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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.

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 $IL\text{-}21R^+$ cells significantly increase in the peripheral blood of RA patients, it

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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 ± 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.

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 ± SD)	48.5 ± 9.85	45.8 ± 6.94
Treatment:		
Methotrexate (DMARD) median dose, mg/wk	7/5–15	
Prednisone (steroid) median dose, mg/d	5–10	
Hydroxychloroquine (DMARD) median dose, mg/d	200–400	
Sulfasalazine (DMARD) median dose, g/d	1–2	

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

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 ± 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 ± 9.19%) compared with those in healthy controls (21.73 ± 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 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′-CCGCCGCCCGTCCACACCCGCC-3′ Forward: 5′-AGCCTCGCCTTTGCCGA-3′ Reverse: 5′-CTGGTGCCTGGGGCG-3′
IL-21	Probe: 5′- TCTGCCAGCTCCAGAAGATGTAGAGACAAA-3′ Forward: 5′-TGTGAATGACTTGGTCCCTGAA-3′ Reverse: 5′-AGCAGGAAAAAGCTGACCACTCA-3′

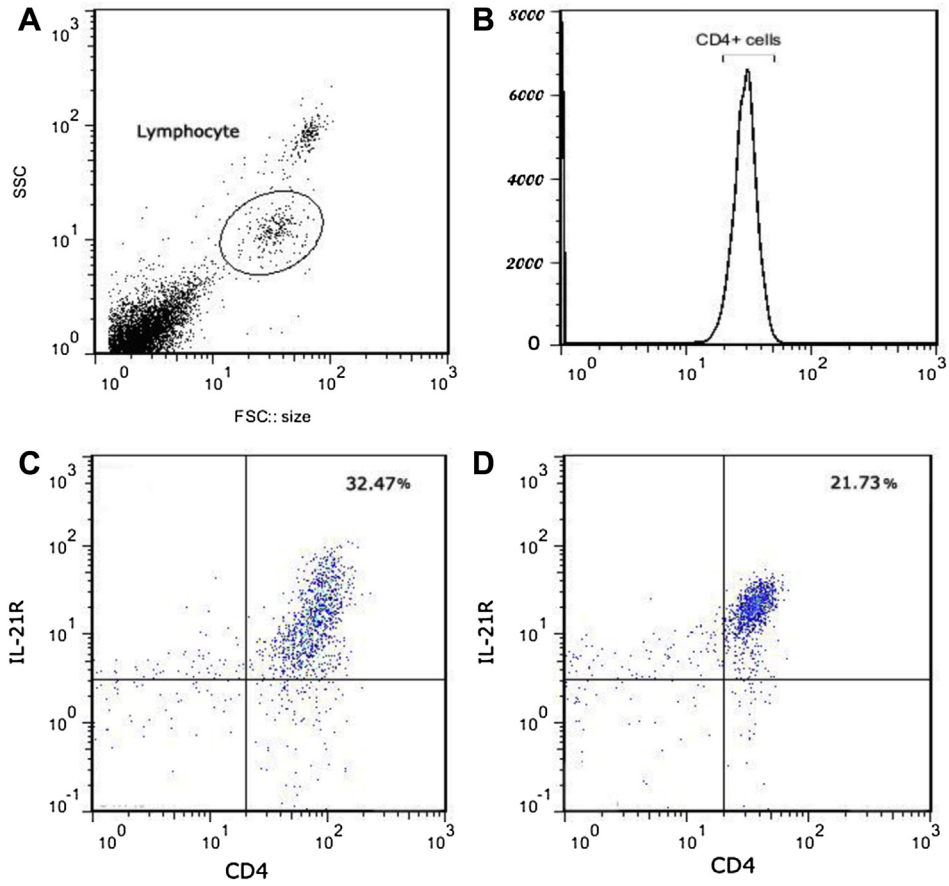

Figure 1 Expression of IL-21R in peripheral blood on a flow cytometric analysis. Whole blood from either RA patients or healthy controls were stained with either mouse anti-human CD4 or interleukin-21 receptor (IL-21R) monoclonal antibodies for 15 minutes at 37°C followed by 15 minutes at room temperature, then erythrocytes were lysed by adding 1.1 mL of fluorescence-activated cell sorter lysing solution. After washing three times with phosphate-buffered saline, cells were examined for calculating the percentage of IL-21R expression on $CD4^+$ cells. (A) Lymphocytes were gated based on the forward and side-scatter properties. (B) At least 5000 $CD4^+$ lymphocytes were analyzed. Shows dot plot quadrant of IL-$21R^+$/$CD4^+$ expressing cells of (C) RA patients and (D) healthy controls.

expression of IL-21 in RA patients was 0.31 ± 0.58 fold in comparison to healthy controls (1 ± 1.4), whereas statistical analyses demonstrated no significant difference between two mentioned groups.

Our findings indicate that the medicines taken might affect expression of IL-21 mRNA but did not influence IL-21R expression in $CD4^+$ cells.

4. Discussion

This study was undertaken to evaluate the expression of IL-21R in the peripheral blood of RA patients. Therefore, we examined IL-21R expression on the surface of $CD4^+$ cells. Our results show that the

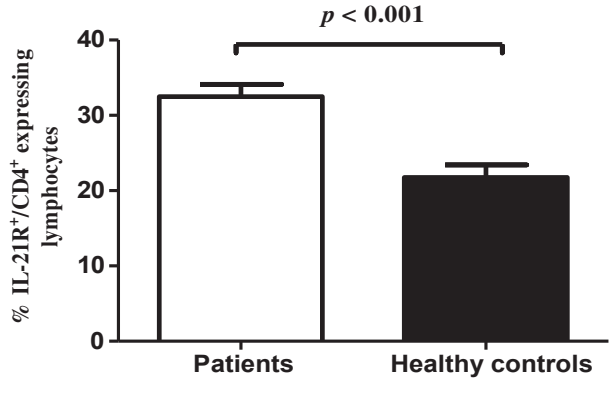

Figure 2 Expression of IL-21R on $CD4^+$ cells. The proportion of interleukin (IL)-21R expression on $CD4^+$ cells from RA patients and healthy controls is indicated. It shows the 32 patients in comparison with 20 age-matched healthy controls. The *p* value was calculated using nonparametric Mann–Whitney test and is significant at <0.05.

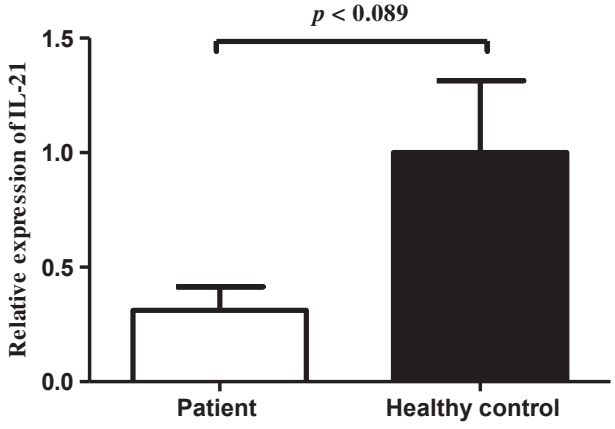

Figure 3 Level of mRNA for interleukin (IL)-21 was detected by quantitative real-time polymerase reaction. Gene expression was normalized to β-actin mRNA levels in each sample. The *p* value was calculated using nonparametric Mann–Whitney test and is not significant at $p < 0.05$.

expression of IL-21R is significantly increased in RA patients compared with controls. IL-21 is a pleiotropic cytokine influencing $CD4^+$, $CD8^+$ T, B, and NK cells.[22,34,35] Phenotypic observations showed that IL-21R is predominantly expressed in the cell surface of $CD4^+$ cells[19] and is not found on nonlymphoid cells in physiologic situations.[36] It has been shown that IL-21R is highly expressed in synovial fluid, cartilage, and bone of RA patients.[25] Fröhlich et al[37] displayed the over expression of IL-21R in inflamed joints of RA patients. Also Liu et al[38] showed that IL-21R expression was markedly higher in $CD4^+$ cells EAU mice. Other studies have shown that IL-21R is upregulated on $CD4^+$ cells.[12,34,39] In a similar study, Liu et al[39] compared the numbers of IL-21R-expressing cells in inflammatory bowel disease patients who took different medicines (e.g., sulfasalazine and corticosteroids) and found that these medicines did not have an impact on IL-21R expression. These observations suggest that IL-21R may be effectively involved in RA progression.

We examined the IL-21 expression in RA patients and found results provided no evidence of significant difference at the mRNA level using TaqMan real-time PCR in RA patients compared to controls. IL-21 has been observed to be effective in a variety of disease conditions.[40] Niu et al[41] showed that IL-21 was produced at high levels in the synovial fluid and serum of RA patients and found a possible role of IL-21. In contrast to the study of Jüngel et al,[25] Andersson et al[42] detected significant higher mRNA level for IL-21 in RA synovial tissues. Additionally Geri et al[43] found a remarkably increased serum level of IL-21 in peripheral blood from patients with Behçet disease. Their study showed that IL-21 could increase the number of Th17 cells and could suppress Treg cells by decreasing FoxP3 expression.[43] Jang et al[44] showed that IL-21 could produce a positive autocrine feedback regulating homeostasis of activated $CD4^+$ cells; therefore, subsequently it might play an essential role in the development of autoimmune arthritis. Fantini et al[45] explored high levels of IL-21 expression in inflammatory bowel disease, inhibiting the induction and expansion of Treg cells. Because a large quantity of patients respond or display little response to treatment,[46] understanding critical roles of various proinflammatory cytokines in the pathogenesis of autoimmune diseases, especially RA, has led to new therapeutic innovations.[47] In this respect, neutralizing of IL-21 activity with an IL-21R−Fc fusion protein could prevent interferon-γ,[26,48] IL-6[25] and increase the expression of FoxP3.[49] Carbone et al[50] indicated that blockade of the IL-6R in RA patients with tocilizumab is associated with a decreased production of IL-21 by memory/activated CD4 T cells. In a correlated study, the effective amount of the IL-21R−Fc fusion protein required to induce a significant effect was reported as 50−100 μg/mL.[30] In another study, blockade of the IL-21 pathway with IL-21R-Fc regained the balance between TH17 cells and Treg cells.[43] Blockade of the IL-21R could ameliorate disease severity in animal studies. Therefore, targeting against IL-21R may be attempted as a potential therapeutic in the management of RA patients.[26,37,51]

In conclusion, this study demonstrated increased proportions of $IL\text{-}21R^+CD4^+$ cells in RA patients, which indicates that IL-21R may play a pivotal role in RA progression and represents a novel target for innovative therapy. Further investigations are necessary to confirm and expand the current results.

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