

## Increased expression of PD-1 on CD4+ T cells subsets in Rheumatoid Arthritis patients

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### Abstract

**Background and objectives:** Rheumatoid arthritis (RA) is a common chronic and systemic autoimmune disease, characterized by inflammation and the destruction of the joints. It is well known that CD4+ T cells play a major role in the pathogenesis of RA. Expanded subpopulations of CD4+ T cells have been reported in RA patients. Here, we investigated the expression of PD-1 on subsets of CD4+ T cells (CD4+CD28- and CD4+CD28+ T cells) in the peripheral blood (PB) and synovial fluid (SF) of patients with RA.

**Methods:** The frequency of CD4+CD28+ T cells was significantly increased in SF versus PB in ND and RL patients. In contrast, the percentage of CD4+CD28- T cells was elevated in PB of ND and RL patients comparison to SF. Expression of PD-1 on CD4+CD28+ and CD4+CD28- T cells in PB of ND and RL patients was significantly higher than the healthy controls. Furthermore, PD-1 expression on CD4+CD28+ and CD4+CD28- T cells in SF versus PB of RL patients were significant increased.

**Results:** We demonstrated Oxidative Stress Balance was elevated at the time of admission in comparison to normal subjects. ROC curve analyze revealed that Oxidative Stress Balance (AUC = 0.7337; P<0.0001) was acceptable diagnostic value to discriminate IS patients from normal subjects. Kaplan-Meier survival analyze shown that Oxidative Stress Balance (P=0.8584) had no prognostic value.

**Conclusion:** These data suggest that CD4+ T cells subsets in RA patients were resistance to PD-1 mediated effects and PD-1 has insufficient ability to suppression of CD4+Tcells.

**Keywords:** CD4+CD28+ T cells; CD4+CD28- T cells; PD-1; Rheumatoid Arthritis

## Introduction

Rheumatoid arthritis (RA) is a common chronic inflammatory disease characterized by synovial inflammation and progressive destruction of the cartilage and bone of joints (1). The etiology and pathophysiology of this disease are not well understood, but it is possible that dysregulation of lymphocyte activation and also cytokine disorders play important roles in the onset, maintenance of inflammation and progression of the disease (2). Numerous animal and clinical studies have proven the pivotal contribution of CD4+ T cells in the immunopathogenesis of RA on multiple levels (3-5). Increased number of activated CD4+ T lymphocytes can be found in peripheral blood (PB), synovial membrane (SM) and synovial fluid (SF) of RA patients (6), and it's observed that in model of collagen-induced arthritis (CIA) and RA patient's depletion of T cells ameliorates disease activity (3). Moreover, treatment with anti-cytokine agents that prevents T cell activation is effective in RA patients (3). In RA patients, CD4+ T cells include subset of cells that lack the phenotypic and functional properties of classic T helper cell and characterized by a defect in CD28 expression (7). Moreover, RA patients display different levels of CD28 (8). Despite the functionally distinct phenotypes of CD4+CD28+ and CD4+CD28- T cells, the role of these cells in the pathogenesis of RA has been proven in various studies (9, 10).

Programmed death-1 (PD-1; CD279) is a co-inhibitory receptor and a crucial factor responsible for peripheral tolerance and immune regulation, which is expressed on CD4+ T cells, CD8+ T cells, B cells, natural killer T cells and monocytes (11). The T cells responses such as proliferation and cytokine

production were down-regulated or inhibited by PD-1 binding to its ligands (PDL-1 and PDL-2), and defect in expression or function of this receptor contributes to T cell hyperactivity (12). Studies have shown that PD-1 play critical roles in the development of autoimmune diseases such as RA (3), and over-expression of PD-1 on synovial T cells and macrophages have been reported in RA patients(3, 12).

According to the important role of PD-1 in the function of T cells and considering the little information about the function of T cells expressing CD28 or not, and also the central role of CD4+ T cells in pathogenesis of RA, in this study we examined the frequency and phenotypic characteristics of CD4+CD28+ and CD4+CD28- T cells in SF and PB of RA patients.

## Material and methods

### Study population

Forty-two RA patients including ten newly diagnosed (ND) and thirty two relapsed (RL) cases were recruited from Sayyad-Shirazi hospital, Gorgan, Golestan, Iran. All study patients diagnosed by an expert rheumatologist based on the criteria of the American College of Rheumatology/European League against Rheumatism (ACR/EULAR2010) (13). Moreover, twenty age and sex matched healthy subjects with no history of inflammatory or autoimmune disorders were also enrolled as healthy controls. Disease history was recorded for all patients, including presenting symptoms, affected joint counts and medication history. The disease activity was evaluated by calculation of 28-joint Disease Activity Score (DAS28) on the day of sample collection. DAS28 was

determined by the swollen and tender joint count and the erythrocyte sedimentation rate (ESR) test results(13). This study was approved by the ethics committee of Golestan University of Medical Sciences and a written informed consent following the declaration of Helsinki was obtained from all participants.

### **Samples collection and Cell purification**

A total volume of 5 mL paired SF and PB samples from ND and RL patients, and also PB from healthy controls were collected in heparinized tube. Sample were kept at 4°C and immediately transported to the laboratory. Blood samples were centrifuged and plasma were separated. SF samples were treated with 20 µg/ML hyaluronidase (BCN, Barcelona, Spain) for 30 min at 37°C, supernatants were collected and then cells were washed with phosphate buffered saline (PBS) (Bio-Idea, Tehran, Iran).SF supernatants and blood plasma of all samples immediately stored at -80°C until use. Using density gradient centrifugation on Ficoll-Paque (Baharafshan, Tehran, Iran),as previously described(14), SF mononuclear cells (SFMCs) and PB mononuclear cells (PBMCs) were isolated.

### **Immunostaining and flow cytometry**

PBMCs and SFMCs were stained with the following anti-human monoclonal antibodies(Biolegend, San Diego, USA): APC-labeled anti CD3, FITC-labeled anti CD8, APC labeled anti CD28 and PE labeled anti CD279 (PD-1), or with appropriate isotype-matched control antibodies, for 45 min at 4°C according to the manufacturer's instructions. Appropriate isotype control antibodies were used for marker settings. To consolidate the flowcytometry records, 2 × 10<sup>4</sup> mononuclear cells were gated in primary plots of all samples for further analyses.

Lymphocytes were identified from other cells by forward and side scattered gating, followed by CD3+ gating and only the gated cells were analyzed. All antibodies were titrated to determine their optimal concentrations prior to use. Stained cells were run on BD accuri c6 flow cytometer (BD PharMingen, San Diego, USA) and analyzed using BD accuri C6 flow software.

### **Statistical analyses**

Statistical software SPSS 22.0 (IBM Corp, USA) and GraphPad Prism 7 (GraphPad Software, USA) were used for data analysis and preparation of graphs. One-way ANOVA with Tukey's post hoc test or nonparametric Kruskal-Wallis with Dunn-Bonferroni post hoc tests were used to compare the means of multiple samples. Independent Samples t-test or nonparametric Mann-Whitney U test was used to compare the means of two groups. Two-tailed Spearman's rank correlation was performed for correlation analyses. P-values less than 0.05 were considered as statistically significant.

## **RESULT**

### **Characteristics of the participants**

The demographic characteristics of all individuals are shown in Table1. The mean DAS28 scores of ND and RL patients were 4.68±0.89, 4.85±1.06, respectively.

**Table 1. Demographic details of the study populations**

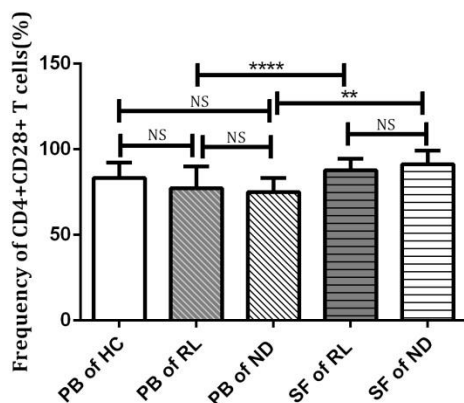
	Newly-diagnosed (N=10)	Relapsed (N= 32)	Healthy control (N=20)
Age (years)	46.42±16.5 9	55.25±15.7 2	45.26±12.7 5
Male	57.15%	9.37%	35%
Female	42.85%	90.63%	65%

\*Data are presented as means ± SE (standard error) for continuous measures and number (%) for categorical variables

**Frequency of CD4+ T cell subsets in PB and SF**

We analyzed the percentage of CD4+ T cell subsets in PB and SF of RA patients and PB of healthy controls. Our results showed that the frequency of CD4+CD28+ T cells was significantly increased in SF versus PB in ND (p=0.006) and RL (p<0.0001) patients (Fig 1). In contrast, the percentages of CD4+CD28- T cells were elevated in PB of ND (p=0.006) and RL (p<0.0001) patients comparison to SF (Fig 2). Additionally, our data indicated the increase number of CD4+CD28- and decrease number of CD4+CD28+ in PB of both RA subgroups in comparison to healthy controls, although these differences was not statically significant (Fig 1,2).

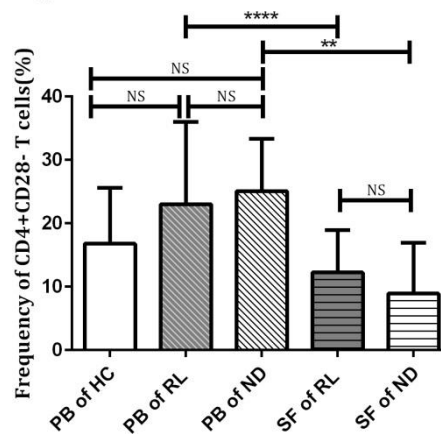
**Figure 1**



**Figure 1.** Analysis of CD4+CD28+ T cells frequency in RA patient subgroups and healthy controls by flow cytometry. Box dot plot data demonstrates means ±SE frequency of CD4+CD28+ T cells in each group.

P-values lower than 0.05 were considered as statistically significant. PB: Peripheral Blood; SF: Synovial Fluid; ND: Newly Diagnosed; RL: Relapsed; NS= not significant. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

**Figure 2**



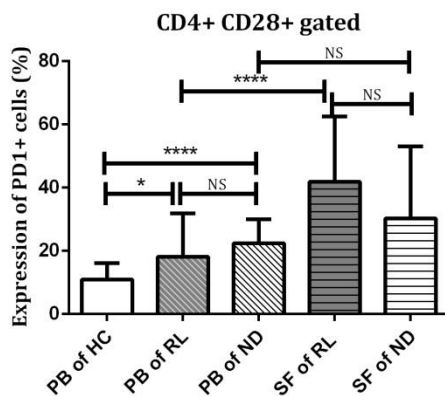
**Figure 2.** Analysis of CD4+CD28- T cells frequency in RA patient subgroups and healthy controls by flow cytometry. Box dot plot data demonstrates means ±SE frequency of CD4+CD28- T cells in each group.

P-values lower than 0.05 were considered as statistically significant. PB: Peripheral Blood; SF: Synovial Fluid; ND: Newly Diagnosed; RL: Relapsed; NS= not significant. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

### PD-1 Expression on CD4+ T cell subsets

The frequency of CD4+CD28+ T cells expressing PD-1 in PB of ND and RL patients was significantly higher than the healthy subjects ( $p<0.0001$  and  $p=0.039$ ) (Fig 3). Similarly, a higher percentage of CD4+CD28- T cells expressing PD-1 was observed in PB of both ND and RL patients as compared with healthy control ( $p<0.0001$  and  $p=0.011$ ) (Fig 4). Analysis demonstrated a significant increase in PD-1 expression on CD4+CD28+ and CD4+CD28- T cells in SF versus PB of RL patients ( $p<0.0001$  and  $p<0.0001$ ) (Fig 3,4).

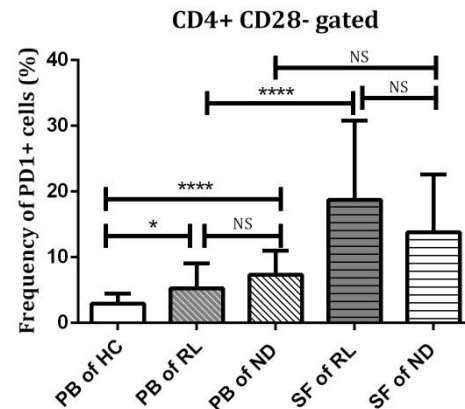
**Figure 3**



**Figure 3.** Analysis of PD-1 expression on CD4+CD28+ T cells. Flow cytometry data are presented as frequency of PD1+CD4+CD28+ T cells.

P-values lower than 0.05 were considered as statistically significant. Data of each bar demonstrates means  $\pm$ SE. All correlations were calculated using Spearman's test. PB: Peripheral Blood; SF: Synovial Fluid; HC: Healthy Control; ND: Newly Diagnosed; RL: Relapsed; NS= not significant. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , \*\*\*\* $p<0.0001$ .

**Figure 4**



**Figure 4.** Analysis of PD-1 expression on CD4+CD28- T cells.

Flow cytometry data are presented as frequency of PD1+CD4+CD28- T cells.

P-values lower than 0.05 were considered as statistically significant. Data of each bar demonstrates means  $\pm$ SE. All correlations were calculated using Spearman's test. PB: Peripheral Blood; SF: Synovial Fluid; HC: Healthy Control; ND: Newly Diagnosed; RL: Relapsed; NS= not significant. \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ , \*\*\*\* $p<0.0001$ .

No correlation was detected between the expression of PD-1 on CD4+28+ and CD4+CD28- T cells and the DAS28 index or serological variables (ESR and CRP).

### Discussion

The important role of CD4+ T cells in pathogenesis of RA was demonstrated on multiple levels. The expansion and accumulation of CD4+ T cells subpopulation in PB and SF of RA patients have been proved in several studies, which some

subtypes are associated with disease activity (3, 6, 15). CD4+CD28- T cells are unusual subtypes of CD4+ T lymphocytes that are frequently found in the patients with RA (9). Because of their pro-inflammatory features, it is suggested that CD4+CD28- T cells contribute to systemic inflammation and development of RA (9). In the present study, we investigated the frequency of CD4+CD28+ and CD4+CD28- T cells in the newly diagnosed and relapsed RA patients. We also evaluated the expression of PD-1 (as an immune checkpoint receptor) on these cells.

CD4+CD28- T cells are functionally distinct from conventional CD4+CD28+ T cells, however it is likely that 2 types of CD4+ T cells contribute differently to the pathogenesis of RA (16). Previous studies noted the increase frequency of CD4+CD28- T cells in PB and/or SF of RA patients, which linked with disease severity and extra-articular disease manifestation (17, 18). CD4+CD28- T cell subset possesses high pro-inflammatory and tissue damaging properties through the production of IFN- $\gamma$  and cytolytic proteins, perforin and granzyme B (19). Our findings showed that the frequency of CD4+CD28+ T cells was significantly increased in SF versus PB in ND and RL patients (Fig 1). In contrast, the percentages of CD4+CD28- T cells were elevated in PB of ND and RL patients' comparison to SF. Our data could indicate that CD4+CD28+ T cells are pivotal cells in inflammatory joints of RA patients and their preferential migration to ST during onset and progression of the disease. The available data regarding frequency of CD4+ CD28+ and CD4+CD28- T cells in RA patients are conflicting (20, 21), but the general agreement is that fewer CD4+CD28+ and correspondingly more CD4+CD28- T cells in

PB of RA patients compared to the healthy subjects (20). This is consistent with our finding although these differences were not statically significant between patients and healthy controls.

PD-1 is a critical receptor for the suppression of T cell activity and induction of tolerance (22). Numerous studies have already demonstrated the important role of PD-1 in RA pathogenesis (3, 23, 24). However, there are conflicting data concerning the level of PD-1 expression and its role in T cell function in the condition of RA (3, 11). In accordance with previous study (22, 25), we show that the frequency of CD4+CD28+ and CD4+CD28- T cells expressing PD-1 in PB of ND and RL patients was significantly higher than the healthy subjects. Also our data showed a significant increase in PD-1 expression on CD4+CD28+ and CD4+CD28- T cells in SF versus PB of RL patients. Continuous T cell activation and highly production of inflammatory cytokines in rheumatoid patients can be lead to up-regulation of PD-1 expression on CD4+ T cell subsets (12). However, despite this over-expression, persistence of inflammation in RA patients especially in the joints, suggesting that PD-1 may not be capable of suppressing T cells function (22). Contrary to our findings, some other studies showed the decrease PD-1 expression and its necessity for suppression of T cells function in RA patients (3). The different results are possibly due to differences in sensitivity of the employed assays or may be due to differences in the selection of patients (e.g. disease activity, duration and medication). It is noteworthy that in our study the MFI of PD-1 on CD4+ T cells subsets in SF was significantly higher than PB (data not shown). Based on our findings, we can conclude that the immune

system react to inflammation by up-regulating negative cost imulatory molecules such as PD-1, but seems that PD-1 pathway is impaired in RA patients (22). Resistance of CD4+ T cells to PD-1 mediated suppression and increased level of sPD-1 can be effective in PD-1 inefficiency (23). Further investigations are needed to clarify the precise physiological and immunological role of PD-1 in RA patients.

In summary, our findings reveal that despite increased expression of PD-1 on T lymphocytes in RA patients, this pathway do not seem to function properly to regulate T cell activation. Better understanding of the role of PD-1 in disease processes in RA conditions may help the development of new therapeutic strategies.

## **Declarations**

### **Ethics approvals and consent to participate**

Code of Ethics: IR.GOUMS.REC.1396.138

### **Conflict of interest**

None

### **Authors' contributions**

All authors contributed equally to this work.

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