hybridization in skin biopsies after venom immunotherapy [28].

Since no double staining were performed we could not exclude that the changes in expression of costimulatory molecules was because of the changes in number of cells bearing those molecules on their surface. We have therefore examined the number of the main APCs in allergic tissue, i.e. macrophages and Langerhans cells. Contrary to the findings of Varney et al. [11], immunotherapy reduced significantly the numbers of macrophages in the present study. The lower amount of macrophages in the LPR biopsies from SIT-treated patients could be related to a decreased influx of monocytes from the bloodstream. One of the mechanisms responsible for monocyte trafficking to the tissue is chemoattraction. Intradermal injection of eotaxin results in a significant increase in CD68⁺ cells peaking after 24 h [29]. There are reports suggesting that the immunotherapy treatment affects the levels of chemokines, for example, patients treated with specific birch immunotherapy had decreased concentration of eotaxin in serum [30].

The lack of changes in the numbers of CD1a⁺ Langerhans cells is in agreement with previous studies showing unaltered numbers of CD1a⁺ cells in skin LPR [20] as well as in biopsies from patients treated with rush venom immunotherapy for 2 years [31].

We were not able to show the effect of immunotherapy on the numbers of EG2⁺ eosinophils. This effect varies in different studies, probably depending on the duration of immunotherapy treatment, intensity of pollen season, characteristics of the patients and the examined tissue.

SIT treatment reduces but, most often do not abolish the allergic symptoms that could be related to the presence of eosinophils in patient's tissue [4].

Wilson showed that 2 years of immunotherapy treatment significantly decreased the number of eosinophils in nasal biopsies [32]. In a skin study, however, with a similar design and number of patients as the present study Varney et al. [11] did not succeed to show any significant difference in eosinophil numbers after 9 months of immunotherapy. When cells positive for IL-4, IL-5 and GM-CSF mRNA were

analysed no reduction in mRNA in immunotherapy-treated patients could be seen.

From clinical point of view the relationship between eosinophil numbers and symptoms of late asthmatic reaction is not clear as illustrated by results from a study with monoclonal anti-IL-5 antibody. The treatment drastically reduced the numbers of eosinophils in blood and sputum; however the asthmatic symptoms of late reaction did not improve [33].

In conclusion, we suggest that one of the important factors modulating the immune responses following SIT is decreased costimulation during antigen recognition leading to decreased activation of CD4⁺ T cells. IL-10 seems to be involved in the immunosuppressive action of the immunotherapy through down-regulation of expression of costimulatory molecules.

Acknowledgements

The study was supported by Swedish Asthma and Allergy Associations Research Foundation.

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