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PD-1 Expression on CD8+CD28- T cells within inflammatory synovium is associated with Relapse: A cohort of Rheumatoid Arthritis

Running head: Role of PD-1+CD8+CD28- T cells in RA relapse

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Highlights

- Different CD8+ T cell immunophenotypes were observed in early and established RA states.
- The higher frequency of CD8+CD28- T cells was observed in PB vs. SF of relapsed patients.
• Relapsed patients also showed higher CXCR3 and especially PD-1 expression on their CD8+CD28- T cells.
• Higher level of PD-1+CD8+CD28- T cell at inflammatory synovium may be associated with RA relapse.
• IFN-γ elevation in the RA synovium probably poses a key role in disease initiation rather than progression.

Abstract

Defect in T lymphocyte homeostasis could implicate initiation and development of rheumatoid arthritis (RA). Since PD-1 plays a key role in the regulation of T lymphocytes, its expression pattern in various CD8+ T cell subsets could be so effective in RA pathogenesis. Here, we investigated the expression of PD-1 and CXCR3 on CD8+CD28- T cells in association with the IFN-γ levels in patients with RA. A total of 42 RA patients, including 10 newly-diagnosed (ND) and 32 relapsed (RL) cases and also 20 healthy donors were enrolled. Phenotypic characterization of CD8+ T cells derived from peripheral blood (PB) and synovial fluid (SF) was performed by flow cytometry. The plasma and SF IFN-γ levels were also assessed by ELISA. The frequency of CD8+CD28- T cells showed no significant differences
between patients and controls while its higher levels were observed in PB, versus SF of RL patients. Relapsed patients also showed higher CXCR3 and especially PD-1 expression on their CD8+CD28- T cells. The IFN-γ concentration was elevated in SF of ND patients while its plasma level was significantly lower in RL subgroup than controls. Although PD-1 could induce immune suppression in effector T cells, it is upregulated during inflammation and its overexpression on CD8+CD28- T cells within inflammatory synovium is associated with severity of disease in our cohort of RA patients.


**Keywords:** CD8+ T-cells; CXCR3; Immunophenotype; PD-1; Relapse; Rheumatoid Arthritis

**1. Introduction**

Rheumatoid arthritis (RA) is a systemic and chronic autoimmune disease (1), characterized by inflammation of synovium, resulting in cartilage and bone damage, and joint destruction (2). Despite the complex pathogenesis of RA, dysregulation of lymphocyte activation is involved in the onset and progression of the disease (3). Numerous studies have observed the accumulation of T cells within the inflamed joints and their participation in RA pathogenesis (4). The antigen-specific activation of T cells could possibly result in the breakdown of tolerance, synovial inflammation, and autoantibody production (5). The involvement of IFN-γ as a
major pro-inflammatory cytokine which mainly produce by activated T lymphocytes (6), has been highlighted in the pathogenesis of RA (7, 8). However, its association to the synovitis onset along with the higher number of IFN-γ producing T cells in RA patients could accentuate its crucial role in the disease initiation (6).

Migration, recruitment and activation of effector T lymphocytes to the sites of inflammation including synovium is mediated by the expression of certain chemokine receptors. The ligands/CXCR3 is considered as a major axis in the migration and recruitment of effector T cells within the milieu of an inflamed synovium (9, 10). The increased expression of CXCR3 and its ligands has been shown in serum, synovial fluid (SF) and synovial tissue of RA patients and also animal models (11, 12). CXCR3 has been also expressed by the most effector CD8+ T cells and linked to their recruitment to inflamed joints (13).

Programmed death receptor-1 (PD-1; CD279) is a cell-surface co-inhibitory receptor which is expressed on activated and exhausted T cells, B cells, monocytes and natural killer T cells (4, 5). T lymphocyte activation and IFN-γ production modulate PD-1_PD-L ligation or related signaling by different ways (14, 15). Any defect in these molecules could contribute to T cell hyperactivity and loss of self-tolerance (16). PD-1 is considered to possess a crucial role in the pathogenesis of autoimmune diseases such as RA, systemic lupus erythematosus (SLE), type 1 diabetes mellitus (T1D) and multiple sclerosis (MS) (4, 5). Moreover, increased expressions of PD-1 on synovial T cells and macrophages have been reported in RA patients (4, 17). Despite the significance in the regulation of T cells and induction of peripheral tolerance (5), its regulatory activity in CD8+CD28- T cells is not well studied.
Chronic inflammation, immune exhaustion and senescence could also be associated to the production of certain types of CD8+CD28- T cells (18-20). On the other hand, there are some evidences that CD8+CD28- phenotype could be one of the subsets of CD8+ regulatory T cells (Tregs) which have been already observed in the peripheral blood and synovium of RA patients (21-23). Although some recent investigations related to CD8+CD28- T cells, they could be a hybrid population and little is known about their role in RA pathogenesis.

At present, we investigated the expression of PD-1 and CXCR3 molecules on CD8+CD28- T cells in the peripheral blood (PB) and synovial fluid (SF) of newly-diagnosed (ND) and relapsed (RL) RA patients, and also assessed IFN-γ levels to evaluate their association with the disease development.
2. Materials and Methods

2.1. Patients and controls

A total of 42 patients with RA were recruited from Sayyad-Shirazi hospital, Gorgan, Golestan, Iran. An expert rheumatologist confirmed the diagnosis according to the criteria of the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR 2010) (24). Patients were divided into ND and RL subgroups. ND patients had not received treatment. All RL patients were treated with conventional glucocorticoid drugs (prednisolone) and standard disease modifying anti-rheumatic drugs (DMARDs) (such as methotrexate, hydroxychloroquine, sulfasalazine, and azathioprine). None of the patients had any other inflammatory or infectious disorder. Twenty age- and sex-matched healthy subjects were also enrolled as healthy controls. Healthy subjects with any history of inflammatory or autoimmune disorders, pregnancy, and glucocorticoid intake during last 6 months were excluded. The disease activity was also evaluated using 28-joint Disease Activity Score (DAS28) on the day of sample collection. DAS28 was assessed by the swollen and tender joint count and the erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) test results (25). This study was approved by the ethics committee of Golestan University of Medical Sciences (No. IR.GOUMS.REC.1396.138). We obtained a written informed consent following the declaration of Helsinki from all patients for relative assessments and data publication. The strong effect size of PD-1 in CD8+CD28- T cells was approved by Cohen’s test results (Cohen’s d: 0.89; Hedge’s g: 0.85).
2.2. Samples collection and preparation

Paired SF and PB samples from 10 ND and 32 RL patients, and also PB from 20 healthy donors were collected in heparinized tubes. Specimens were kept at 4°C and immediately transferred to the laboratory. Blood samples were centrifuged and plasma samples were separated. SF samples were treated with 20 μg/ML hyaluronidase (BCN, Barcelona, Spain) for 30 min at 37°C, supernatants were collected and cells were then washed with phosphate buffered saline (PBS) (Bio-Idea, Tehran, Iran). SF mononuclear cells (SFMCs) and PB mononuclear cells (PBMCs) were isolated by Ficoll-Paque (Baharafshan, Tehran, Iran) density gradient centrifugation as described (26). SF supernatants and plasma from all samples were stored immediately at -80°C after collection.

2.3. Antibodies and flow cytometry

PBMCs and SFMCs were stained with the combinations of the anti-human monoclonal antibodies for 45 min at 4°C, according to the manufacturer’s instructions. Isotype-matched control antibodies were appropriately used to eliminate the background staining. All monoclonal antibodies and isotype controls were purchased from biolegend (Biolegend, San Diego, USA). Stained cells were analyzed using BD accuri c6 flow cytometer (BD PharMingen, San Diego, USA) and analyses were performed with BD accuri C6 software. According to the application of multiple fluorochromes in each tube, all data were compensated to avoid false results. To consolidate the flow cytometry records, $2 \times 10^4$ mononuclear cells were gated in primary plots of all samples for further analyses. According to the limit of
fluorescence scatter (FL) in BD accuri c6, we could only have four fluorescence dyes simultaneously in each tube. Thus, cells from each sample were stained by APC-labeled anti-CD3 (Clone: UCHT1) and FITC-labeled anti-CD8 (Clone: RPA-T8) in the first tubes to investigate CD3 expression (pan T cell marker) on the surface of CD8+ cells, and in the second tubes cells were stained with FITC labeled anti-CD8, APC labeled anti CD28 (Clone: CD28.2), PE labeled anti-CD279 (PD-1) (Clone: EH12.2H7) and PerCP/CY5.5 labeled anti-CD183 (CXCR3) (Clone: G025H7) to analyze different phenotypes of CD8+ T cells. Based on the isotype control signals, frequency of positive cells considered as cell percent and mean fluorescence intensity (MFI) of positive cells intended to assess relative mean of expression.

2.4. Assessment of IFN-γ

Concentration of IFN-γ level in the plasma and SF was measured by commercial ELISA kits (Biolegend) according to the manufacturer's protocol. Stat Fax 2100 microplate reader (Awareness, USA) was used to obtain the optical density of each sample at the 450 nm against 630 nm reference wavelengths, as described (27).

2.5. Statistical analyses

All data were analyzed using SPSS 22.0 (IBM Corp, USA) and GraphPad Prism 7 (GraphPad Software, USA). 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. The Pearson or Spearman correlation analyses were performed to calculate the correlation coefficient. *P*-values less than 0.05 were considered statistically significant.
3. Results

3.1. Characteristics of the participants

Demographic features of all individuals and some clinical and laboratory findings of the RA patients are presented in Electronic supplementary Table S1. The mean DAS28 scores of ND and RL patients were 4.68±0.89, 4.75±1.06, respectively. All patients were diagnosed with active form of disease but correlation analysis showed that DAS28 was significantly associated with CRP (r=0.56, \(p=0.023\)) and ESR (r=0.55, \(p=0.018\)) only in RL patients. The expressions of all variables were analyzed on the basis of gender and in different subgroups of treatment strategies. No significant difference in these variables was demonstrated (Table S2, S3).

3.2. Evaluation of CD3 expression on CD8 positive cells

Analysis of CD8 positive mononuclear cells from all samples showed two different regions based on CD8 MFI, \(\text{CD8}^{\text{low}}\) (about less than 10% of all CD8+ cells) and \(\text{CD8}^{\text{high}}\) (about more than 90% of all CD8+ cells) (Electronic supplementary Fig. S1). These regions were further analyzed, according to CD3 expression to investigate T lymphocytes within CD8+ mononuclear cells. Analyses revealed that more than 99% of \(\text{CD8}^{\text{high}}\) cells co-express CD3 in all samples (Electronic supplementary Fig. S1). Hence, \(\text{CD8}^{\text{high}}\) cells were gated as CD8 T lymphocytes for further phenotypic assessments.
3.3. Frequency of CD8+CD28- T cells

We analyzed the percentages of CD8+CD28- T cells in PB and SF of RA patients and PB of healthy controls. Our results showed that there was a significant correlation between PB and SF regarding CD8+CD28- T cells frequency, only in ND patients (r=0.89, p=0.016). The frequency of CD8+CD28- T cells was almost similar in the PB and SF of ND patients, while RL subgroup showed higher numbers of CD8+CD28- T cells in PB versus SF (p<0.0001) (Fig. 1). In contrast, we observed no significant differences in the frequency of CD8+CD28- T cells between PB of both RA subgroups and healthy controls (Fig. 1). Additionally, no association was found between the percentages of CD8+CD28- T cells in PB or SF with production of rheumatoid factor (RF) and CRP in patients. We also did not find any age association of CD8+CD28- T cell in both patients and healthy subjects.

3.4. CD8+CD28- T cells expressed higher level of CXCR3 in RL patients

Analysis of CD8+CD28- T cells expressing CXCR3 of RL patients demonstrated a significant increase in frequency versus healthy donors in PB (p=0.002), while this elevated number was not significant in comparison to ND patients, in both PB and SF samples (Fig. 2). The mean expression of CXCR3 on CD8+CD28- T cells (MFI) was lower in ND patients than healthy control in PB (p=0.025), and also than RL patients in SF (not significant). Correlation analyses showed no association between CD8+CD28- T cells and CXCR3 expression in each subgroup.
3.5. Increased frequency of PD-1+CD8+CD28- T cells in RL patients

The frequency of CD8+CD28-T cells expressing PD-1 in RL patients was significantly higher in both PB (vs. normal controls; \( p=0.006 \)) and SF (vs. ND patients; \( p<0.0001 \)) (Fig. 3), although this increase was also observed in other T cell subsets of RL patients (Table 1, 2). Analyses exhibited that the MFI of PD-1 was elevated on T cells in RL patients (data not shown). Besides, there was no correlation between PB and SF regarding PD-1+CD8+CD28- T cells frequency in the patients.

3.6. Co-expression assessment of PD-1 and CXCR3 on CD8+CD28- T cells

The frequency of PD-1+CXCR3+CD8+CD28- T cells in PB of RL patients have been increased significantly in comparison to ND patients (\( p=0.027 \)) and healthy subjects (\( p=0.001 \)) (Fig. 4). Additionally, the frequency of these cells in RL subgroup was significantly lower in SF than PB (\( p=0.002 \)) (Fig. 4). Flow cytometric scatter plots also have represented the CD8+CD28- T cells co-expressing PD-1 and CXCR3 in PB of a healthy control, PB of a ND patient, PB of a RL patient, SF of a ND patient and a SF of RL patient (Electronic supplementary Fig. S2-S6).

As demonstrated in PB of RL patients, there was a significant correlation between the expression of PD-1 and CXCR3 on CD8+CD28- T cells (\( r=0.70, p<0.0001 \)) (Fig. 5), but not in ND patients. Also there was a significant reverse correlation between frequency of PD-1+CXCR3+CD8+CD28- T cells with ESR and CRP of RL patients (\( r=-0.49, p=0.036 \) and \( r=-0.56, p=0.021 \)).
3.7. Higher level of IFN-γ is significantly related to ND patients

As shown in Fig. 6, the plasma levels of IFN-γ in RL patients were significantly lower than healthy controls ($p<0.0001$), while there was no significant difference between ND patients and healthy subjects. Additionally, the IFN-γ concentration was significantly elevated in SF versus PB of our patients ($p<0.01$), though it was considerably higher in ND subgroup ($p=0.012$) (Fig. 6). Correlation analyses indicated a strong significant reverse association between the plasma IFN-γ concentration and PD-1 MFI in PB CD8- T cell of ND patients ($r=-0.86, p<0.01$).

3.8. Correlations analysis between frequency of the cells and laboratory findings

Analysis showed that there was no significant association between the rate of CD8+CD28- T cells, CD8+CD28-PD-1+ and CD8+CD28-CXCR3+ T cells in PB and SF of ND patients with CRP, RF and ESR, as inflammatory indicators. Moreover, no correlation was detected between these indicators and IFN-γ concentration in both plasma and SF.
4. Discussion

Defective function and deregulated immune response could stimulate the initiation and development of RA (28). Several studies have shown the significance of different phenotypes of CD8+ T cells in RA pathogenesis, as introduced “the unusual suspects” (29). Although the subsets and relative roles of CD8+CD28- T cells in RA patients are still unclear, some studies demonstrated their association with the T cell exhaustion (18, 20) and also immune regulation (23, 30).

CD8+CD28- T cells have also considered for their critical roles in various chronic immune disorders, such as Graves's disease, RA and type 1 diabetes mellitus (31). These cells exhibit weak immune responsiveness and shortened telomeres that often considered aging associated senescence, probably because of the continuous exposure to foreign antigens or chronic inflammation (31). Despite the contradictory data about function of CD8+CD28- T cells, recently some evidences have also been studied regarding regulatory activity of this subset (23). At present, we investigated the frequency of CD8+CD28- T cells in the ND and RL Iranian patients with RA. We also assessed the co-expression of PD-1 (as an immune checkpoint receptor) and CXCR3 (as a homing receptor) on these cells in association with IFN-γ levels (as a pro-inflammatory factor) for the first time.

However, our study had two main potential limitations. Since various medications of RA could probably exert different effects on the expression of PD-1 and CXCR3, comprehensive research on drug effects could be complementary to our study. Another limitation is low sample size of ND patients (n=10). According to the paired sample (PB and SF) collection in this study, we could only select the new cases with
inflamed synovium which are rarely found. On the other hand, our findings from ND patients showed minimum data variation which implies data reliability in this case.

The similar frequency of PB CD8+CD28- T cells between our patients and normal controls and also amongst ND and RL patients (Fig. 1) suggests that this subset is not basically affected in the PB by disease conditions (32). In contrary, we observed significant lower rate of these cells in the SF of RL subgroup (Fig. 1). Since the disease is more severe and chronic in RL patients, this reduction in the site of inflammation could be an indication of CD8+ T cell exhaustion (18) and/or defect in immunoregulation (23, 30, 33). Accordingly, this could be associated with the progression and relapse, not to the onset of the disease.

Little is known about the expression pattern of CXCR3 on CD8+CD28- T cells and related functions. It could be expressed on various CD8+ T cell subsets with activated and effector phenotypes and so promote their homing into the inflammatory tissues (34, 35). In contrast, we showed lower amount of CD8+CD28-CXCR3+ T cells in the SF vs. PB of patients, in spite of being significant (Fig. 2). However, we observed less CXCR3 (MFI) on PB CD8+CD28- T cells with more frequency in SF of ND patients (Table 1, 2). We also demonstrated no association between CD8+CD28- T cells and CXCR3 expression. Consequently, our analyses could indicate dependent role of CXCR3 in CD8+CD28- T cells homing into the swollen synovium, which is also controversial (36).

PD-1 plays a critical role in the regulation and suppression of T lymphocytes (5, 16). Several animal and clinical studies have shown the significance of PD-1 in RA immunopathogenesis (4, 5, 17). As demonstrated in Fig. 3, the expression of PD-1
was significantly higher in CD8+CD28- T cells of RL patients than healthy donors (in PB) and also than ND patients (in SF). The higher expression was also observed in CD8+CD28+ T cells of these patients (Tables 1, 2). In the view of these results, we could not expect suppressive role for PD-1, especially in CD8+CD28- T cells of RA patients. Otherwise, higher PD-1 expression on the effector T cells should have reduced disease activity while RL patients are recognized by higher severity, even more than ND (33, 37). Contrary to our findings, a previous research showed necessity of PD-1 for the regulatory function of CD8+CD28- T cells in lupus-prone mice (38). Previous assessments also exhibited that expression of PD-1 upregulated during chronic activation or viral infections which could be related to the T cell exhaustion (19, 39, 40). According to the higher PD-1+CD8+CD28- T cells in relapsed RA (Fig. 3) and also exclusion of patients with chronic infection from our study, this phenotype may be related to the CD8+ T cell over-activation in RL patients. Some other studies have also shown that PD-1 signaling is capable of inducing exhaustion of both CD4+ and CD8+ Tregs (41-43). On the other hand, the regulatory and suppressive functions of CD8+CD28- T cells in RA were also shown (21-23). Hence, CD8+ Treg suppression by higher PD-1 expression may partially explain disease relapse in our RL patients.

Interestingly, we observed positive correlation between PD-1 and CXCR3 expression exclusively in CD8+CD28- T cell subset, not any other phenotypes (data not shown), which is only related to the PB of RL patients (Fig. 5). According to the increased expression of PD-1 and CXCR3 on CD8+CD28- T cells in PB of RL patients (Fig. 4) and reverse correlations of their frequency with inflammatory indicators (ESR and CRP), they could probably be defined as an exhausted cells.
Higher concentrations of IFN-γ in SF of our RA patients, in consistent with other studies (8, 44), are strongly related just to the ND patients (Fig. 6). IFN-γ is an important factor concerning function of T lymphocytes, activation of local macrophages and pathogenesis of the RA (6, 7, 44). IFN-γ elevation in the SF of ND patients could specify its key role especially in the initiation of local inflammatory process. Even though, the exact role of IFN-γ in initiation and/or progression of RA is still controversial. Our results also showed a strong reverse correlation between plasma IFN-γ and PD-1 expression only in CD8 negative T cells (data not shown) while a previous study showed similar correlation in CD8 positive T cells (15). This could suggest a relative association effect of IFN-γ with the T cell phenotypes and maybe with the diverse disease situations.

5. Conclusion

Our findings reveal different immunophenotypes of CD8+ T cells in early and established RA states, related to their homing and regulation. Although PD-1 could induce immune suppression in effector T cells and is upregulated during inflammation and chronic T cell activation, its overexpression on CD8+CD28- T cells in inflammatory synovium may be associated with RA progression and clinical relapse and it is probably unrelated to the IFN-γ level. Functional analyses related to the different CD8+ T cell phenotypes could be helpful in the characterization of their role in RA pathogenesis in various disease states. It would be constituted a novel approach in favor of targeted immunotherapy.
Declaration of Competing Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Authors’ Contributions

FA and AM did conception and design; FA and MA select patients and collect specimens; FA, SM, and NB did statistical analyses; FA, HS and AM managed study, interpreted data and did drafting the manuscript; NB, MA, SM, and AM edited the manuscript. All authors approved the final version of the manuscript.

Ethical approval

The present study which involved human participants was approved by the ethical committee of Golestan University of Medical sciences (Code of Ethics:
A written informed consent following the declaration of Helsinki was given by all participants.

References:


**Fig. 1.** Analysis of CD8+CD28- T cells frequency in RA patient subgroups (n=42) and healthy controls (n=20) by flow cytometry.

Box dot plot data demonstrates means ±SE frequency of CD8+CD28- T cells in each group.

*P*-values lower than 0.05 were considered as statistically significant. All data were analyzed using *t*-test. 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 1**

**Fig. 2.** Analysis of CXCR3 expression on CD8+CD28- T cells.
Flow cytometry data are presented as frequency of CXCR3+CD8+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 2**

![Graph showing expression of CXCR3+ cells in different conditions](image)

**Fig. 3.** Analysis of PD-1 expression on CD8+CD28- T cells.
Flow cytometry data are presented as frequency of PD1+CD8+CD28- T cells.

P-values lower than 0.05 were considered as statistically significant. Data of each bar demonstrates means ±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 3**

![CD8+ CD28- gated](image)

**Fig. 4.** Analysis of CXCR3 and PD-1 co-expression on CD8+CD28- T cells.
Flow cytometry data are presented as frequency of PD1+CXCR3+CD8+CD28- T cells. P-values lower than 0.05 were considered as statistically significant. Data of each bar demonstrates means ±SE. 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**

**Fig. 5.** Correlation between PD-1 and CXCR3 expression on CD8+CD28- cells.

Positive correlation between PD-1 and CXCR3 expression on CD8+CD28- cells in PB of RL patients (n=32) (r = 0.7, p<0.0001).
P-values lower than 0.05 were considered as statistically significant. Data of each bar demonstrates means ±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.

Figure 5

**Fig. 6.** Assessment of IFN-γ concentration.

Higher level of IFN-γ in SF, especially in ND patients (n=10).
P-values lower than 0.05 were considered as statistically significant. 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 6

Tables:

Table 1. The Mean of positive cells percent in each gate from peripheral blood samples

<table>
<thead>
<tr>
<th>Cells %</th>
<th>ND patients (n=10)</th>
<th>RL patients (n=32)</th>
<th>Normal controls (n=20)</th>
</tr>
</thead>
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<td>CD8+T</td>
<td>CD8+CD28+</td>
<td>CD8+CD28-</td>
<td>CD8+T cells</td>
</tr>
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<td>T cells</td>
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<tr>
<td>------------------</td>
<td>-----------</td>
<td>----------</td>
<td>---------</td>
</tr>
<tr>
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<tr>
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<td>1.41</td>
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</tbody>
</table>

PB: peripheral blood; ND: newly diagnosed; RL: relapsed
Table 2. The Mean of positive cells percent in each gate synovial fluid samples

<table>
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<th>Cells % in SF</th>
<th>ND patients (n=10)</th>
<th>RL patients (n=32)</th>
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<td>PD1+</td>
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<tr>
<td>PD1+CXCR3+</td>
<td>1.33</td>
<td>4</td>
</tr>
</tbody>
</table>

SF: synovial fluid; ND: newly diagnosed; RL: relapsed