EFFECTS OF IMMUNOTHERAPY ON THE DISTRIBUTION AND CLONALITY OF TCR V\textsubscript{g} AND V\textsubscript{d} SUBFAMILY T CELLS IN ALLERGIC RHINITIS PATIENTS

UTICAJ IMUNOTERAPIJE NA DISTRIBUCIJU I KLONALNOST T \textit{}CELIJA IZ POTPORODICE TCR V\textsubscript{g} I V\textsubscript{d} KOD OBOLELIH OD ALERGIJSKOG RINITISA

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Summary: The aim of this study was to investigate the changes in the peripheral specific IgE level, distribution of TCR V\textsubscript{g} and V\textsubscript{d} subfamily T cells and mRNA expressions of TCR V\textsubscript{g} I–III following specific immunotherapy (SIT) with house-dust-mite extract in allergic rhinitis (AR) patients. Ten AR patients undergoing SIT with house-dust-mite extract for 1 year were recruited. Quantitative analysis of immunofluorescence was performed to detect the serum specific IgE (sIgE) level before and after SIT; RT-PCR-genescan analysis was employed to detect the mRNA expressions of TCR V\textsubscript{g} (I–III) and V\textsubscript{d} (1–8) in the peripheral mononuclear cells followed by analysis of T cell clonality. Real-time quantitative PCR was applied to detect the expressions of TCR V\textsubscript{g} I–III genes. Ten healthy volunteers served as controls. For AR patients, SIT treatment could improve the symptoms, but the serum sIgE level was not markedly decreased. Before SIT, the expressions of TCR V\textsubscript{g} I–III gene were similar between AR patients and controls (P>0.05) but markedly decreased after SIT in AR patients (P<0.05 in TCR V\textsubscript{g}I and V\textsubscript{g}II). The expressions of TCR V\textsubscript{d} (1–8) before and after SIT were 5.3±0.82 and 4.9±0.57, respectively, and that in healthy controls was 5.2±1.40. V\textsubscript{d}1, 2, 3 and 6 were the most common genes found in these patients. Significant difference in the TCR V\textsubscript{d}6 subfamily T cells was found between the two groups. Polyclonal or biclonal proliferation was found in the T cells of patients before SIT and in healthy controls, but oligoclonal proliferation in only 1 subject before SIT. After SIT, the proportion of patients with oligoclonal proliferation of T cells (6/10) was markedly increased (P<0.05). SIT for 1 year could alter the expressions of TCR V\textsubscript{g} I–III genes, the
distribution of TCR Vg and Vd T cells and the ways in which T cells proliferate. The early improvement of symptoms following immunotherapy might not be related to the serum sIgE content in AR patients, but associated with the TCR gd T cells, especially the TCR Vd6 T cells.

**Keywords:** allergic rhinitis, specific immunotherapy, TCRgd T cells, T cell receptor, clonality

**Introduction**

Based on the types of T cell receptors (TCR), T cells can be classified into TCR\(\alpha\)T cells and TCR\(\gamma\delta\) T cells. In the peripheral blood, the TCR\(\gamma\delta\) T cells are the predominant type and can specifically recognize antigens. However, TCR\(\gamma\delta\) T cells account for only 1–10% of T cells in the peripheral blood but for as high as 20–50% in the intestinal and respiratory mucosa and they can directly recognize antigens (1). TCR\(\gamma\delta\) T cells have been regarded to bridge the natural immunity and acquired immunity, and play an important role in the immune response of respiratory mucosa (2). Studies also reveal TCR\(\gamma\delta\) T cells can be classified into Th1 cells and Th2 cells. In the different stages of respiratory allergic inflammation, different TCR\(\gamma\delta\) T cells exert positive or negative regulatory effects on the immune function, and specific immunotherapy (SIT) can rectify the imbalance between T cell subsets (3–8). The knowledge on TCR\(\gamma\delta\) T cells is still insufficient as compared to TCR\(\alpha\)T cells, and few studies report the role of TCR\(\gamma\delta\) T cells in allergic rhinitis (AR) and SIT. The present study aimed to investigate the changes in the peripheral specific IgE level, distribution of TCR Vg and Vd T cells and expressions of TCRVg I–III following SIT for 1 year.

**Materials and Methods**

**Sample collection**

A total of 10 AR patients receiving SIT with standardized house-dust-mite extract (standardized immunotherapy protocol of the University of Copenhagen, Denmark (9)) in the Third Affiliated Hospital of Sun Yat-Sen University were recruited. The therapy was effective (the score of nasal symptoms was improved by >30% (10)). The median age of these patients was 23 years (range: 18–42 years), and patients were coded A1–A10. In addition, 10 healthy volunteers served as controls and were coded N1–N10. The peripheral blood was obtained followed by collection of serum and mononuclear cells.

**Detection of serum specific IgE**

The automatic system of quantitative analysis of immunofluorescence (UniCAP 100E; Pharmacia, Sweden) was used to detect the serum specific IgE (sIgE) against the house-dust-mite allergen before and after SIT.

**RNA extraction and cDNA synthesis**

Total RNA was extracted with TRIZol reagent (Invitrogen, USA) and then reverse-transcribed into first-stand cDNA using random primers and the PowerScriptTM Reverse kit (BD, USA). RT-PCR was performed to detect the expression of \(\beta2\) microglobulin which was then used to determine the quality of cDNA.

**Primers**

The forward primers for 3 TCR Vg subfamily members (Vg1–III) and 8 TCR Vd subfamily members (Vd1–8) were designed and two reverse primers were designed according to the region shared by Cg1/Cg2 (Cg) and Cd region (Cd). In addition, two fluorescein conjugated primers (Cg-fam and Cd-fam) were also designed according to the sequence in the up-stream of Cg and Cd sequence and used to mark the PCR product and for gene scanning. All primers were synthesized in TIBMOLBIOL (Germany) (11).

**RT-PCR**

The RT-PCR was performed according to previously reported methods (8). The reaction mixture (20 µL) included 1 mL of cDNA, one primer for Vg or Vd, reverse primer for Cg or Cd (0.5 µmol/L), 0.1 mmol/L dNTP, 1 U of Taq DNA polymerase, 1.5 mmol/L MgCl$_2$ and 1×PCR buffer (Promega, USA). PCR was carried out on an amplifier (Biometra, Germany) and PCR conditions included predenaturation at 94 °C for 3 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min, and a final extension at 72 °C for 6 min. The PCR products were stored at 4 °C.

**mRNA expressions**

Relative quantitative method was employed to determine the relative mRNA expression of each gene in the mononuclear cells. \(\beta2\)M served as an internal reference and the relative expression of each gene was calculated according to the Ct value as follows: relative expression = \(2^{-\Delta\text{Ct}} \times 100\%\) where \(\Delta\text{Ct} = \text{Ct}(\text{TCR Vg}) - \text{Ct}(\beta2\text{M})\) (12).

**Ključne reči:** alergijski rinitis, specifična imunoterapija, TCR gd T celije, T čelijski receptor, klonalnost
Analysis of cell clonality

Labeling PCR clonality. The 10 μL-mixture consisted of 2 μL of unlabeled PCR products; 0.1 μmol/L primer for Cg-fam or Cd-fam, 1.5 mmol/L MgCl2, 0.2 mmol/L dNTP, 1 U of Taq DNA polymerase and PCR buffer. The PCR was done for 35 cycles and the conditions were identical to those above except for annealing at 66 °C. The remaining procedures were the same as those described above.

Genescan analysis (CDR3 size analysis). In brief, 2 μL of Fam conjugated PCR products were mixed with Hi-DirFormamide (ABI) and standards (GENESCANTM-500-LIZ™, PerkinElmer, ABI, including LIZ conjugated DNA fragments of different sizes) at a volume ratio of 20:1 (final volume: 10 μL). The mixture was denaturated at 94 °C for 4 min and the samples were added to a 96-well plate which was then loaded onto the sequence analyzer containing Performance Optimized Polymer-4 (POP-4; ABI) gel followed by electrophoresis. The data were input into a computer, and the size of products and the fluorescence intensity were analyzed with the GeneScan® Analysis Software. During the development of T cells, reorganization of V and J segments occurs forming the functional TCR gene. In the reorganization, different amounts of nucleotides (N) are inserted between V and J segments and the length of D region changes. This process may lead to the formation of a hypervariable region, also known as complementary determining region 3 (CDR3). CDR3 is the site that specific antigens bind to and determine the specificity of TCR. The size and sequence of CDR3 varied in T cells with different clonalities. Therefore, the primers were designed according to the upstream sequence of V region and downstream sequence of C region and used for PCR amplification. Based on the size of PCR products, the size of CDR3 was then determined. The size and content of PCR products varied in T cells with different clonalities. Thus, the T cell clonalities can be determined according to the PCR products. Fluorescein conjugated products were subjected to analysis with GeneScan analysis software. The location and height of the peak represent the size and content of PCR products, and the shape of the peak represents the clonality. A single peak suggests the PCR products are derived from T cells with identical CDR3 size (monoclonality); a major peak and several small peaks suggest the PCR products are derived from oligoclonal T cells (oligoclonality); multiple peaks suggest multiclonality.

Statistical analysis

Statistical analysis was performed with SPSS version 13.0 statistic software package. Comparisons between groups were done with t test or chi square test. A value of P<0.05 was considered statistically significant.

Results

sIgE levels before and after SIT in AR patients

In the 10 patients, the score of nasal symptoms was improved by >30% after SIT for 1 year which was defined as effectiveness. However, the serum sIgE level was not markedly decreased after SIT treatment (22.89±9.60 kU/L vs 19.62±7.63 kU/L, P>0.05).

mRNA expressions of TCR Vγ subfamilies

The expressions of TCR Vγ I and VγII in AR patients were lower than those in healthy controls, but TCR VγIII expression was higher than in controls. There were no marked differences in the expressions of TCR Vγ I–III between AR patients before SIT and healthy controls (P>0.05). After SIT, the expressions of TCR Vγ I–III were decreased in AR patients, especially the expressions of TCR Vγ I and Vγ II (Vγ I: t=–2.904, P=0.00; Vγ II: t=–2.217, P=0.039; Vγ III: t=–1.850, P=0.079) (Figure 1).

Expressions of TCR Vγ and Vδ in the T cells of AR patients and healthy controls

TCR Vγ expression was found in all samples. The expression of TCR Vδ in AR patients before and after SIT was 5.3±0.82 and 4.9±0.57, respectively, and that in controls was 5.2±1.40. The expressions of Vδ 1, 2, 3 and 6 were found in the majority of subjects and those of Vδ 4, 5, 7 and 8 were only noted in several individuals.

TCRVγ and Vδ T cell clonality in AR patients and healthy controls

Genescan analysis showed 3 AR patients had consistent T cell polyclonality and the clonal prolifer-
The efficacy of SIT might be reflected by the changes in the serum sIgE level. In the present study, the score of nasal symptoms was improved by >30% and some symptoms even resolved. The improvement of symptoms, however, was not accompanied by the decrease of sIgE and the sIgE level was not markedly decreased after SIT for 1 year. Thus, we speculate that the humoral immune response to the house-dust-mite allergen still exists during the SIT because the patients are not isolated from the allergens leading to the sustained production of sIgE. Therefore, in the early stage of SIT, the pathway mediating the production of sIgE is not blocked and the improvement of nasal symptoms may not be closely associated with the sIgE content but benefit from the T cell mediated cellular immunity. Another research also proposed that the sIgE represented the patients’ idiosyncrasy and the control of clinical symptoms was not linearly related to the sIgE level (13). The decrease of sIgE level may lag behind the improvement of symptoms. Thus, we should be well aware of the long term SIT in clinical practice (14). Whether the normal sIgE can be used as a criterion for determining the efficacy or discontinuation of SIT should be further clarified in evidence-based medicine.

The TCR \( \gamma \delta \) T cells and their subsets have relatively high abundance in the respiratory mucosa and play pivotal roles in the allergic inflammation of the respiratory tract (1). Exploring the expressions of TCR\(\gamma\) and TCR \(\delta\) in the peripheral blood or nasal mucosa and the clonality of T cells and investigating the potential functions of these cells in allergic diseases may therefore be helpful for the understanding of the pathogenesis of AR. The peripheral TCR \( \gamma \delta \) T cells are derived from early thymocytes. These cells do not undergo classic thymus differentiation and directly move from the thymus into the peripheral blood before expressing CD4 and CD8 molecules. Therefore, these cells are largely negative for CD4 and CD8 (except for a few cells positive for CD8). The TCR \( \gamma \delta \) gene is composed of reorganized V(D)J segments and the C region, which is similar to TCR \( \alpha\beta \) and immunoglobulin genes. Although the diversity of V region in the \( \gamma \) and \( \delta \) sites is different from that of \( \alpha \) and \( \beta \) sites, the diversity of the junctional region of TCR \( \gamma \) and \( \delta \) renders it to have the potential of diversity (16). TCR\(\gamma\) and TCR\(\delta\) can form heterologous dimers which are expressed on the T cells. Thus, T cells with the expression of TCR \( \gamma \delta \) are defined as TCR \( \gamma \delta \) T cells. The TCR \( \gamma \) chain gene consists of a variable domain (V), a junctional domain (J) and a constant domain (C) and can be divided into V\(\gamma\) I–III subfamilies. TCR \( \delta \) chain gene is composed of a V domain, a J domain, a C domain and a diversity domain (D) and can be divided into 8 subfamilies. During the reorganization of the TCR gene, the formed CDR3 is highly diverse and the size and sequence of CDR3 vary in different clonal T cells during the reorganization of the TCR gene. Based on the characteristics,
RT-PCR and genescan analysis were employed to detect the distribution and clonality of TCR Vγ and Vδ subfamily T cells. Both methods are sensitive in the detection of T cell clonality (8, 11). Previous studies seldom reported the role of TCR γδ T cells in the peripheral cells due to their small amount. Real time fluorescent quantitative RT-PCR has the characteristics of rapidity and high sensitivity in the detection of genes and was employed to detect TCR γδ T cells in the present study. We measured the mRNA expressions of TCR Vγ I–III genes. Our results showed the AR patients had relatively high expressions of TCR Vγ I and Vγ II but low TCR Vγ III expression, which were consistent with those previously reported. Of note, there were no marked differences in the expressions of TCR Vγ I–III between AR patients before SIT and healthy controls, and SIT significantly decreased the expressions of TCR Vγ I–III. This finding suggests SIT in the early stage may inhibit the pro-inflammatory effects conferred by TCR γδ T cells leading to the improvement of AR clinical symptoms.

In the present study, genescan analysis showed the cluster expressions of Vγ subfamily genes in the peripheral T cells of AR patients and healthy controls. The expressions of Vγ subfamily genes were found in almost all subjects and no significant differences were noted in all genes except for Vδ 6 between patients and healthy controls. In respect of the proliferation, Vδ 6 T cells mainly had polyclonal or biclonal proliferation in the AR patients before SIT and the healthy subjects, and oligoclonal proliferation was found in only a few Vδ 6 T cells. After SIT, the proportion of cells with oligoclonal proliferation was significantly increased, and this preferable expression of selected genes may be related to certain functions. The T cells in healthy individuals usually present polyclonal proliferation (17). Only after the stimulation by specific antigens the body generates clonal T cells as a response to the specific antigens. In the present study, genescan analysis showed the majority of peripheral TCR Vδ subfamily T cells presented polyclonal proliferation in AR patients and healthy volunteers, but there still were 3 healthy subjects having clonal Vδ 4 or 6 T cells. Shen et al. (16) also showed similar results and they postulated that the presence of TCR Vδ clonality in a few T cells is a normal phenomenon and a result of non-specific random proliferation. A previous work showed TCR Vδ 1 was preferably expressed in the human intestinal epithelial cells, and its expression was also frequently found in autoimmune diseases, tumors and bronchial asthma (18). Our results revealed the TCR Vδ 6 T cells had an increased trend toward oligoclonal proliferation following SIT. Based on this finding, we speculate that TCR Vδ 6 T cells may be a group of cells exerting resistance to the allergic response after SIT with standardized house dust mite. The preferable expression of TCR Vδ 6 may be closely related to the improvement of AR symptoms. This should be further confirmed in future studies.

Taken together, our study investigated the distribution and clonality of TCR Vγ and Vδ subfamily T cells in the peripheral T cells of AR patients. These findings may provide evidence for the investigations on the pathogenesis of AR. More studies with a large sample size are required to confirm the role of TCR γδ T cells in the nasal mucosa of AR patients.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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