



ORIGINAL ARTICLE

Interleukin-21 Receptor Might be a Novel Therapeutic Target for the Treatment of Rheumatoid Arthritis



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Background: Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by the synovial inflammation of the joints. Various cells and cytokines have been identified that may contribute to RA pathology. Interleukin (IL)-21 is a proinflammatory cytokine mediating pleiotropic functions through the IL-21 receptor (IL-21R). Blockade of IL-21R may represent a hopeful therapeutic approach in RA. The aim of this study was to determine the percentage of IL-21R expressing CD4⁺ cells and IL-21 mRNA expression in peripheral blood of RA patients.

Methods: Surface expression of IL-21R on CD4⁺ cells in peripheral blood of RA patients ($n = 32$) compared to healthy control participants ($n = 20$) was evaluated by flow cytometry. Simultaneously, mononuclear cells were taken apart from the peripheral blood of individuals on a density gradient. The expression of IL-21 mRNA was assessed by real-time polymerase chain reaction.

Results: IL-21R-expressing CD4⁺ cells from RA patients showed a significantly higher percentage of IL-21R compared with healthy controls ($p < 0.05$). Moreover, real-time polymerase chain reaction showed that there was no significant difference between patients and healthy controls.

Conclusion: Our results indicate higher expression of IL-21R in RA patients and suggest that targeting of the IL-21R may be a novel therapeutic idea for the treatment of RA.

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1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease with unclear etiology and pathology, characterized by the infiltration of various inflammatory cells into the joints, inflammation of synovial tissues, cartilage destruction, and bone erosions.^{1–6} RA prevalence is about 1% of the population worldwide.^{7,8} The milieu of secreted cytokines is critical in the differentiation and expansion of pathogenic cells.^{9,10} Hence the inflammatory conditions in RA are controlled by various cytokines, especially interleukin (IL)-21.¹¹ IL-21 is a newly discovered proinflammatory cytokine contributing towards autoimmune diseases such as systemic lupus erythematosus, multiple sclerosis, type 1 diabetes, and, especially, in rheumatoid arthritis.^{12–14} It enhances the differentiation of Th17 cells

and intensify IL-17 production.^{15,16} IL-21 is a member of the IL-2 family of cytokines chiefly produced by activated CD4⁺ T cells, comprising Th1, Th2, and Th17 cells.^{17,18} However, its receptor (IL-21R) has been observed on various kinds of cells, influencing on both innate and adaptive immunity systems.^{18,19} It augments proliferation and differentiation of CD4⁺/CD8⁺ T cells and also reinforces the activation and propagation of natural killer cells.^{1,12,20,21} In addition to regulating the production of antibody, containing all immunoglobulin G isotypes, IL-21 has a substantial role in activation, maturation, and clonal expansion of B cells.^{22–24} Heterodimeric receptor of IL-21 contains IL-21 specific receptor plus common γ -chain receptor which is structurally associated with IL-2R, IL-4R, and IL-15R.^{1,22,23} The role of IL-21 and IL-21R in human diseases has not been determined precisely.²⁵ However, blocking of IL-21 and IL-21R has ameliorated synovitis and articular cartilage damages in animal models of arthritis, such as collagen-induced arthritis.^{19,26,27} Therefore, this study was designed to investigate IL-21-expressing CD4⁺ cells and the expression of IL-21 mRNA in RA patients in comparison to healthy controls. If IL-21R⁺ cells significantly increase in the peripheral blood of RA patients, it

Conflicts of interest: All contributing authors declare no conflicts of interest.

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may represent a substantial therapeutic target for the treatment of human RA.

2. Methods

2.1. Patients and sampling

In this study, 32 patients with RA who fulfilled the 1987 revised criteria of the American College of Rheumatology were totally included.²⁸ This group consisted of 28 female and 4 male individuals. The mean \pm standard deviation age of the patients was 48.5 ± 9.85 years. Twenty healthy individuals matched for sex ratio and mean age (16 females and 4 males, age 45.8 ± 6.94 years) were also included as a control group. Written consent was obtained from all participating individuals prior to sample collection. At the time of sample collection, recruited patients had been treated with disease-modifying antirheumatic drugs and steroids.²⁹ However, these are not a certain cure for RA; medications conventionally used can relieve inflammation and inhibit mitigation of the inflammatory cells to the tissue resulting in joint damage reduction.³⁰ The clinical details and medication of patients and healthy controls who donated whole blood are presented in Table 1. Whole blood samples were collected from peripheral veins. The protocols of flow cytometry and RNA extraction were carried out simultaneously and cDNA was immediately synthesized, and stored at -70°C until it was used for real-time polymerase chain reaction (PCR).

This study was approved by the Ethics Committee of Shahrekord University of Medical Sciences, Shahrekord, Iran.

2.2. Real-time PCR

After gathering heparinized whole blood, peripheral blood mononucleated cells were isolated by Ficoll–Hypaque density centrifugation (Sigma-Aldrich, St Louis, MO, USA). Then, total RNA was isolated from peripheral blood mononucleated cells using RNX-Plus solution (Sinaclon, Tehran, Iran). Genomic DNA was removed from total RNA using RNase-free DNase Set (Qiagen, Chartsworth, CA, USA). Reverse transcription reaction was conducted at 25°C for 5 minutes, 42°C for 60 minutes and 70°C for 5 minutes in a 12 μL mixture containing 4 μg of total RNA, using Revert Aid First cDNA synthesis kit (Revert Aid First, Fermentas, Finland). Each real-time PCR was prepared in a 20 μL reaction mixture containing 10 μL TaqMan Universal PCR Master Mix, 3 μL cDNA, 0.4 μL primers (10pM each of forward and reverse primers) and 0.2 μL probe (10pM) in capillary tubes and conducted on a Rotor Gene 3000 (Corbett, Mortlake, NSW, Australia). Cycling conditions were: initial denaturation 5 minutes at 95°C for the activation of polymerase, followed by 40 cycles of 15 seconds at 95°C , and 60 seconds at 60°C . Results

were normalized on the basis of values for β -actin cDNA. The primer and probe sets (5'-FAM-labeled) were purchased from Applied Biosystems (Foster City, CA, USA). BLAST searches were conducted on them to ensure gene specification. The sequences of the primer and probes are summarized in Table 2. All samples of RA patients and healthy controls were assayed in duplicate. Relative gene expression was measured by the previously published method.^{31,32} Negative controls were also included, containing all the elements of the reaction mixture other than template DNA.

2.3. Flow cytometry

Antibodies used for flow cytometry were purchased from BD Pharmingen (San Diego, CA, USA): anti-CD4 conjugated to PE-Cy5 and anti-IL-21R conjugated to allophycocyanin. Briefly, aliquots of 110 μL of the whole blood containing K_3EDTA were added and stained with 20 μL of each monoclonal antibody for 15 minutes at 37°C and 15 minutes at room temperature, then erythrocytes were lysed by adding 1.1 mL of fluorescence-activated cell sorter lysing solution (Becton Dickinson, Lincoln Park, NJ, USA). After staining, the cells were washed three times in phosphate-buffered saline (PBS) and immediately analyzed using flow cytometry (PARTEC, Münster, Germany). Lymphocytes were gated based on the forward- and side-scatter properties and at least 5000 CD4^+ lymphocytes were observed. The flow cytometry results were analyzed using Flow Jo software version 7.6 (Tree Star, Ashland, OR, USA).

2.4. Statistical analysis

Statistical analyses were performed with SPSS version 15.0 (SPSS Inc., Chicago, Illinois, USA). Data are expressed as mean \pm standard deviation. Differences between the two groups were analyzed with nonparametric Mann–Whitney test and presented using Prism software (GraphPad, La Jolla, California). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. IL-21R expressing CD4^+ cells

In order to calculate the percentage of IL-21R expressing CD4^+ cells, the collected peripheral blood from RA patients and healthy controls were analyzed using flow cytometry.³³ As shown in Figures 1 and 2, peripheral blood CD4^+ cells of RA patients revealed a significantly higher percentage of IL-21R ($32.47 \pm 9.19\%$) compared with those in healthy controls ($21.73 \pm 7.45\%$; $p < 0.05$). On the basis of this finding, IL-21R may play an important role in the pathogenesis of RA.

3.2. Expression of IL-21 in total RNA extracts

To understand the potential function of IL-21 in RA, total RNA extracts derived from peripheral blood of RA patients and also healthy controls were included in the study. As shown in Figure 3, the

Table 1 Basic characteristics and medications of RA patients and healthy controls included in the study

	RA patients	Healthy controls
Total number	32	20
Men/women	4/28	4/16
Age, y (mean \pm SD)	48.5 ± 9.85	45.8 ± 6.94
Treatment:		
Methotrexate (DMARD)	7/5–15	
median dose, mg/wk		
Prednisone (steroid)	5–10	
median dose, mg/d		
Hydroxychloroquine (DMARD)	200–400	
median dose, mg/d		
Sulfasalazine (DMARD)	1–2	
median dose, g/d		

DMARD = disease-modifying anti-rheumatic drug; SD = standard deviation.

Table 2 Sequences of TaqMan primers and probes for the β -actin and interleukin-21 (IL-21) genes

Gene	Primer sequences and probes
β -actin	Probe: 5'-CCGCCGCCGTCCACACCCGCC-3' Forward: 5'-AGCCTCGCCTTTGCCGA-3' Reverse: 5'-CTGGTGCCTGGGGCG-3'
IL-21	Probe: 5'-TCTGCCAGCTCCAGAAGATGTAGAGACAAA-3' Forward: 5'-TGTGAATGACTTGGTCCTGAA-3' Reverse: 5'-AGCAGGAAAAAGCTGACCACTCA-3'

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