Evaluation of IL-17 Producing Memory Regulatory and Effector T Cells Expressing CD26 Molecule in Patients with Psoriasis

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ABSTRACT

Memory regulatory T cells (Tregs) has been demonstrated to produce IL-17 in Psoriasis. Forkhead box P3 (Foxp3) has been demonstrated not to be reliable marker to evaluate Treg cells. Effector CD4+T cells also express Foxp3 after activation. Human T helper-17 cells (Th-17) express high level of surface CD26, while regulatory T cells are CD26 negative or low and this phenotype is stable even after activation of Treg cells. In this study, we aimed to analyze IL-17 producing Treg cells using CD26.

Memory T cells were isolated from 10 patients with psoriasis and 10 controls. Ex vivo stimulated IL-17 producing regulatory (Forkhead Box P3 (Foxp3)+CD25+CD26-/low) and effector (Foxp3+CD25+CD26hi) memory T cells were analyzed by flow cytometry. IL-23, IL-6, TNFα, TGFβ and IL-17 cytokine levels were also evaluated.

No significant difference in IL-17+ memory regulatory T cells was seen between patients and controls (p=0.19). A significant decrease in the percentage of IL-17 producing CD26hi effector memory T cells was observed in patients (p=0.04). However, the percentage of these cells was not different between patients with mild or severe form of psoriasis compared to controls (p=0.13). We could not find any significant difference regarding IL-23, IL-6, TNFα, TGFβ and IL-17 cytokine levels in plasma and cell culture supernatant samples between patients and controls.

Taken together, our results showed a reduced IL-17 producing effector memory CD26hi T cells in patients with psoriasis compared to controls. However, IL-17 producing memory regulatory CD4+T cells of patients showed no significant difference from that of controls.

Keywords: CD26; Foxp3; Interleukin-17; Psoriasis; Regulatory; T-lymphocytes

INTRODUCTION

Psoriasis is a chronic, inflammatory and autoimmune skin disorder,1,2 with prevalence rate
between 0.9% in the USA and 8.5% in Norway. Although the etiology of psoriasis is unclear, the clinical studies and experimental models have provided evidence indicating that psoriasis is a T cell-mediated disease in which Th-1, Th-17 and Th-22 and their related pro-inflammatory cytokines have a fundamental role in its pathogenesis. Furthermore, regulatory T cells (Tregs) isolated from psoriatic lesions and blood of patients have abnormalities in frequency and function.

Regulatory T cells have an important role in maintaining self-tolerance and homeostatic immune responses. Initially, these cells were detected with the CD4+CD25+phenotype, but discovery of transcription factor called Foxp3 resulted in more accurate detection of regulatory T Cells. Foxp3 is a transcription factor that acts as a master regulator for the development and function of regulatory T cells. There are controversial results with respect to stability of Foxp3 expression and plasticity of Foxp3 regulatory T cells. Recent studies have indicated that plasticity in regulatory T cells occurs and these cells can differentiate to different types of effector cells and acquire inflammatory phenotype. IL-17 producing regulatory T cells have been identified in some inflammatory disorders like psoriasis and inflammatory bowel disease (IBD). For the first time, Foxp3+/IL-17+ cells were observed in mice and in the subsequent studies it was found that a portion (4-8%) of regulatory T cells in peripheral blood samples of healthy subjects produce IL-17 under ex-vivo stimulation. Other studies, however, showed that the Treg cells were stable in Foxp3 expression and rejected the plasticity in Treg cells. In another study, it was demonstrated that the plasticity originated from a small population of non-Treg Foxp3+ T cells. A few studies showed that IL-17 producing regulatory T cells maintain their suppression function. Otherwise, a study demonstrated that IL-17/Foxp3 regulatory T cells contribute to the pathogenesis of rheumatoid arthritis (RA). It has been suggested that this plasticity along with functional defect in regulatory T cells may be involved in psoriasis pathogenesis.

Due to the absence of definite markers to identify regulatory T cells, such data interpretation is problematic. More evaluation is needed to confirm that IL-17/Foxp3 cells are only regulatory cells or contaminated with non-regulatory cells. In most studies the CD4+CD25+ Foxp3+ cells were considered as regulatory T cells. Meanwhile, activation of T cells leads to induction of Foxp3. CD25 is also expressed at high level on activated effector T cells, while activated regulatory T cells exhibit variable expression level of CD25.

CD26 (a member of serine proteases family with dipeptidyl peptidase IV activity) is widely expressed on immune cells, predominantly on memory T cells and up-regulated following stimulation. CD4+ T cells subsets display different levels of CD26 expression. Human T helper-17 cells (Th-17) express high level of surface CD26, while regulatory T cells are CD26 negative or low.

The aim of the present study was to evaluate the IL-17 producing regulatory and effector memory T cells (IL-17+Foxp3+CD25+CD26low and CD26+ memory T cells, respectively) from patients with psoriasis following ex-vivo stimulation. Also, to investigate the inflammatory responses that can affect regulatory T cells differentiation, the level of IL-23, IL-6, TNFα, TGFβ and IL-17 cytokines were monitored in plasma and cell culture supernatant samples by ELISA.

**MATERIALS AND METHODS**

**Patients**

10 patients with chronic plaque-type psoriasis (30% male and 70% female) were included in this study. All patients were referred to Skin and Stem cell Research Institute of Tehran university of Medical Sciences from April 2016 through February 2017. 10 sex and age matched healthy subjects with no history of skin or autoimmune disorders were included as controls. This study was approved by Ethics committee of Tehran university of Medical Sciences (93-04-30-27699-136553). All participants were informed about the purposes of the study, then an informed consent was obtained. Clinical evaluation was performed by a dermatologist. Clinical severity of psoriasis was scored by psoriasis area and severity index (PASI). Based on the PASI scoring, patients were classified into mild (PASI<10) and moderate to severe (PASI>10). A clear inclusion and exclusion criteria were considered for selection of patients. In this regard, Patients did not use any topical/systemic anti-inflammatory or Immunomodulatory treatments as well as anti-oxidant supplements for at least 4 weeks before sampling. The exclusion criteria were as follows: had history of liver and kidney disease,
malignancies, thyroid disorders, smoking and alcohol consumption and/or in the cases of comorbidity with other autoimmune–inflammatory condition including diabetes, RA, multiple sclerosis (MS).

Isolation of Memory CD4+T Cells

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient sedimentation from whole blood samples using Lymphodex (Inno-Train, Germany). Memory CD4+CD45RO+T cells were purified from PBMCs by negative selection using magnetic cell sorting kit (Miltenyi Biotec, USA) according to instruction manuals (purity greater than 94%).

Phenotypic and Intracellular Cytokine Assay

Fresh isolated memory T cells (1x10^6 cells/mL) were cultured for 48h in RPMI 1640 (Gibco-USA) containing 10% (V/V) FBS (Biosera - France) and 1% Penicillin-Streptomycin Solution (Biosera – France) and stimulated with soluble anti-human CD3/CD28 (0.1 µg/mL) (Mabtech, Sweden). Recombinant human IL-2 (rhIL-2, 25U) (Peprotech, USA) was added after 24 hours. To evaluate the IL-17 producing cells following 48 hours, the cells were stimulated with phorbol-12-myristate 13-acetate (PMA, 10 ng/mL) (CAS 16561-29-8) and ionomycin (500 ng) (CAS 56092-82-1) in the presence of Brefeldin A (5 µg/mL, Bio Legend) for 5 hours. Then, the cells were analysed by flow cytometry (BD FACSCanto™ II, US). Briefly, 1x10^6 cells were washed and stained using recommended amount of anti-human CD25- Alexaflour 647 and CD26- Perpcy5.5 monoclonal antibodies (mAb) (Biolegend, USA) for 20 minutes in 4°C. Afterward, the cells were fixed and made permeable with Fix and perm reagent (Biolegend, USA) following instruction manuals and intracellular staining for Foxp3 and IL-17 was performed with anti-human FOXP3-Alexa Fluor 488 and IL-17A-PE mAbs (Biolegend, USA). Approximately 250,000 events were acquired for each sample. Unstained cells, isotype and single stain controls were used for setting the flow cytometer. Flow cytometry data were analyzed using Flowjo software (version 7.6).

Cytokine ELISA Assay

The levels of TNFα, IL-23, IL-6, TGF β and IL-17 cytokines were measured in plasma and cell culture supernatants by Sandwich enzyme-linked immunosorbed assay (ELISA) kits (affymetrix eBioscience, USA) according to manufacture instructions. Memory T cells (1x10^6 cells/mL) were expanded using anti-CD3/CD28 (0.1 µg/mL) and after 48h, the culture supernatants were collected and stored at −70°C until their analysis. The minimum detection limits used in assays were 4 pg/mL for TNFα, 15 pg/mL for IL-23, 2 pg/mL for IL-6, 8 pg/mL for TGFβ and 1.6 for IL-17.

Statistical Analysis

Statistical analysis was performed using SPSS (IBM SPSS statistics version 22, USA). Independent Samples T Test or Mann Whitney U Test was used to compare two Independent groups. One Way-ANOVA or Kruskal Wallis was used to compare K Independent groups. A p values less than 0.05 were considered statically significant.

RESULTS

Study Population

Demographic and clinical data of patients are summarized in Table 1.

83% of patients with moderate to severe form were overweight (30> BMI ≥25 kg/m²). Eighty percent of patients had a positive family history (first degree and second degree relatives) of psoriasis and or autoimmune disorders. All of patients were categorized as early onset psoriasis. A history of sleep problem was reported only by two patients who suffered from mild type of psoriasis.

CD26low is Representative of Foxp3+ Cells in PBMCs

For evaluation of Foxp3 expression in memory CD26 negative or positive T cells, PBMCs were stained with anti-CD4, CD45RO, Foxp3 and CD26 monoclonal antibodies and analyzed by Flow cytometry. As depicted in Figure 1A, our results showed that CD26low memory CD4+T cells expressed Foxp3. While, CD26hi memory CD4+T cells were Foxp3 negative. Interestingly, analysis of Foxp3+T cells in anti CD3/CD28 activated CD26low and CD26hi memory CD4+T cells revealed that Foxp3+cells present in both groups. (Figure 1B).
IL-17 Producing Foxp3\(^+\)CD25\(^-\)/low (Regulatory) and Foxp3\(^+\)CD25\(^+\)CD26\(^{hi}\) (Effector) Memory CD4\(^+\)T Cells

In order to measure the IL-17 producing regulatory and effector memory CD4\(^+\)T cells, the activated memory T cells were gated based on forward (FSC) and side scatter (SSC) parameters. Afterwards, the cells were divided into regulatory and effector memory T cells based on CD25 and CD26 expression and Foxp3\(^+\)IL-17 cells were analyzed in each subsets. Accordingly, Foxp3\(^+\)CD25\(^-\)/low and CD26\(^{hi}\) cells considered as regulatory and effector memory CD4\(^+\)T cells, respectively (Figure 2A).

Figure 1. Activated memory regulatory (CD26\(^{-}\)/low) and effector (CD26\(^{hi}\)) CD4\(^+\)T cells express Foxp3 following ex-vivo stimulation using anti-CD3/CD28. (A) Human PBMCs were isolated from peripheral blood and stained for surface CD4 (anti-human CD4-APC-Cy7), CD45RO (anti-human CD45RO-PE), CD26 (anti-human CD26-PerCP-Cy5.5) as well as intracellular Foxp3 (anti-human Foxp3- Alexafluor 488). Lymphocytes were gated based on forward (FSC) and side scatter (SSC) properties. After gating of CD4\(^+\)T cells, the CD4\(^+\)CD45RO\(^+\)T cells were gated. Then, Foxp3 expression was measured in CD26\(^{-}\)/low and CD26\(^{hi}\) subsets. Histograms show the frequency of Foxp3\(^+\) cells in each selected compartment. The blue lines represent the expression profile related to isotype controls, whereas the solid red lines indicate the percentage of Foxp3\(^+\) cells in each subpopulations. (B) FACS analysis of Foxp3 expression in CD26\(^{-}\)/low or CD26\(^{hi}\) compartment in activated memory CD4\(^+\)T cells. Memory CD4\(^+\)T cells were isolated from PBMCs and stimulated with anti-CD3/CD28 for 48 hours, then the cells were stained for surface CD26 and intracellular Foxp3.
IL-17’Memory CD4+ T Cells Expressing CD26 in Psoriasis

Figure 2: Ex vivo stimulated effector memory CD4+ T cells from patients with psoriasis show decreased IL-17 production compared to controls. To measure intracellular IL-17 producing regulatory or effector memory CD4+ T cells, following activation with anti CD3/CD28 mAb for 48 hours, the cells were stimulated with PMA, ionomycin in the presence of Brefeldin A for 5 hours. Then, the cells were subjected to surface and intracellular staining and analyzed by FACS. (A) Representative flow cytometry plots and gating strategy of IL-17-producing memory CD4+ T cells analysis. Summarized data showing the percentage of the IL-17 producing effector (B) and regulatory (C) memory CD4+ T cells in patients with psoriasis (n=10) compared with healthy controls (n=10), (p=0.04 and p=0.19, respectively). Data show Mean±SEM. Independent Samples T Test was used for analysis of data. p<0.05 was considered to be statistically significant.

Our data showed a significant decrease of IL-17’Foxp3’CD25’CD26+ memory CD4+ T cells in patients compared to controls (Mean±SEM; 16.5±2.7, n=10; 26.5±3.7, n=10, respectively, p=0.04) (Figure 2B). Nevertheless, the percentage of these cells showed no significant difference in patients with mild or moderate to severe type of psoriasis compared to healthy subjects (p=0.13).

However, no significant difference was found in the percentage of IL-17’Foxp3’CD25’CD26low memory CD4+ T cells between patients and controls (mean±SEM; 5±1.2 n=10; 7.4±1.2, n=10, respectively, p=0.19) (Figure 2C). According to PASI score, data were reanalyzed to identify any difference between patients with mild or moderate to severe types of psoriasis compared with controls. No significant difference was found in the percentage of these cells among patients with mild or moderate to severe form of psoriasis compared to controls. (p=0.35).
Table 1. Demographic and clinical data of the patients with psoriasis in the study on the IL-17 producing memory regulatory and effector T Cells expressing CD26 molecule

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients with psoriasis (n)</th>
<th>Control</th>
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<tr>
<td></td>
<td>Mild (4)</td>
<td>Moderate to Severe (6)</td>
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<tr>
<td>PASI</td>
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<td>&gt;10</td>
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<td>Age, years (Mean ±SD)</td>
<td>35.2± 9.6</td>
<td>28.3± 8.4</td>
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<td>Disease duration (Mean ±SD)</td>
<td>18.5 ± 3.1</td>
<td>13.5 ± 7.8</td>
</tr>
<tr>
<td>BMI (Mean ±SD)</td>
<td>20.2±1.7</td>
<td>26.7± 6 kg/m²</td>
</tr>
<tr>
<td>Family history:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Psoriasis</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Diabetes</td>
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<td>1</td>
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<tr>
<td>Thyroid disease</td>
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PASI: Psoriasis area and severity index, BMI: Body mass index

Figure 3. Ex vivo activated memory Foxp3^{hi} CD25^{+}/IL-17^{+} T cells from patients with psoriasis are decreased compared to controls. (A) For data analysis, activated memory cells were gated, then IL-17/CD25^{+} cells were investigated in Foxp3^{hi} subpopulation. (B) Bars represent mean percentage±SEM of memory Foxp3^{hi} IL-17^{+}/CD25^{+} T cells from patients compared with controls. *p=0.002 in patients (n=10) compared with controls (n=10) analyzed by independent sample T test.
Decreased Foxp3hi CD25+/IL-17hi Memory CD4+ T Cells in Patients with Psoriasis

In this part of work, IL-17 producing T cells in activated memory CD4+ T cells were measured using Foxp3 and CD25 markers. Following gating of activated memory CD4+ T cells, CD25+/IL-17hi cells were evaluated in gated Foxp3hi cells (Figure 3A). The results demonstrated a significant decrease in Foxp3hi CD25+/IL-17hi cells in patients compared with controls. (Mean±SEM; 22.7±3.8%, n=10; 37.8±4.7, n=10, respectively; p=0.02) (Figure 3B). However, no significant difference in frequency of these cells was observed among patients with mild or moderate to severe form of psoriasis compared to controls, (p=0.06).

Correlation between IL-17 Producing Regulatory and Effector Memory CD4+ T Cells and Disease Phenotype

Pearson’s correlation analysis was performed to identify the relationship between IL-17 producing regulatory or effector memory CD4+ T cells and age, BMI and disease duration. Our results showed no significant correlation between IL-17 producing regulatory and effector memory CD4+ T cells with age, BMI as well as disease duration (r=-0.182, p=0.6; r=0.05, p=0.8; r=-0.517, p=0.1)(r=0.08, p=0.8; r=-0.044, p=0.2), respectively. Furthermore, no significant difference in IL-17 producing memory T cells subsets and different clinical subtypes of psoriasis was observed (p=0.5 and p=0.7 for IL-17 producing regulatory and effector memory CD4+ T cells, respectively).

IL-23, IL-6, TGFβ, TNFα and IL-17 Cytokine Levels Not Different between Patients and Controls

The levels of IL-23, IL-6, and IL-17 were undetectable in plasma. Plasma concentrations of TNFα and TGFβ showed no significant difference in patients compared to controls (p=0.5 and p=0.3, respectively). We also found no significant difference of IL-23 (p=0.5), IL-6 (p=0.8), IL-17 (p=0.1), TNFα (p=0.8) and TGFβ (p=0.5) levels in cell culture supernatants between patients and controls (Figure 4).

Figure 4. IL-23, IL-6, IL-17, TNFα and TGFβ cytokines profile in plasma and cell culture supernatants from patients with psoriasis compared to controls: plasma and cell culture supernatant samples (1×10⁶ cells/mL) were collected and IL-23, IL-6, IL-17, TNFα and TGFβ cytokines levels were assessed by ELISA. Data represent the Mean±SEM or Median [Interquartile Range]. No significant differences in cytokine levels were seen in patients compared to controls, p>0.05.
DISCUSSION

Aberrant Th-17 response and suppression function of regulatory T cells have a major role in development of autoimmune and inflammatory disorders including psoriasis.7, 33, 34 Foxp3+/IL-17+ T cells were shown in the CD4+CD25hi T cell populations in peripheral blood and colitic tissues of patients with ulcerative colitis which had immunosuppressive function.35 Zaruhhi Hovhannisyan et al also identified the presence of IL-17 producing regulatory T cells with suppressive function in intestinal mucosa of IBD patients.15 Bovenschen HJ et al observed that CD4+CD45RA-CD25hi Treg cells from patients with psoriasis differentiate into IL-17 producing T cells under ex vivo stimulation. They also found that IL-17+/CD45RO regulatory T cells were found in skin lesions of patients with psoriasis.14

In most of the studies, regulatory T cells have been investigated using non-specific markers that share between regulatory T cells and activated effector T cells like Foxp3 and CD25.23,36 Recently, Francisco J. Salgado et al introduced a new marker to analyze regulatory T cells called CD26. Their results demonstrated that resting regulatory T cells display a negative or low expression of CD26 and this phenotype (CD26low) is kept stable for activated regulatory T cells following ex vivo stimulation.29 To date, CD26 was used as a negative marker to evaluate regulatory T cells in numerous studies.37,38

In the present study, we evaluated IL-17 producing cells using the CD26. Our primary results showed that CD26 is a helpful marker to evaluate activated regulatory T cells. As shown in Figure 1, activated CD26hi CD4+ T cells that are considered as effector T cells express a high level of Foxp3. It means that Foxp3 could not be considered as a reliable marker for analysis of activated regulatory T cells.

Our results regarding IL-17+ memory CD4+ T cells showed that IL-17+ cells were present in both regulatory and effector memory CD4+ T cell subsets. We found a significant decrease in IL-17+ effector memory CD26hi T cells of patients with psoriasis compared to controls. However, no significant difference in IL-17+ regulatory memory CD4+ T cells was observed between patients and controls.

Published results showed that the IL-17 producing regulatory T cells increased in patients with psoriasis. We reanalyzed our data without using of CD26. Our results revealed a significant decrease in IL-17 producing cells in memory Foxp3hi CD25+/IL-17+/CD4+ T cells of patients compared to controls. These results showed that the use of CD26 can help to better characterize the IL-17 producing CD4+ T cells.

Decrease in IL-17 producing CD26hi effector memory CD4+ T cells in patients with psoriasis needs further investigation. Infiltrated immune cells into the skin play fundamental role in pathogenesis of psoriasis (39). Recently, the results obtained from a study of the CD26low T cells revealed that CD26hi T cells subsets are naturally able to migrate toward tumor through increased expression of chemokine receptors (40). Therefore, this hypothesis can be raised that major number of IL-17 producing CD26hi memory CD4+ T cells in patients with psoriasis might be moved toward skin to involve the inflammatory response.

TNFα is an important inflammatory cytokine and recognized as a therapeutic target in psoriasis.41 Serum level of TNF in patients with psoriasis was observed to be higher than controls.42,43 Measurement of TNFα concentration in activated memory T cells supernatant and plasma showed no significant difference between patients and controls. TGFβ in combination with IL-6 and IL-23 involves in development of IL-17 producing T cells.44,45 IL-6 was shown to be increased in inflamed skin and serum of patients with psoriasis.42, 46 It was demonstrated that IL-6 involves in defective suppression function of regulatory T cells against effector T cells in psoriasis.47 Expression of IL-23 has been seen to be increased in inflamed skin of patients with psoriasis.48 Nockowski P et al reported increased level of serum TGFβ in patients with psoriasis.49 In consistent with our result, Zaher H et al observed that the serum concentration of TGFβ was non-significantly elevated in patients compared to controls.50 In our study, assessment of the level of IL-6 and IL-23 in cell culture supernatant showed no significant difference in patient compared to controls as well. The results regarding the IL-17 concentration in cell culture and serum are controversial. Priscilla Stela Santana de Oliveira et al and Li Zhang et al reported a significant increase in serum level of IL-17 in patients with moderate to severe type of psoriasis compared to controls.51,52 However, according to result of a study by Ozer Arican et al, serum level of IL-17 was not
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significant difference between patients and controls.42 In our study, plasma level of IL-17 was undetectable. The result of IL-17 assay in cell culture supernatant showed no significant difference between patients and controls. Due to the lack of significant difference in circulating IL-17 producing regulatory memory T cells and decreased in effector subset, our results with respect to cytokines levels in cell culture supernatants were completely logical.

In summary, we found no significant difference in IL-17 producing memory regulatory T cells between patients with psoriasis and healthy subjects. Our results showed that CD26 could be a useful marker for evaluation of the regulatory and effector memory T cells as well.

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