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Imbalance of Peripheral Blood T Helper Type 17 Responses in Patients with Vitiligo

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ABSTRACT

There is growing evidence to suggest that Th cells play pivotal roles in a variety of chronic inflammatory diseases, including vitiligo. However, the exact role of different subsets of Th cells in the pathogenesis of vitiligo is still a question. The purpose of present study was to determine the mRNA expression level of Th17 master transcription factor retinoic acid receptor-related orphan receptors gamma (ROR γ t) and cytokine mRNA and protein expression profiles of Th17 cells.

22 patients with vitiligo and 22 normal subjects were enrolled in the study. Gene expression profiles of freshly isolated peripheral blood mononuclear cells (PBMCs) were determined by quantitative real-time reverse transcriptase PCR (qRT-PCR). Plasma concentrations of IL-17A and IL-22 were also assayed using ELISA kits.

The results showed that ROR γ t, IL-17A and IL-22 mRNA expression were increased in patients remarkably compared to healthy controls ($p < 0.05$). Furthermore, plasma IL-17A and IL-22 levels were also higher in vitiligo patients versus controls ($p < 0.001$).

These data suggest that a deregulated Th17 adaptive immune response may contribute to the pathogenesis of vitiligo.

Keywords: Autoimmunity; Cytokines; Gene expression profiling; T lymphocyte; Vitiligo

INTRODUCTION

Vitiligo is one of the most prevalent pigmentation disorder, characterized by the partial or complete loss of melanin-forming cells. The etiology of disease is

unclear, though researchers suspect that a combination of genetic and environmental factors contribute to the development of inappropriate immune responses against melanocytes.¹

There is ample evidence, that both arms of the innate and adaptive immune responses, play a role in onset and progression of vitiligo.² Although, most attention has so far been focused on the adaptive immune elements specially T cells that seem to play an important role in disease pathogenesis.^{3,4} These cells

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are composed of functionally heterogeneous subpopulations and can be distinguished based on the expression of the specific cell surface markers, by their particular effector molecules and transcriptional regulators.⁵

Recent studies show a discrete population of CD4⁺ Th17 cells, named Th17 are associated with many human autoimmune disorders.⁶ These cells constitute approximately 1% of all human peripheral CD4⁺ T cells and exhibit distinct developmental requirements. They are characterized by the secretion of specific cytokines, in particular IL-17A and IL-22 and ROR γ t is the primary transcription factor that controls their differentiation.⁷ Although there is evidence that Th17 cells can play pathogenic roles in vitiligo, there is no clear consensus in the literature on this issue.⁸⁻¹⁰ As a result, identification of molecular mechanisms underlying lineage commitment of human Th17 cells in vitiligo patients will extensively clarify our insight into disease pathogenesis.

Thus, the objective of this project was, (1) to define Th17-specific pattern of gene expression (ROR γ t, IL-17A, and IL-22) in PBMCs of patients with vitiligo and healthy controls, (2) to explore plasma levels of IL-17A and IL-22 in patients and compare with those of healthy subjects, (3) analysis of relationships between variables measured in this study.

MATERIALS AND METHODS

Sampling

Blood specimens (5 mL) were collected in EDTA-containing tubes and processed for PBMC preparation using Ficoll density-gradient centrifugation (Pharmacia, Uppsala, Sweden). The separated plasma was also stored at -80°C until analysis. A total of 22 patients admitted to dermatology outpatient clinic who were diagnosed with vitiligo formed the study group. One patient (4.5%) had segmental vitiligo, 17 (77.3%) had generalized vitiligo, and 4 (18.2%) had whole body vitiligo.

All the patients were drug-naïve at the time of testing or were off medication for at least three weeks of the study. Treatment-experienced patients had received previous therapy with topical steroid (n=1) and tacrolimus as topical immunomodulator (n=2). All patients had inactive or stable vitiligo with spontaneous disappearance of hypopigmented skin lesions and or no appearance of one or more new lesions or spreading of

older lesions for at least 3 months

The mean age of the patients (male 11, female 11) was 33.91±9.86 years and the mean disease duration was 14.36±11.20 years. Patients with inflammatory/autoimmune disorders were excluded from the study.

Age- and gender-matched 22 healthy subjects with no history of underlying malignancy, autoimmune or inflammatory diseases were included as the control group. They consisted of 8 men and 14 women with a mean age of 39.91±14.52 years. All participants gave informed written consent before enrollment to the study. The protocol was approved by the Research Ethics Board at Tehran University of Medical Sciences.

Quantitative Real-time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) Assay

Total RNA was extracted from PBMCs using RibospinTM (GeneALL, Seoul, South Korea) according to the manufacturer's instructions. The quantity and quality of purified RNA solutions were determined by Nanodrop spectrophotometer (Thermo Scientific, USA) and agarose gel electrophoresis, respectively. cDNA synthesized from mRNA of patients and controls using the first strand cDNA synthesis kit (Fermentas, Germany) and was used for amplification of target regions. qRT-PCR were performed with a Rotor-Gene 6000 instrument (Corbett Life Science, Australia), using SYBR Premix EX Taq II (Takara, Japan).

OLIGO software (National Biosciences, USA) was used for designing oligonucleotide primers and purchased from TAG Copenhagen (Denmark). PCR primer sequences used for quantification of the 3 candidate genes are indicated in Table 1. For the normalization of qPCR data, the expression of β -actin was used as the internal reference, as previously described.¹¹

Each reaction was performed in 10 μ L with 5 μ L SYBR Premix, 1 μ L cDNA at a concentration of 200 ng/ μ L, 0.5 μ L of forward and reverse primers, and 3 μ L double distilled water.

PCR conditions were 2 minute at 95°C followed by 38-45 cycles of 5s at 95°C and final step of 20s at 62°C for ROR γ t, 45s at 57°C for IL-17A, 45s at 60°C for IL-PCR conditions were 2 minute at 95°C followed by 38-45 cycles of 5s at 95°C and final step of 20s at 62°C for ROR γ t, 45s at 57°C for IL-17A, 45s at 60°C for IL-22, and 20s at 63°C for β -actin. Relative expression values

Th17 Cells in Vitiligo

Table 1. Primer sequences of T helper type 17 (Th17)-related genes used in the real-time reverse transcriptase PCR to investigate imbalance of Th17 responses in patients with vitiligo

ROR γ t	F: 5'AGGAAGTCCATGTGGGAG3' R: 5'AGCACAATCTGGTCATTCTGG3'
IL-17	F: 5'CTCATTGGTGTCACTGCT3' R: 5'CGGTTGTAGTAATCTGAGG3'
IL-22	F: 5'CAACAGGCTAAGCACATGTCATATTG3' R: 5'CAGGCATTTCTCAGAGACATAAACAG3'
Beta-actin	F: 5'AGACGCAGGATGGCATGGG3' R: 5'GAGACCTTCAACACCCCAGCC3'

F: Forward primer, R: Reverse primer, ROR γ t: Retineic-acid-receptor-related orphan nuclear receptor gamma, IL-17: Interleukine-17, IL-22: Interleukine-22

were then calculated by subtracting the threshold cycle (Ct) value of β -actin from the Ct value of the sample (Δ Ct). Higher Δ CT values represent lower mRNA levels.

Cytokines Assay

Circulating levels of IL-17A and IL-22 in plasma samples of individual participants were determined by sandwich enzyme-linked immunosorbent assay method according to the manufacturer's instructions (Bender Med Systems, San Diego, California, USA).

Statistical Analysis

Data analysis was done with the SPSS, version 11.0 (SPSS, Inc., Chicago, IL, USA). Statistical comparisons were performed by the T-tests or Pearson test. Difference with a *p*-value less than 0.05 was considered significant. Results are expressed as means \pm SD.

RESULTS

Expression of Th17-Related Genes in Patients with Vitiligo

Gene expression levels were measured in PBMCs from patients with vitiligo and healthy controls. The comparison of mRNA expression of Th17-related genes between the patient and control groups are shown in Figures 1-3. There was a significant difference in mRNA expression of ROR γ t (*p*=0.038), IL-17A (*p*=0.027) and IL-27(*p*=0.04) between the groups. In general, patients had a higher expression of Th17 cells related transcription factor (ROR γ t) and cytokines (IL-17A and IL-22) than the control group.

Circulating Cytokine Levels in Patients with Vitiligo

As seen in Figure 4, the levels of IL-17A and IL-22 were significantly higher in plasma taken from patients with vitiligo than in normal subjects (*p*<0.001).

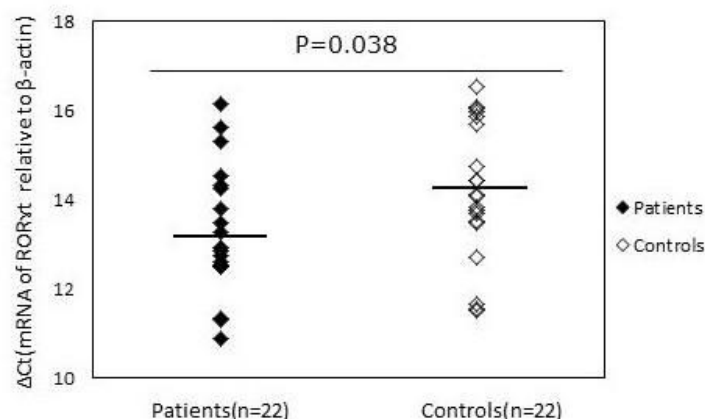


Figure 1. Detection of ROR γ t mRNA in peripheral blood mononuclear cells from patients with vitiligo and healthy persons by reverse transcription-quantitative polymerase chain reaction. ROR γ t mRNA levels were significantly higher in patients than normal persons. Please note that a higher Δ Ct value corresponds to a comparably lower expression level.

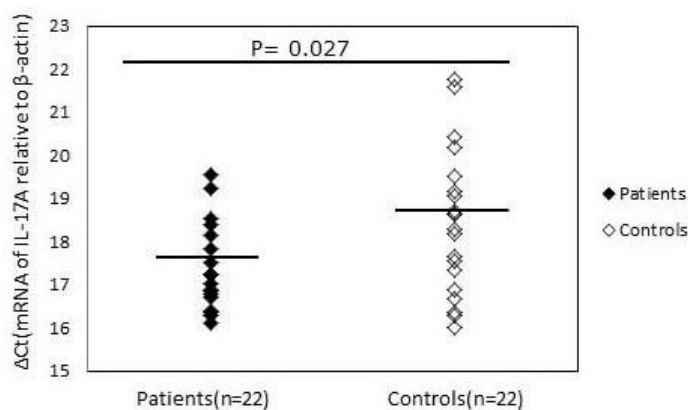


Figure 2. Detection of IL-17A mRNA in peripheral blood mononuclear cells from patients with vitiligo and healthy persons by reverse transcription-quantitative polymerase chain reaction. IL-17A mRNA levels were significantly higher in patients than normal persons. Please note that a higher Δ Ct value corresponds to a comparably lower expression level.

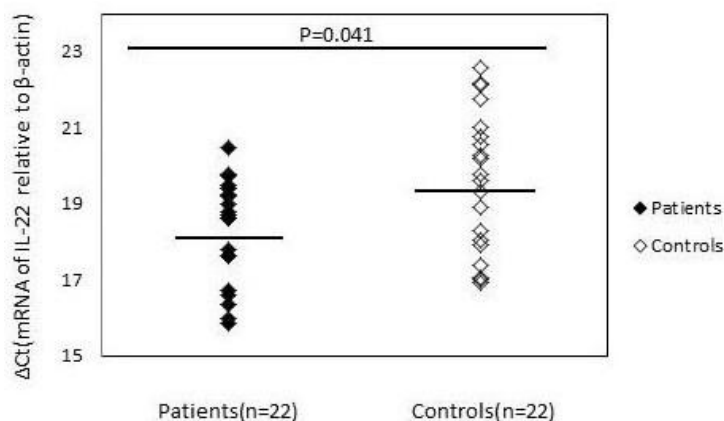


Figure 3. Detection of IL-22 mRNA in peripheral blood mononuclear cells from patients with vitiligo and healthy persons by reverse transcription-quantitative polymerase chain reaction. IL-22 mRNA levels were significantly higher in patients than normal persons. Please note that a higher Δ Ct value corresponds to a comparably lower expression level.

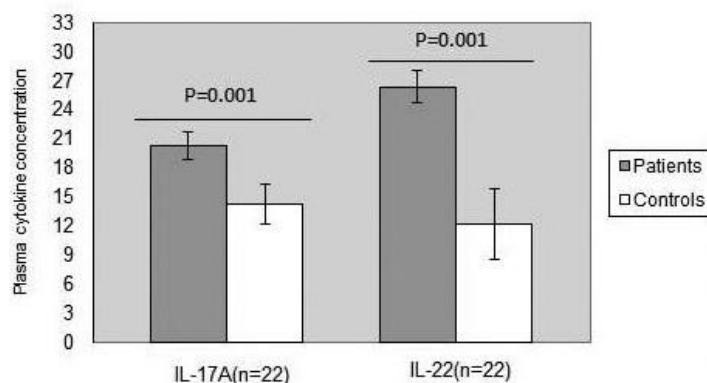


Figure 4. Detection of IL-17A and IL-22 in samples obtained from subjects with vitiligo and healthy subjects, using enzyme-linked immunosorbent assay. Circulating plasma levels of IL-17A and IL-22 were significantly higher in patients compared with the control group.

Correlation Analysis

The mRNA expression of ROR γ t correlated positively with mRNA expression of IL-17A ($r=0.678$, $p=0.001$) and IL-22 ($r=0.483$, $p=0.023$). Statistical analysis indicated that in patients, IL-17A mRNA level was significantly correlated with IL-22 mRNA level ($r=0.758$, $p=0.001$). There was also good correlation between IL-17A and IL-22 mRNA expression and plasma IL-17A and IL-22 secretion respectively ($p<0.001$).

DISCUSSION

Th17 cell is a newly recognized population of IL-17-producing CD4⁺ Th cell and is an important arm in the progression of inflammatory and autoimmune diseases. The identification of Th17 cells provides new insights into cellular mechanisms that are responsible for the initiation and progression of the autoimmune process. For instance, they have been implicated in different types of autoimmune diseases that were previously considered to be pure Th1-mediated responses, including psoriasis and multiple sclerosis.^{12,13} However, inappropriate over activation of Th17 responses do not occur in all immune or inflammatory disorders.¹⁴

These cells are distinguished by distinct developmental pathways and unique biologic functions.^{15,16} Therefore, quantitative gene expression analysis of cytokines and transcription factors involved in differentiation and effector function of Th17 cell subset may help to better understand, the immunopathological responses of vitiligo.

The first part of this study, hence, was devoted to assess the expression pattern of ROR γ t as the master regulators of Th17 lineage commitment, in PBMCs of patients with vitiligo. The results from present study showed that the level of ROR γ t mRNA was increased significantly in patients compared to normal controls. To the best of our knowledge, this is the first report for expression analysis of ROR γ t in PBMCs of patients with inactive vitiligo. This result is in line with the previous studies that have demonstrated increasing numbers of Th17 cells in the patient's skin samples.¹⁷ However, there has been a report about the frequency of Th17 cells in the peripheral blood of vitiligo patients. In this trial, flow cytometry was used to assess frequencies of Th17 cells in PBMCs from 5 patients with vitiligo and 25 healthy donors and the results

showed no statistically significant difference between the two groups.¹⁸ These opposing findings may be the result of several factors, such as treatment conditions, different experimental techniques and different sample size for experiments.

The next step of this study was used to identify the genes expression pattern of Th17-related cytokines. Moreover, circulating levels of IL-17A and IL-22 proteins were measured in the plasma collected from peripheral blood draws.

Our results indicated that the expression of IL-17A and IL-22 transcripts were significantly increased in PBMCs of patients with vitiligo compared to normal controls. Moreover, the plasma levels of examined cytokines in patients with vitiligo were significantly higher than that of healthy subjects. These results are consistent with available experimental results, indicating increased expression of IL-17A and IL-22 transcripts and their elevated serum levels in patients with vitiligo.^{9,19,20} However, there is no agreement among studies that investigated IL-17 and IL-22 levels between vitiligo patients and controls. For instance, Basak et al. were unable to detect differences in the amount of blood serum levels of IL-17 in vitiligo subjects versus controls.²¹ Moreover, Shi et al. reported low expression of IL-17 in experimental model of vitiligo.¹⁰ Many different variables such as age of disease onset, involvement of the body area, treatment and course or type of disease may influence the outcome of these various experiments.^{10,21,22}

Moreover till now there is no comprehensive understanding of the biological significance of increased Th17-related cytokine with respect to the various immunological changes triggered and mediated by them. Nonetheless, several investigators have observed a quantitative relationship between cytokine production and induction or maintenance of vitiligo. The effector cytokines of Th17 cells, act in many target cells and can provide a number of different functions. For instance, expression of microphthalmia transcription factor (MITF) and melanogenesis-related molecules can be down-regulated by Th17 factors IL-22 and IL-17A in cultured melanocytes.^{17,23} Moreover, Th17 cells can directly (via IL-17A) or indirectly (by adjacent lesional fibroblasts and keratinocytes) down-regulate melanogenic activity.²³ IL-17A can stimulates the production of multiple cytokines including IL-6.²⁴ On the one hand, this cytokine play crucial roles in determining Th17 lineage commitment.²⁵ On the other

hand, it is able to inhibit melanocyte activity.^{26,27} Therefore, autoimmune derangement of melanocyte might be the consequence of an increased Th17 activity and their products. Our findings provide some evidence in support of the newly proposed concept on vitiligo pathogenesis. Based on this notion, the downregulation of melanocyte activity depend on perturbed balance of the local cytokine network.²⁸

In this scenario, Th17-secreted IL-17A acts as an initiator for the production of inhibitory cytokines from dermal fibroblasts and keratinocytes. It can also repress melanocyte activity and may induce destruction of melanocytes. Increased expression of IL-17A has also provided new insights into the other contributing mechanisms that have been suggested to explain the pathogenesis of vitiligo. For instance, IL-17A can potently stimulate the chemokine CCL20 production that promote recruitment of cytotoxic CD8+ T cells from the systemic circulation into peripheral tissues.²⁹ It must be mentioned that cytotoxic melanocyte antigen-specific CD8+ T cells play a major role in autoimmune destruction of melanocytes in patients with vitiligo.³⁰ Therefore, chemokine-dependent attraction of CD8+ T cells by IL-17 may be important in disease pathogenesis.³¹

The expression of molecules such as E-selectin, P-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 that mediate leukocyte-endothelial cell adhesion are also stimulated by IL-17.³² Moreover, keratinocytes as the most abundant cells in the epidermis produce several chemokines under the influence of IL-17 and promote influx of T cell, neutrophil, macrophage(MΦ), and dendritic cell.³³ It has been shown that the accumulation of MΦs and T cells is accompanied by the loss of melanocytes.³⁴ Furthermore, the influx of neutrophil promotes production of several reactive oxygen species (ROS) that may contribute to melanocyte destruction.³⁵ These results show a possible relationship between oxidative stress(as a popular hypothesis concerning emergence of vitiligo)³⁶ and Th17 cell products.

This study had some limitations which have to be pointed out. First, we did not evaluate gene and protein expression markers of Th17 cells in the response to medical treatments. Another limitation was the absence of any experimental design for the assessment of gene expression patterns of Th17 cells in the skin lesions of patients with vitiligo. Therefore, further study is

required to explain exact mechanism linking Th17 cells and immunologic processes in vitiligo patients.

The present paper indicates the increased expression of critical transcription factor for Th17 development and their related cytokines in PBMCs of patients. These data constitute more evidence for the potential role of Th17 cells in the pathogenesis of vitiligo. However, further studies require to be done to explain the dysregulated mechanism of Th17 activation in vitiligo, and its impacts on the other immune and non-immune cells of patients.

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Th17 Cells in Vitiligo

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