Transfer of T cells from intranasal ovalbumin-immunized mice ameliorates allergic response in ova-sensitized recipient mice

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ABSTRACT

Mucosal immunotherapy is suggested as a treatment strategy for tolerance induction in allergic diseases. The purpose of this study was to determine the effect of transferred splenic T cells from intranasal ovalbumin (OVA)-immunized mice to naive mice before sensitization on its impact of cytokine production and airway histopathology. BALB/c mice in group I received intranasal immunotherapy (days1–6), carboxylfluorescein succinyl ester (CFSE)–labeled splenocytes or splenic T cells were i.v. transferred to naive recipients (group II) before OVA sensitization. Acute murine asthma model was established by two i.p. OVA injections (days 21 and 28) and seven OVA nebulizations (days 42–48) in groups I, II and III. Groups III and IV served as asthma model and control, respectively. CFSE-labeled cells in splenocytes and lymph node lymphocytes, lung histopathology, IL-4, IL-10, and interferon (IFN) gamma cytokines of recipients were analyzed 24 hours after OVA nebulization challenge. CFSE-labeled T cells from group I were detected in spleen and regional lymph nodes of the OVA-sensitized recipients (group II). Smooth muscle and thickness of airways were less in intranasal OVA immunotherapy and OVA-sensitized recipients when compared with the asthma model ($p < 0.05$). Area of inflammation was significantly suppressed in OVA-sensitized recipients compared with the asthma model ($p < 0.01$). IL-10 and IFN-gamma levels in splenocyte supernatants were significantly increased in intranasal immunotherapy and OVA-sensitized recipients compared with asthma model and controls ($p < 0.01$). IL-4 levels were significantly less in intranasal immunotherapy group and the OVA-sensitized recipient group when compared with asthma the model group ($p < 0.05$). This study suggests that intranasal immunotherapy with allergens regulates T-cell responses and ameliorates airway histopathology in sensitized mice, hence, encouraging mucosal tolerance induction as a suitable treatment of allergic diseases.

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Key words: Allergy, IFN-gamma, IL-10, intranasal immunotherapy, mucosal immunotherapy, murine, ovalbumin, T-helper cells, TH1 cells, TH2 cells

Allergen SIT by the subcutaneous route is a common and effective treatment in many cases of allergic diseases.[1] Despite the subcutaneous route of desensitization is still preferable, intranasal and oral (local) routes have been considered as alternatives.[2]

Mucosal tolerance, characterized by specific immunologic unresponsiveness to subsequent antigenic challenge, may be achieved by either the induction of anergy, deletion of responsive cells, or active cellular regulation *via* T regulatory (Tr) cells.[3] Among $CD4^+$ T cells, Tr1 cells produce primarily IL-10,[4] whereas Th3 cells act *via* transforming growth factor (TGF)–β, IL-4, and/or IL-10.[5] Naturally occurring $CD4^+CD25^+$ Tr are characterized by the expression of Foxp3.[6]

Intranasal immunization has long been used as a route for vaccine administration and shown to be effective in inducing both systemic and generalized mucosal immune response.[7] Recently, it has been shown that intranasal immunotherapy with Bet V1 inducees long-term effects of tolerance induction and is associated with enhanced expression of IL-10, transforming growth factor (TGF)-β and Foxp3-mRNA in $CD4^+$ T cells.[8]

Experimental models of adoptive transfer have helped to define the role of T cells and their cytokines in orchestrating allergic asthma. Several studies clearly showed that adoptively transferred antigen-specific $CD4^+$ T cells induce airway inflammation in part by activating other effector cells that participate in airway inflammatory responses.[9–11] In this study we aimed to determine the effect of adoptive transfer of splenic T cells from mice immunized with OVA intranasally to naive mice before sensitization on Th1 (interferon [IFN] γ), Th2 (IL-4), and regulatory T-cell (IL-10) cyto-

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kine production and parameters of airway histopathology in the recipient.

METHOD

Mice

Five- to 6-week-old BALB/c mice were obtained from TUBITAK (The Scientific and Technical Research Council of Turkey) and housed according to the National Institutes of Health Guidelines. Experimental protocols were ethically approved and complied with the Animal Research Committee of Marmara Medical University (March 12, 2006, Istanbul, Turkey).

Study Groups

BALB/c mice were divided into four groups, with six to eight mice per group. Group I (nasal immunotherapy group) received 30 μL of phosphate-buffered saline (PBS) containing 100 μg of ovalbumin (OVA) (type V; Sigma, St. Louis, MO) by intranasal (i.n.) route on 6 consecutive days (days 0–6). On the day before sensitization with OVA, group II (OVA-sensitized recipient) received carboxylfluorescein succinyl ester (CFSE)–labeled splenocytes or splenic T cells from group I (transfer group, day 21) by i.v. route. Group III asthma model group (OVA sensitized and recipient of naive T cells) received CFSE-labeled splenocytes from untreated naive mice and served as positive controls. An acute asthma model was established in groups I, II, and III by administration of two doses of i.p. 10-μg OVA in 1.5 mg of alum injection (sensitization, days 21 and 28) and seven subsequent 1% OVA nebulizations (challenge, days 42–49). Group IV served as the healthy control (Fig. 1). All determinations were performed 24 hours after the last OVA challenge.

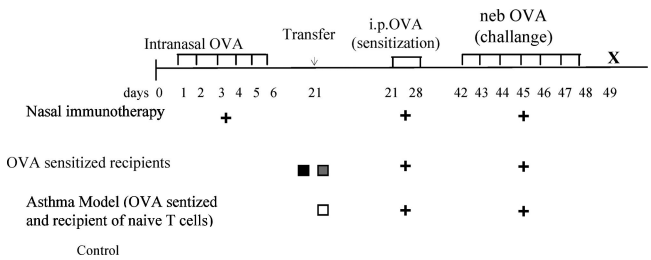

Figure 1. Study design. Age-matched BALB/c mice divided into four groups. Intranasal OVA immunotherapy group received 100 μg of OVA intranasally on days 1–6 (one time per day). Naive recipients received CFSE labeled 1×10^6 splenocytes (black box) or splenic T cells (gray box) i.v. from the intranasal OVA immunotherapy group. The asthma model group received CFSE-labeled splenocytes from untreated naive mice (ova sensitized and recipient of naiveT cells; white box). The asthma model was established by i.p. OVA injections and OVA nebulization. The control group did not receive any treatment.

Adoptive Transfer of Splenocytes and T cells

On the same day of the first i.p. OVA sensitization (day 21), spleens from the nasal immunotherapy group were collected, and single cell suspensions were prepared by passing the cells through a cell strainer. Splenocytes were suspended in lysing buffer (8.29 gr/lt of NH_4Cl, 1 gr/lt of $KHCO_3$, 37 mg/lt of EDTA, and 1 lt of distilled water). Cells were washed three times and resuspended in RPMI-1640 medium supplemented and 10% heat inactivated FCS, 2 mM of L-glutamine, and penicillin/streptomycin (all from Sigma, St. Louis, MO). After isolation of splenocytes, pure T cells were isolated with mouse pan T-cell isolation kit (Miltenyi Biotec AG, Bergisch Gladbach, Germany). Briefly, anti-CD14, anti-CD16, anti-CD19, anti-CD56, anti-CD36, anti-CD123, and anti-CD235a were added to splenocytes for the depletion of B cells, natural killer cells, dendritic cells, monocytes, granulocytes, and erythroid cells. Control antibodies were phycoeruthrin- or fluorescin isothiocyanate-conjugated mouse IgG1 and mouse IgG2 (BD, PharMingen, San Jose, CA). Fluorescence analysis was performed on an EPICS XL (Beckman Coulter Corp., Fullerton, CA) with argon laser (488 nm). Isolated cells were labeled with CFSE (Molecular Probes, Inc., Eugene, OR). Briefly, splenocytes and splenic T cells (1×10^6cell/mL) were resuspended in 1 mL of RPMI-1640/10% FCS. CFSE was added to make a final concentration of 5 M, and the cells were gently mixed and incubated for 12 minutes at +4°C protected from light. Labeling of cells was stopped by adding cold PBS with 2% fetal bovine serum, and then the cells were washed and resuspended in complete medium. Recipient naive mice received 50 μL of saline containing CFSE labeled 2×10^5 splenocytes or splenic T cells i.v. on the day before the first i.p. OVA sensitization (day 21). In the asthma model, group splenocytes from naive mice were labeled with CFSE and transferred to naive mice before establishment of the acute asthma model (Fig. 1). CFSE-labeled cells were analyzed in splenocytes and lymph node lymphocytes of recipients 24 hours after nebulization challenge. In this experiment although our main aim was to determine the effect of splenic T cells on the tolerance induction, we also transferred splenocytes simultaneously to other groups of naive mice to test the migration of both splenocytes and splenic T cells locally and systemically in recipients.

Bronchoalveolar Lavage (BAL) Fluid

Animals were anesthetized with rompun/ketalar (0.03/0.07 mL). The trachea was cannulated and BAL was collected by injecting 0.4 mL of PBS three times into the lung. Total cell counts were performed on cytospin slides stained with Wright Diff-Quik (Baxter

Bade AG, Dudingen, Switzerland) and 200 cells were counted.

Histopathological Analysis

Twenty-four hours after the last OVA nebulization (day 49) mice in all groups were killed. Lungs were fixed and inflated by intratracheal instillation of buffered formol solution. Then, lungs were removed and placed in buffered solution. Subsequently, lungs were embedded into paraffin for preparation of histopathological sections. Sectioned paraffin-embedded tissues (3–5 μm) were stained with hematoxylin and eosin for the evaluation of epithelial thickness and with periodic acid Schiff to analyze smooth muscle thickness and hyperplasic goblet cells. All histological analyses were performed with MS Basic Image Analyser Software, which was adapted on a model BH2-RFCA microscope (Olympus Optical Co., Ltd., Tokyo, Japan). The airways were classified as small (<500 μm), medium (500–1000 μm), and large (>1000 μm) according to their circumferences. Measurements were made on distal airways cut in transverse sections and free of branching. Measurement of thickness of epithelial, smooth muscle, and number of hyperplasic goblet cells and severity of inflammation on the airways were recorded. Semiquantative analysis was achieved by scoring for inflammation severity according to number of inflammatory cell lines surrounding the airway.

Cytokines Determination

Splenocytes and lymph node lymphocytes were isolated, stimulated with OVA (40 μg/mL). Supernatants were obtained 24 hours later for IL-4, IL-10, and IFN-γ cytokine analysis. Cytokine levels of culture supernatants were determined using commercial ELISA kit (Endogen, Rockford, IL).

Statistical Analyses

For statistical analyses, SPSS package (release 10.1; SPSS, Inc., Chicago, IL) program was used. Kruskal-Wallis test was used for comparison of all groups. When differences were statistically significant, Mann-Whitney *U* test was used for between-group comparisons. A value of $p < 0.05$ was considered statistically significant.

RESULTS

CFSE-Labeled Cells in the Spleen and Lymph Nodes of Recipients

After transfer of CFSE-labeled splenocytes from nasal immunotherapy group to naive mice, splenocytes were detected in the spleen and regional lymph nodes (14.9 ± 1.9% and 12.2 ± 1.8%, respectively) of the OVA-sensitized recipient. Also, T-cell transfer from intranasal OVA immunotherapy group to naive mice CFSE-labeled T cells were detected in the spleen ad regional lymph nodes (7.3 ± 0.9% and 6.9 ± 1.0%, respectively). On the other hand, transfer of splenocytes from untreated mice to naive mice resulted in statistically less CFSE-labeled cells in the spleen and regional lymph nodes (1.6 ± 0.3% and 2.8 ± 0.8%, respectively) compared with splenocytes and T cells of OVA-sensitized recipients ($p < 0.05$; Fig. 2).

Histopathological Analyses

The smooth muscle and whole thickness of large airways were found to be less in the nasal immunotherapy group (group I, 35 ± 13.2 μm and 58.3 ± 17.5 μm, respectively) and the OVA-sensitized recipients (group II, 30 ± 7 μm and 55 ± 14.1 μm, respecitvely) when compared with the asthma model group (group III, 71.7 ± 22 μm and 96.2 ± 31.1 μm, respectively; $p < 0.05$; Figs. 3 *A* and 4). In addition, there was no statistically significant difference in the thickness of the smooth muscle ($p = 0.34$) and whole thickness ($p = 0.08$) in all sized airways in intranasal OVA immunotherapy and OVA-sensitized recipients groups when compared with healthy controls (group IV, 26.6 ± 2.8 μm and 43.3 ± 5.7 μm, respectively). Adoptive transfer of T cells from intranasal OVA immunotherapy group to naive mice significantly suppressed the area of inflammation ($p < 0.01$) compared with the asthma model group (Fig. 3 *B*). In BAL analyses, there were less inflammatory cell numbers in intranasal OVA immunotherapy (68 ± 9.8) and OVA-sensitized recipient groups (56 ± 16.9) compared with the asthma model group (98 ± 25.4; Fig. 3 *C*).

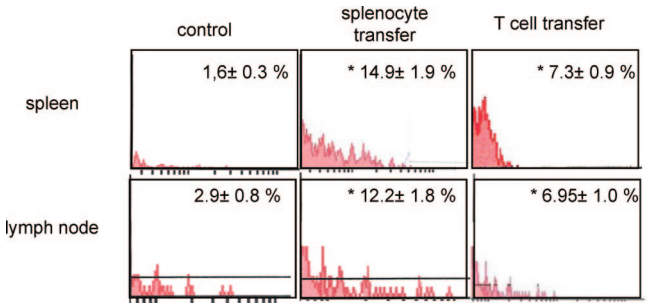

Figure 2. *CFSE-labeled splenocytes or splenic T cells in the spleen and lymph node of OVA-sensitized recipient mice after adoptive transfer. Splenocytes and T cells from the intranasal immunotherapy group were isolated and labeled with CFSE and transferred to naive mice. The asthma model was established by i.p. and nebulized OVA. Migration of CFSE-labeled splenocytes and T cells to the spleen and lymph node were analyzed by flow cytometric analyses. *Significant difference (p < 0.01) of the OVA-induced recipient group (recipients) compared with the asthma model (sensitized and recipient of naïve T cells).*

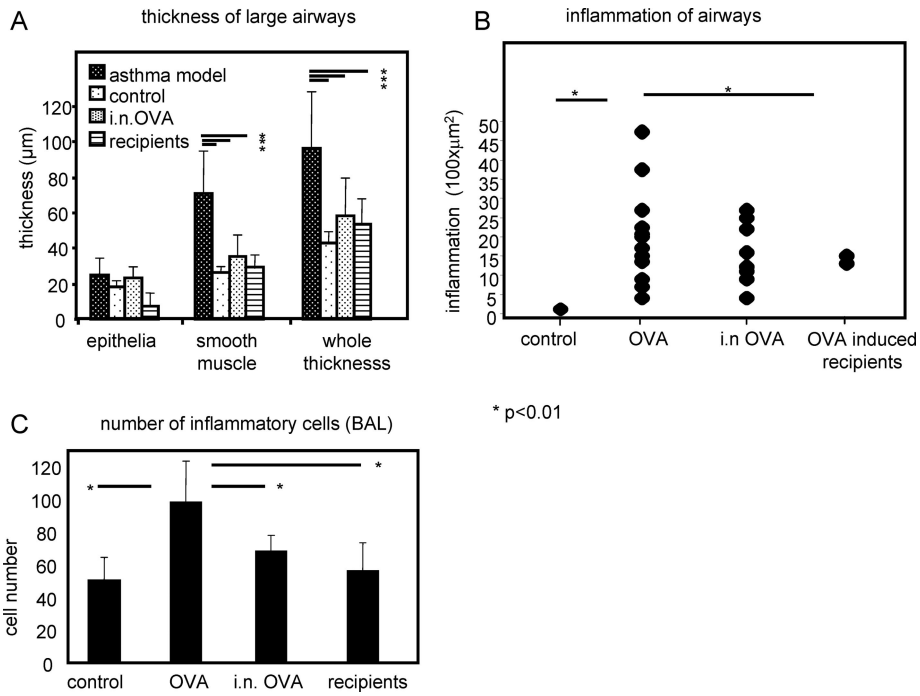

*Figure 3. Histopathological analyses of airways. Lungs of all groups were taken 1 day after last OVA nebulization. They were fixed in formaldehyde and embedded to paraffin. Sections were stained with periodic acid Schiff (PAS) and analyzed under light microscope with 40× magnification. (A) Epithelial, smooth muscle, and whole thickness of airways were measured. (B) Infiltration of inflammatory cells to perivascular and peribronchial parts of the lung was measured. (C) Number of inflammatory cells in the BAL fluid was counted. Values represent the mean ± SD, *Significant difference ($p < 0.01$) of the asthma model versus the intranasal immunotherapy (i.n. IT) or OVA-induced recipient group (recipient) or control.*

Cytokine Levels of Splenocytes and Lymph Node Lymphocytes

When compared with healthy controls, OVA stimulated IL-10 levels in splenocyte culture supernatants significantly increased in the nasal immunotherapy group and OVA-sensitized recipient group ($p < 0.05$). Spontaneous cytokine levels detected in supernatants of regional lymph node lymphocytes revealed increased IL-10 levels in the intranasal OVA immunotherapy and OVA-sensitized recipient groups when compared with the asthma model and healthy control groups, while the IL-4 level decreased in all groups with an exception of asthma model group ($p < 0.01$ and $p < 0.05$, respectively; Fig. 5).

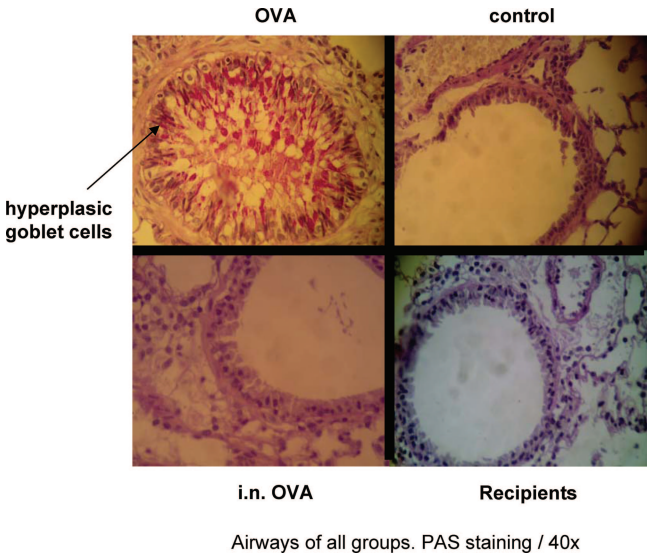

Figure 4. PAS-stained lung sections of all groups. (40×).

Spontaneous and OVA-stimulated IL-4 levels in splenocyte supernatants were significantly less in the nasal immunotherapy group and OVA-sensitized recipient group when compared with the asthma model group ($p < 0.05$). OVA-stimulated IFN-γ levels were increased in the intranasal immunotherapy and OVA-sensitized recipient groups compared with the asthma model and control groups ($p < 0.01$). Likewise, in the OVA-sensitized recipient group spontaneous IFN- γ levels were significantly higher compared with other groups ($p < 0.05$).

DISCUSSION

The present study provides evidence that, transfer of splenic T cells from intranasal OVA-immunized mice is able to ameliorate histopathological changes and increase secretion of IL-10 and IFN-γ from splenocytes and lymph nodes of recipients. Adoptive transfer of antigen-specific $CD4^+$ T cells is sufficient to induce

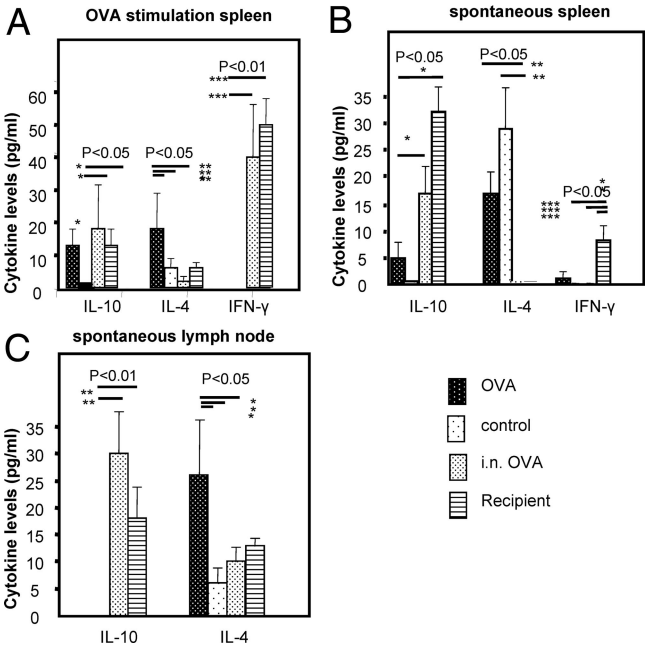

*Figure 5. OVA-stimulated and -unstimulated culture supernatants were collected and IL-10, IL-4, and IFN-γ levels were detected by ELISA. (A) The value of *p < 0.05, **p < 0.05, ***p < 0.01 intranasal immunotherapy (in. IT) group and OVA-sensitized recipients (recipient) versus control and/or asthma model, respectively. (B) The value of *p < 0.05, ***p < 0.05 in. IT group and OVA-sensitized recipients (recipient) versus control and asthma model, respectively. The value of **p < 0.05 control and asthma model versus in. IT or OVA-sensitized recipients (recipient). (C) The value of *p < 0.05, asthma model versus control or in. IT group or OVA-sensitized recipients (recipient). The value of **p < 0.01 in. IT group and OVA-sensitized recipients (recipient) versus control or/and asthma model.*

allergic airway inflammation after antigen challenge. The degree of inflammation following T-cell adoptive transfer is similar to that produced in actively sensitized animals, providing evidence that $CD4^+$ T cells are sufficient for the induction and maintenance of inflammation in experimental asthma.[9] Phenotypically, the cells responsible for transfer of allergic responses are activated, antigen-specific T cells that express high levels of CD4 and produce Th2 cytokines.[12–15]

In our study, we transferred T cells from the nasal immunotherapy group to naive recipients. This adoptive transfer managed to suppress histopathological changes in large airways and decreased inflammation in the recipient lung. Thus, these results may suggest that intranasal OVA immunization may induce $CD4^+$ T cells, hence, transferring their modulatory capacity to naive cells *in vivo*. In a previous study, it was shown that mucosal Tr cells, which can be isolated from the spleen, protects against the development of both delayed type hypersensitivity and IgE responses, irrespective of initial or ongoing cytokine polarization.[16]

Mucosal administration of soluble proteins (antigens) may enhance peripheral immunologic tolerance, which plays an important role in preventing or controlling unwanted immune responses to both self- and non–self-antigens *via* regulating homeostasis. This approach has been recognized as a promising way to prevent allergic, autoimmune, or infection-induced immunopathological reactions.[17] Recently, interest has focused on the delivery of allergen orally, particularly by sublingual administration. There have been a number of articles written concerning the allergen immunotherapy and the main concern is of a better knowledge of the mechanisms of mucosal tolerance leading to appropriate dosage, schedules, and safety in high-risk groups as emphasized in recently published reviews.[18,19] In humans, a meta-analysis of the double-blinded, placebo-controlled trials that have been performed in the past 10 years has shown that sublingual immunotherapy is clinically efficacious.[20]

Studies with experimental murine models revealed that intranasal exposure of house-dust mites,[21] purified protein derivative,[22] and intragastric administration of *Mycobacterium vaccae*[23] prevents allergic sensitizations and inflammation in mice. With the hypothesis of these studies in mind we attempted to evaluate whether intranasal allergen (OVA) immunotherapy prevents smooth muscle and whole thickness of airways. In addition, we aimed to detect whether intranasal OVA immunotherapy has a down-regulating effect on the number of inflammatory cells in BAL. Our results indicated that these parameters were significantly less in the intranasal immunotherapy group when compared with the OVA-sensitized group. Moreover, by regulating the cytokine secretion profile this model of allergen immunotherapy delivered before establishing the asthma model was able to decrease Th2-type immune response *via* suppression of IL-4 levels both in splenocytes and in lymph node lymphocytes. While down-regulating Th2-type immune response, intranasal application of OVA upregulated OVA-stimulated IFN-γ secretion from splenocytes, as well as IL-10 secretion both from splenocytes and lymph node culture supernatants. These results point to the ameliorating effect of intranasal OVA immunotherapy on the histopathologic changes in the airways and cytokine polarization away from Th2-type in OVA sensitized mice.

The current study evaluated whether tolerance can be transferred by using a labeled technique for follow-up of the transferred splenocytes and T cells. This provides a path of mechanism caused by tolerated T cells. Moreover, the modulatory effect of those transferred T cells on changes of airway histopathology on antigen challenge is investigated.

It was previously shown that nasal tolerance induction involves active suppression mediated by $CD4^+$ Tr cells by adoptive transfer experiments.[24,25] To understand whether we can transfer this protective effect of intranasal immunotherapy harnessed by probably T cells, we transferred splenic T cells of the treated group to naive mice before sensitization with OVA. In our study adoptive transfer of T cells from the immunotherapy group were able to decrease OVA-induced IL-4 levels in both the spleen and the lymph node lymphocytes. Moreover, this resulted in increased OVA-induced IL-10 and IFN-γ levels. It was also hypothesized that expression of IL-10 and possibly other cytokines in the airway environment may contribute to the maintenance of the normal state of allergen nonresponsiveness.[26] Kearly *et al.* clearly showed that transfer of $CD4^+CD25^+$ T cells suppress the Th2 cell-driven response to allergen *in vivo* by an IL-10–dependent mechanism.[27]

In conclusion, we demonstrated that transfer of intranasal tolerance induced T cells to naive mice before establishing asthma model abrogates the features of allergic airway disease *in vivo*. These include preventing smooth muscle, whole thickness, and inflammatory cell infiltration to peribronchial and perivascular areas of large airways; decreased inflammatory cells in BAL fluid; and reduced IL-4 levels. Moreover, down-regulation of inflammation was observed with a concomitant increase in IL-10 secretion from splenocyte culture supernatants. Transfer of T cells from mice receiving immunotherapy through the mucosal route regulates T-cell responses and controls histopathological changes of airways in recipient mice.

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