

EFFECTS OF IMMUNOTHERAPY ON THE DISTRIBUTION AND CLONALITY OF TCR V γ AND V δ SUBFAMILY T CELLS IN ALLERGIC RHINITIS PATIENTS

UTICAJ IMUNOTERAPIJE NA DISTRIBUCIJU I KLONALNOST T ČELIJA IZ POTPORODICE TCR V γ I V δ 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 γ and V δ subfamily T cells and mRNA expressions of TCR V γ 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 γ (I–III) and V δ (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 γ 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 γ 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 γ I and V γ II). The expressions of TCR V δ (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 δ 1, 2, 3 and 6 were the most common genes found in these patients. Significant difference in the TCR V δ 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 γ I–III genes, the

Kratka sadržaj: Cilj ove studije bio je da se istraže promene u nivou specifičnog IgE u perifernoj krvi, distribuciji T ćelija iz potporodice TCR V γ i V δ i ekspresiji TCR V γ I–III u mRNK posle specifične imunoterapije (SIT) sa ekstraktom grinja iz kućne prašine kod pacijenata sa alergijskim rinitisom (AR). Uključeno je 10 pacijenata sa AR lečenih specifičnom imunoterapijom sa ekstraktom grinja iz kućne prašine tokom 1 godine. Obavljena je kvantitativna analiza imunofluorescencijom kako bi se odredio nivo specifičnog IgE (sIgE) u serumu pre i posle SIT; primenjena je RT-PCR-genescan analiza radi određivanja ekspresije TCR V γ (I–III) i V δ (1–8) u mRNK u perifernim mononuklearnim ćelijama posle čega je obavljena analiza klonalnosti T ćelija. Kvantitativna PCR analiza u realnom vremenu sprovedena je kako bi se odredila ekspresija TCR V γ I–III gena. Deset zdravih dobrovoljaca služilo je kao kontrolna grupa. Kod pacijenata sa AR, tretman SIT olakšao je simptome, ali nivo sIgE u serumu nije se značajno snizio. Pre SIT, ekspresija TCR V γ I–III gena bila je slična kod pacijenata sa AR i kontrolnih subjekata ($P > 0,05$), ali se značajno snizila posle SIT kod pacijenata sa AR ($P > 0,05$ u TCR V γ I i V γ II). Ekspresija TCR V δ (1–8) pre i posle SIT bila je $5,3 \pm 0,82$ i $4,9 \pm 0,57$, a kod zdravih subjekata $5,2 \pm 1,40$. V δ 1, 2, 3 i 6 geni bili su najviše zastupljeni kod ovih pacijenata. Značajna razlika u T ćelijama TCR V δ 6 potporodice utvrđena je između dve grupe. Poliklonalna ili biklonalna proliferacija nađena je u T ćelijama pacijenata pre SIT i kod kontrolnih subjekata, ali oligoklonalna proliferacija pronađena je samo kod 1 subjekta pre SIT. Posle SIT, proporcija pacijenata sa oligoklonalnom proliferacijom T ćelija (6/10) bila je značajno povišena ($P < 0,05$). SIT tokom 1 godine može izmeniti ekspresiju TCR V γ I–III gena, distribuciju TCR V γ i V δ T ćelija kao i načine proliferacije T ćelija. Rano ublažavanje simptoma posle imunoterapije možda nije povezano sa sadržajem sIgE u serumu kod pacijenata sa AR, ali je u vezi sa TCR gd T ćelijama, naročito TCR V δ 6 T ćelijama.

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distribution of TCR V γ and V δ 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 $\gamma\delta$ T cells, especially the TCR V δ T cells.

Keywords: allergic rhinitis, specific immunotherapy, TCR $\gamma\delta$ T cells, T cell receptor, clonality

Introduction

Based on the types of T cell receptors (TCR), T cells can be classified into TCR $\alpha\beta$ 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 $\alpha\beta$ 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 V γ and V δ T cells and expressions of TCRV γ 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.

Ključne reči: alergijski rinitis, specifična imunoterapija, TCR $\gamma\delta$ T ćelije, T ćelijski receptor, klonalnost

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 β 2 microglobulin which was then used to determine the quality of cDNA.

Primers

The forward primers for 3 TCR V γ subfamily members (V γ I–III) and 8 TCR V δ subfamily members (V δ 1–8) were designed and two reverse primers were designed according to the region shared by C γ 1/C γ 2 (C γ) and C δ region (C δ). In addition, two fluorescein conjugated primers (C γ -fam and C δ -fam) were also designed according to the sequence in the up-stream of C γ and C δ 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 μ L) included 1 mL of cDNA, one primer for V γ or V δ , reverse primer for C γ or C δ (0.5 μ mol/L), 0.1 mmol/L dNTP, 1 U of Taq DNA polymerase, 1.5 mmol/L MgCl₂ and 1 \times PCR buffer (Promega, USA). PCR was carried out on an amplifier (Biometra, Germany) and PCR conditions included predenaturation at 94 $^{\circ}$ C for 3 min, 40 cycles of denaturation at 94 $^{\circ}$ C for 1 min, annealing at 60 $^{\circ}$ C for 1 min and extension at 72 $^{\circ}$ C for 1 min, and a final extension at 72 $^{\circ}$ C for 6 min. The PCR products were stored at 4 $^{\circ}$ C.

mRNA expressions

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

Analysis of cell clonality

Labeling PCR products. The 10 μ L-mixture consisted of 2 μ L of unlabeled PCR products; 0.1 μ mol/L primer for C γ -fam or C δ -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 μ L of Fam conjugated PCR products were mixed with Hi-DiFormamide (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 μ L). 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

slgE 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 slgE 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 V γ subfamilies

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

Expressions of TCR V γ and V δ in the T cells of AR patients and healthy controls

TCR V γ expression was found in all samples. The expression of TCR V δ 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 V δ 1, 2, 3 and 6 were found in the majority of subjects and those of V δ 4, 5, 7 and 8 were only noted in several individuals.

TCRV γ and V δ T cell clonality in AR patients and healthy controls

Genescan analysis showed 3 AR patients had consistent T cell polyclonality and the clonal prolifer-

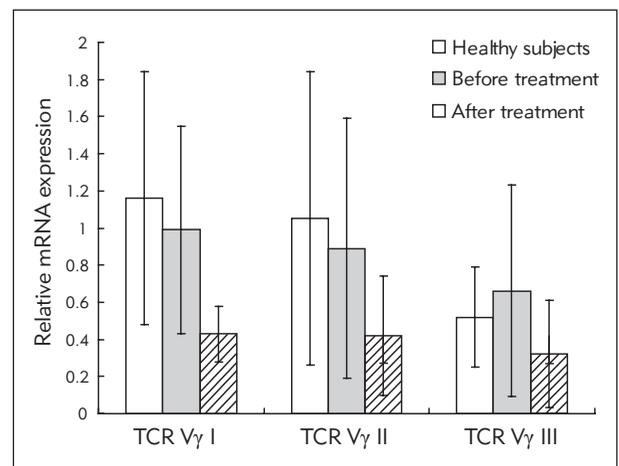


Figure 1 mRNA expressions of TCR V γ genes in AR patients before and after SIT.

		A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
VγI	before treatment										
	after treatment										
VγII	before treatment										
	after treatment										
VγIII	before treatment										
	after treatment										
Vδ1	before treatment										
	after treatment										
Vδ2	before treatment										
	after treatment										
Vδ3	before treatment										
	after treatment										
Vδ4	before treatment	■			■				■		
	after treatment	■			■				■	■	
Vδ5	before treatment	■		■				■			■
	after treatment	■		■				■			■
Vδ6	before treatment	■	■	■	■	■	■	■	■	■	■
	after treatment	■	■	■	■	■	■	■	■	■	■
Vδ7	before treatment			■		■				■	
	after treatment			■		■				■	
Vδ8	before treatment				■				■		
	after treatment				■		■				

Figure 2 Distribution and clonality of TCR Vγ and Vδ subfamily T cells in 10 AR patients before and after SIT.

ation of T cells changed in different subfamilies in 7 patients. For example, A1 had biclonal proliferation in the Vδ 4, 5 and 6; A2 and 8 presented oligoclonal proliferation in the Vδ 6 and 4; A4, 5, 6 and 9 had biclonal proliferation in the Vδ 4 and 6, and polyclonal proliferation in the remaining Vδ subfamilies. After SIT, A1 presented oligoclonal proliferation in the Vδ 4 and 6, and biclonal proliferation in the Vδ 5; A2, 4, 5, 6 and 8 manifested oligoclonal proliferation in the Vδ 6; A4 had biclonal proliferation in the Vδ 4; A9 had biclonal proliferation in the Vδ4 and Vδ6 and polyclonal proliferation in the remaining subfamilies (Figure 2). In the 10 healthy controls, 3 had clonal T cells mainly in the Vδ 4 (n=2) and Vδ 6 (n=1). In respect of proliferation patterns, there was significant difference in the TCR Vδ 6 between groups. The controls and patients before SIT were characterized by polyclonal or biclonal proliferation in the TCR Vδ 6 (only 1 had oligoclonal proliferation) and AR patients following SIT for 1 year by oligoclonal proliferation in the TCR Vδ 6 (6/10, 60%) (P<0.05).

Discussion

For the IgE mediated allergic diseases, SIT with the allergen extract is the unique strategy targeting the pathogenic factor, and its effectiveness has been confirmed (9). However, the mechanism underlying the therapeutic efficacy of SIT is still poorly understood. AR is a type I allergic disease mediated by IgE.

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 γδ 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 TCRγ and TCR δ 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 γδ 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 γδ gene is composed of reorganized V(D)J segments and the C region, which is similar to TCR αβ and immunoglobulin genes. Although the diversity of V region in the γ and δ sites is different from that of α and β sites, the diversity of the junctional region of TCR γ and δ renders it to have the potential of diversity (16). TCRγ and TCRδ can form heterologous dimers which are expressed on the T cells. Thus, T cells with the expression of TCR γδ are defined as TCR γδ T cells. The TCR γ chain gene consists of a variable domain (V), a junctional domain (J) and a constant domain (C) and can be divided into Vγ I-III subfamilies. TCR δ 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 V γ and V δ subfamily T cells. Both methods are sensitive in the detection of T cell clonality (8, 11). Previous studies seldom reported the role of TCR $\gamma\delta$ 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 $\gamma\delta$ T cells in the present study. We measured the mRNA expressions of TCR V γ I–III genes. Our results showed the AR patients had relatively high expressions of TCR V γ I and V γ II but low TCR V γ III expression, which were consistent with those previously reported. Of note, there were no marked differences in the expressions of TCR V γ I–III between AR patients before SIT and healthy controls, and SIT significantly decreased the expressions of TCR V γ I–III. This finding suggests SIT in the early stage may inhibit the pro-inflammatory effects conferred by TCR $\gamma\delta$ T cells leading to the improvement of AR clinical symptoms.

In the present study, genescan analysis showed the cluster expressions of V γ subfamily genes in the peripheral T cells of AR patients and healthy controls. The expressions of V γ subfamily genes were found in almost all subjects and no significant differences were noted in all genes except for V δ 6 between patients and healthy controls. In respect of the proliferation, V δ 6 T cells mainly had polyclonal or biconal proliferation in the AR patients before SIT and the healthy subjects, and oligoclonal proliferation was found in only a few V δ 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 V δ subfamily T cells presented polyclonal proliferation in AR patients and healthy volunteers, but there still were 3 healthy subjects having clonal V δ 4 or 6 T cells. Shen et al. (16) also showed similar results and they postulated that the presence of TCR V δ clonality in a few T cells is a normal phenomenon and a result of non-specific random proliferation. A previous work showed TCR V δ 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 V δ 6 T cells had an increased trend toward oligoclonal proliferation following SIT. Based on this finding, we speculate that TCR V δ 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 V δ 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 V γ and V δ 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 $\gamma\delta$ 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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