Frequency of Th9 Cells in Different Stages of Rheumatoid Arthritis

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ABSTRACT

Background and Objectives: T helper (Th) lymphocytes play a key role in the pathogenesis of autoimmune diseases. As a new subset of lymphocytes, Th9 is thought to be involved in a wide range of disorders including rheumatoid arthritis (RA). In this study, we evaluated frequency of Th9 and Th2 cells and its correlation with disease activity in patients with different stages of RA.

Methods: The frequency of circulating interleukin 9- and/or interleukin 4-producing $CD3^+CD8^-$ T cells was determined among 41 patients with established RA, 14 patients with very early RA (VERA) and 23 healthy controls by flow cytometry analysis. Then, correlation of cell frequencies with disease activity score 28 (DAS-28) was assessed. Serum levels of interleukin 6 and anti-citrullinated peptide antibodies were measured by enzyme-linked immunosorbent assay.

Results: Frequency of Th9 cells was significantly higher in RA patients compared to healthy controls ($P=0.009$). Moreover, mean percentage of circulating Th9 cells in patients with inactive VERA was significantly higher than that in those with active disease ($P=0.046$). In addition, mean percentage of Th9 cells had a negative correlation with the DAS-28 ($r=-0.568$, $P<0.05$). There was no significant correlation between the mean serum level of interleukin 6 and percentage of Th2 and Th9 cells ($P>0.05$).

Conclusion: Our results suggest that Th9 cells may have a potential role in RA initiation. Thus, targeting Th9 cells could be a promising strategy for advanced RA therapies.

Keywords: Rheumatoid arthritis, Th2 cells, Th9 cells.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease that can cause progressive inflammation in peripheral joints (1). It affects 0.5-1% of the world's population (2). Although diverse genetic, immunological and environmental factors are associated with development of RA, auto-reactive T lymphocytes play a particularly important role in the disease initiation and manifestation (3). The role of $CD4^+$ T cells in the pathogenesis of RA is well-established (4). According to the cytokine milieu, $CD4^+$ T lymphocytes may differentiate into diverse populations each of which may play a distinct role in immune responses (5). Th1 cells predominantly mediate immunity against intracellular pathogens, whereas Th2 cells are mainly involved in defense against extracellular parasites, asthma and allergens (6). Th17 cells take part in immunity against some extracellular bacterial and fungal pathogens (7). Although Th1 and Th17 cells are considered as main T cell subsets in RA pathogenesis (8), other T cell subsets such as Th2 and Th9 have also been proposed to be involved in autoimmunity (9). As newly described effector subsets, Th9 cells dominantly produce interleukin-9 (IL-9). These cells are involved in various disorders ranging from autoimmune diseases to allergic diseases (10, 11). As the signature cytokine in Th9 cells, IL-9 is increased in serum and synovial fluid of RA patients (12). It should be noted that IL-9 is an ambiguous and pleiotropic cytokine that can induce survival of different T cell subtypes via its receptor (13). Some studies have suggested the plasticity between Th2 and Th9 cells, and the fact that these subsets can change to each other depending on the microenvironments they are exposed to (14). The anti-citrullinated protein antibody (ACPA) is found in majority of RA patients and is valuable for diagnosis of the disease. Based on the presence of ACPA in serum, RA could be subdivided into two distinct subsets; ACPA-positive and ACPA-negative RA (1). ACPA-positive RA patients are more likely to have an aggressive disease phenotype with higher rates of erosive damage (15). The roles of inflammatory cytokines, Th2 and Th9 in different stages of RA are not thoroughly understood. Hence, we evaluated serum level of IL-6 and frequency of Th2 and Th9 cells in peripheral blood of healthy individuals and RA patients with different disease stages and activities.

MATERIALS AND METHODS

The study included 55 RA patients diagnosed by a rheumatologist based on the American College of Rheumatology/European League Against Rheumatism criteria (2010) (16). Peripheral blood samples were obtained from patients at Sayyad Shirazi Teaching Hospital, Gorgan, Iran. Based on the disease stages, the patients were divided into two subgroups: very early RA (VERA) and established RA. Disease duration in VERA patients was less than three months after the first signs of RA (17). VERA patients were not receiving any treatment. Disease duration of established RA patients was at least three months after the onset of early signs of RA. Established RA patients were receiving prednisolone and disease-modifying antirheumatic drugs (DMARDs) such as methotrexate, hydroxychloroquine and sulfasalazine. The patients were classified as having active or inactive disease according to the disease activity score of 28 joints (DAS-28) (18). None of the patients had chronic infection or inflammatory disease other than RA. Twenty-three age- and gender-matched healthy controls were also randomly selected from the same hospital. This study was approved by the ethics committee of the Golestan University of Medical Sciences (Code of Ethics: 31078693122410), and a written informed consent was obtained from all participants after fully explaining the study objectives and procedures.

Initially, 500 µL of diluted sodium-heparinized whole peripheral blood of each subject were cultured in 2 ml of RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (GIBCO, USA). Then, it was stimulated with phorbol myristate acetate (PMA, 40.5 µM, eBioscience, USA) plus ionomycin (670 µM, eBioscience, USA) for 5 hours in the presence of brefeldin A (5.3 mM) and monensin (1 mM, eBioscience, USA) at 37 ℃ and 5% CO_2. Later, 100 µl of activated cells were incubated with peridinin-chlorophyll protein complex-cyanine5.5-labeled anti-human CD3 (Biolegend, USA) and fluorescein isothiocyanate-labeled anti-human CD8a (Biolegend, USA). Because CD4-positive cells would turn into CD4-

negative cells after the stimulation with PMA, we used anti-human CD8 for surface staining and analyzed CD3$^+$CD8$^-$ cells instead of CD3$^+$CD4$^+$ cells (19). After elimination of erythrocytes using lysis buffer, cells were fixed and permeabilized with 1X intracellular fixation and permeabilization buffer (eBioscience, USA). For intracellular cytokine staining, the cells were mixed with phycoerythrin-labeled anti-human IL-9 (Biolegend, USA) and allophycocyanin-labeled anti-human IL-4 (Biolegend, USA). Isotype controls (Biolegend, USA) were used for confirmation of antibody specificity. The labeled, fixed cells were analyzed the same day for cytoplasmic cytokines with a 4-color BD Accuri C6 flow cytometer (Becton–Dickinson Bioscience, USA) followed by analysis with FlowJo 7.6 (Tree Star, USA). The profile of the CD3$^+$CD8$^-$T cells was defined by their ability to exclusively express IL-9 (Th9 cells) and IL-4 (Th2 cells).

Determination of the serum ACPA (IgG) antibodies was performed using a commercial enzyme-linked immunosorbent assay kit (Euroimmun, Germany). The ACPA of more than 5 U/ml was considered positive. Serum level of IL-6 was determined using commercially available enzyme-linked immunosorbent assay kits (Biolegend, USA). Presence of rheumatoid factor (RF) was assessed in all sera using the standard AVITEX slide latex agglutination test (Omega Diagnostics, UK).

All statistical analyses were carried out using SPSS version 22 and GraphPad Prism 5.04. The Kruskal-Wallis test was used to compare nonparametric means of multiple samples. P-values less than 0.05 were considered statistically significant. Positive r-values represent a positive correlation while negative r-values represent a negative correlation.

RESULTS

Overall, 55 RA patients with a mean age of 48.34 ± 2.62 years and 23 healthy controls with mean age of 41.78 ± 1.30 years were included in the study. Fourteen patients had VERA ($\leq$ 3 months in duration). Twenty-six patients had active disease with a DAS-28 of > 3.2, while 29 patients had quiescent disease (DAS-28 < 3.2). Demographic and clinical characteristics of patients and healthy controls are presented in table 1.

Table 1- Clinical characteristics of RA patients and healthy controls

Characteristics	Very early RA (n=14)	Established RA (n=41)	Healthy controls (n=23)	P-value
Age	50.64 ± 4.41 years	46.04 ± 1.79 years	41.78 ± 1.30 years	0.466
Sex (male/female)	4/10	1/40	3/20	0.093
BMI	18.92 ± 0.56	18.65 ± 1.12	21.11 ± 0.74	0.227
Frequency of anti-CCP positivity	8 (57.14%)	29 (70.73%)	0	-
Frequency of RF positivity	5 (35.71%)	19 (46.34%)	0	-
ESR	22.69 ± 5.18 mm/h	26.61 ± 2.34 mm/h	ND	-
DAS-28 score	3.91 ± 0.45	3.28 ± 0.27	ND	-

RA: Rheumatoid arthritis, BMI: Body mass index, CCP: Cyclic citrullinated peptide, RF: Rheumatoid factor, ESR: Erythrocyte sedimentation rate, DAS-28: Disease activity score using 28 joint counts, ND: Not determined. Data are presented as mean ± standard error.

Figure 1- Frequency of T cells and subpopulations in RA patients and healthy controls. The Kruskal-Wallis with Dunn-Bonferroni post hoc test was used to compare the means of samples. Horizontal lines show median frequencies. Mean percentage of CD3$^+$T cells (A) and CD3$^+$CD8$^-$T cells (B) differed significantly between VERA and established RA patients (**P<0.01).
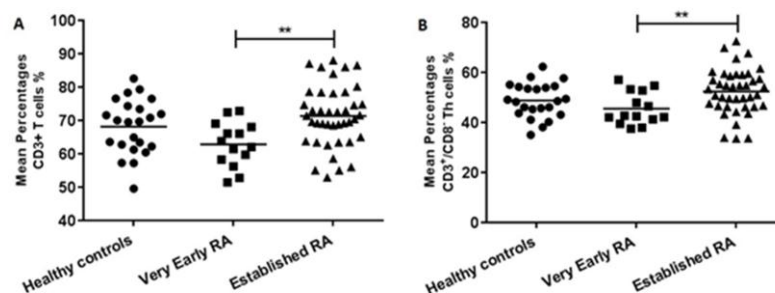
Frequency of T helper 9 cells but not Th2 cells was increased significantly in RA patients

As shown in Figure 2, there was no significant difference in the frequency of circulating Th2 cells between VERA patients (1.71 ± 0.43%), established RA patients (1.52 ± 0.45%) and healthy controls (1.41 ± 0.40%). Moreover, the mean percentage of circulating Th2 cells did not differ significantly between RA patients with and without active disease (Table 2).

As illustrated in Figure 2B, the mean percentage of circulating Th9 cells in VERA and established RA patients was significantly higher than that in the control group (P=0.009). In addition, the mean percentage of Th9 in established RA patients was not significantly different from that in VERA patients (P>0.05). There was no statistically significant difference in the mean percentage of circulating Th9 cells between RA patients with DAS-28 of >3.2 (1.47 ± 0.58%) and those with DAS-28 of <3.2 (1.59 ± 0.65%) (P>0.05). As shown in table 2, mean percentage of circulating Th9 cells in VERA patients with inactive disease was significantly higher than that in those with active disease (P=0.01). There was a negative correlation between the DAS-28 score and the frequency of Th9 subset (r=-0.568, P<0.05). In addition, there was a positive correlation between the frequency of CD8$^-$T cells and the Th9 subset (r=0.295, P=0.008).

The mean percentage of circulating Th2 and Th9 cells did not differ significantly between ACPA-positive and ACPA-negative RA patients (Table 3). Same finding was obtained when comparing VERA and established RA patients. Mean serum level of IL-6 in both VERA and established RA patients was significantly higher than in healthy controls (P<0.01). However, there was no significant correlation between level of IL-6 and percentage of Th2 or Th9 cells (P>0.05).

Figure 2- Flow cytometry analysis of IL-4 and IL-9 expression on gated CD3$^+$CD8$^-$ T cells. The Kruskal-Wallis with Dunn-Bonferroni post hoc test was used to compare the means of samples. Horizontal lines show median frequencies of the data. (A) Frequency of Th2 cells in RA patients and healthy controls. (B) Frequency of Th9 cells in RA patients and healthy controls (**P<0.01).
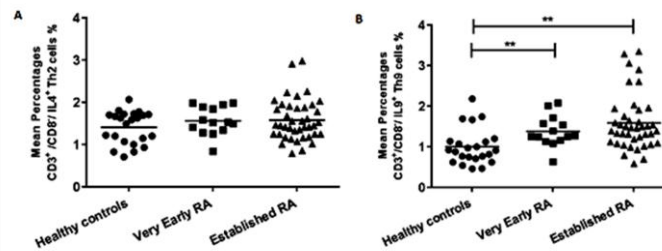

Table 2- Mean percentage of circulating Th2 and Th9 cells based on disease activity (DAS-28 score) in VERA and established RA patients.

Variable	DAS-28 > 3.2	DAS-28 < 3.2	P-value
CD3$^+$ CD8$^-$ IL-4$^+$ T cell in Established RA	1.60 ± 0.48	1.49 ± 0.44	0.32
CD3$^+$ CD8$^-$ IL-4$^+$ T cell in VERA	1.59 ± 0.26	1.93 ± 0.62	0.26
CD3$^+$ CD8$^-$ IL-9$^+$ T cell in Established RA	1.53 ± 0.69	1.60 ± 0.66	0.46
CD3$^+$ CD8$^-$ IL-9$^+$ T cell in VERA	1.17 ± 0.25	1.77 ± 0.57	0.01

DAS-28: Disease activity score using 28 joint counts, CD3$^+$CD8$^-$IL-4$^+$: Th2 cells, CD3$^+$CD8$^-$ IL-9$^+$:Th9 cells. Data are presented as mean ± standard deviation.

Table 3- Mean percentage of circulating Th2 and Th9 cells among ACPA-positive and -negative RA patients.

Variable	Anti-CCP (+)	Anti-CCP (-)	P-value
CD3$^+$ CD8$^-$ IL-4$^+$ T cell in Established RA	1.56 ± 0.44	1.44 ± 0.48	0.87
CD3$^+$ CD8$^-$ IL-4$^+$ T cell in VERA	1.79 ± 0.53	1.60 ± 0.26	0.41
CD3$^+$ CD8$^-$ IL-9$^+$ in T cell Established RA	1.66 ± 0.72	1.51 ± 0.31	0.37
CD3$^+$ CD8$^-$ IL-9$^+$ T cell in VERA	1.35 ± 0.57	1.77 ± 0.57	0.13

CCP: Cyclic citrullinated peptide, CD3$^+$CD8$^-$IL-4$^+$: Th2 cells, CD3$^+$CD8$^-$ IL-9$^+$:Th9 cells. Data are presented as means ± standard deviation.

DISCUSSION

Th2 and Th9 cells are subsets with similar properties in immunity against parasitic infections and induction of allergic diseases (20). More careful analyses showed that Th9 cells are distinct from Th2 cells and can be differentiated from low numbers of activated CD4$^+$T cells in the presence of transforming growth factor β and IL-4 or thymic stromal lymphopoietin (21, 22). Depending on the combined cytokine milieu in which Th9 lymphocytes are generated, these cells can be differentiated by several non-specific transcription factors (23). Nevertheless, few studies have evaluated the role of these cells in patients with different stages of RA (24). In numerous experimental autoimmune disease models, Th9 cells have been found as primary contributors to the inflammatory progression (25). Previously, it had been indicated that Th9 cells are increased in RA patients compared to healthy controls (11, 26, 27). Our results confirmed the findings of earlier studies regarding the significant increase in the frequency of circulating Th9 cells in RA patients. Furthermore, these findings indicated that the percentage of Th9 cells in the inactive group of VERA patients was significantly higher than that in the active group. Thus, Th9 cells seem to have a role in the disease initiation before the activation phase. These findings may be useful for achieving a successful disease remission by initiating an effective treatment in the first six months of RA onset (28). Established RA patients receiving therapy had more Th9 cells (1.62%) than those who were not taking any medication (1.37%), but this difference was not statistically significant (P=0.074). According to recent studies, Th9 cells are involved in the organization of lymphoid centers in synovial tissue and are important in the induction of joints deformities in RA patients (29). It has been proposed that Th9 cells may be also involved in RA pathogenesis by helping B cells produce RA-related autoantibodies, such as ACPAs (24, 30). However, we found no significant difference in the mean frequency of circulating Th9 cells between ACPA-positive and -negative RA patients. Ruocco et al. indicated that activation of Th9 cells mediated

by plasmacytoid dendritic cells could have an immunoregulatory role in the Th17 cells-generated inflammation in multiple sclerosis (31). Nonetheless, the exact role of Th9 might rely on the tissue microenvironment and cytokines present in the milieu (32). Interleukin 6 is a critical cytokine in autoimmune diseases, including RA (33). The results of our study showed that the serum level of this pro-inflammatory cytokine was significantly higher in RA patients than in healthy controls. However, we failed to find a significant correlation between IL-6 and frequency of Th9 lymphocytes. Previous studies revealed that IL-6 does not influence IL-9 expression in naive CD4$^+$T cells (34). Thus, Th9 cells and IL-6 seem to be involved in separate inflammatory pathways in patients with RA. It has been suggested that Th2 may be associated with early steps of RA pathogenesis (6), but in line with a study by Chen et al. (35), we found no significant difference in the frequency of circulating Th2 cells between RA patients and healthy controls. Moreover, we found no significant difference in the mean frequency of circulating Th2 cells between ACPA-positive and -negative RA patients. Previous studies have shown that both Th2 and Th9 cells are involved in human atopic diseases, but our data indicated that while Th9 frequency is increased significantly, frequency of Th2 lymphocyte is not altered significantly in RA patients. We also found that the prevalence of CD3$^+$ T and CD8$^-$CD3$^+$ T cells in the RA group was significantly higher than in the VERA group. Earlier studies have shown that the frequency of T and Th lymphocytes increases in patients with RA (36). Although in our study, the frequency of these cells in the established RA group was more than that in the control group, the difference was not statistically significant.

CONCLUSION

Our findings suggest that targeting Th9 cells could be a promising strategy for advanced RA therapies. However, further studies are required to increase our understandings of the exact role of Th9 cells in the pathogenesis of autoimmune diseases.

CONFLICT OF INTEREST

We declare that there is no conflict of interest regarding publication of this article.

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