Original Article

PD-L1-expressing neutrophils as a novel indicator to assess disease activity of rheumatoid arthritis

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Abstract: Background: It is well-known that increased frequency of neutrophils was found in patients with Rheumatoid arthritis (RA), and neutrophils are the most abundant immune cells in Synovial fluid (SF) of RA patients. Costimulatory molecules plays an important role in determining the activation status and function of immune cells. However, the immunomodulatory roles and mechanisms of costimulatory molecules on neutrophils in RA are poorly understood. Objective: To determine the frequency of PD-L1-expressing neutrophils in patients with RA and tested the hypothesis that their frequency correlated with the activity of RA. Method: The expression of costimulatory molecules including PD-1, PD-L1, Tim-3, CD40, TIGIT, CD80 and CD86 on neutrophils were determined by flow cytometry. The frequencies of PD-L1-expressing neutrophils in patients with RA were further analyzed for their correlation with markers of autoimmune response, inflammation, disease activity and severity of RA. Results: Compared with healthy controls (HC), the frequency of PD-L1-expressing neutrophils in peripheral blood (PB) and synovial fluid (SF) were significantly elevated in RA patients (P = 0.0001) (P < 0.0001). And, the frequency of PD-L1-expressing neutrophils in SF of RA patients was significantly increased than that in autologous PB (P < 0.0001). Furthermore, we found that the frequency of PD-L1-expressing neutrophils in patients with RA was increased significantly in subjects with high RF titre, high levels of inflammatory markers and high Disease activity score 28 (DAS28). Conclusion: The frequency of PD-L1-expressing neutrophils is elevates in patients with RA and correlates with the disease activity of RA.

Keywords: Rheumatoid arthritis, PD-L1, neutrophils, synovial fluid, disease activity score 28

Introduction

Rheumatoid arthritis (RA) is a chronic debilitating systemic autoimmune disease characterized by inflammation and destruction of the joints. About 1% of the population suffers from RA, and many patients develop long-term joint damage, severe illness and disability [1]. Accumulating studies have shown that several factors are involved in the pathogenesis of RA, including genetic, infectious, environmental, and hormonal factors [2-4]. Unbalance of adaptive and innate immune systems driving the excessive immune responses is observed in RA. Due to its heterogeneity and multiplicity, the etiology of RA still remains elusive [5].

Although the etiopathology of RA is not fully understood, it is known that neutrophils, macrophages, synovial fibroblasts, T cells and B cells are involved in the mechanisms that drive the onset of RA [6]. Neutrophils, which are part of the innate immunity, are crucial for pathogenic defense. They are the first cell type to arrive at sites of inflammation [7]. And, neutrophils are by far the most abundant immune cell in SF of the joints of RA patients [8, 9]. Neutrophils have been reported to lead to the pathology of RA by releasing inflammatory mediators and regulating the functions of other immune cells including macrophages, NK cells, dendritic cell (DC), T cells and B cells [10]. Nevertheless, the roles of neutrophils in RA pathogenesis have not been well elucidated, especially, the regulative effect on immune cells.

Programmed death ligand 1 (PD-L1) is one of the costimulatory molecules in the B7 family
which functions as an immunomodulatory molecule. The engagement of PD-L1 with its receptor, programmed death 1 (PD-1), delivers inhibitory signals to target cells such as activated T-cell and B-cell, thus helps to maintain the balance between effective immunity, tolerance and immunopathology [11]. PD-L1 is broadly expressed on a variety of immune cells, including T cells, B cells, dendritic cells, and monocytes. Recent evidence indicates that PD-L1 is also expressed on neutrophils and associated with the development of numerous diseases, including the infection of human immunodeficiency virus [12], sepsis [13], Burkholderia pseudomallei-Infected disease [14], tuberculosis [15] and systemic lupus erythematosus [16]. However, the frequency and roles of PD-L1-expressing neutrophils in RA has not been established.

In the present study, we determined the frequency of PD-L1-expressing neutrophils in patients with RA and tested the hypothesis that their frequency correlated with the activity of RA.

Materials and methods

Subjects

Fresh peripheral blood (PB) was harvested by venipuncture from 67 patients with RA which fulfilled the American College of Rheumatology criteria for RA [17], and 52 healthy controls (HC) that unrelated to the patients, without inflammatory and autoimmune diseases. Synovial fluid (SF) was obtained from 27 cases of the 67 RA patients. RA disease activity was measured using the disease activity score 28 (DAS28) [18]. The study was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University (No. 019) and was carried out in compliance with the Helsinki Declaration. Informed consent was obtained from all the participants before they entered the study.

Flow cytometry analysis

The peripheral blood and synovial fluid were drawn and analyzed immediately for the molecular phenotypes of neutrophils by flow cytometry. The following antibodies were used: ECD-conjugated anti-CD3, PC5-conjugated anti-CD15 (BD Biosciences, San Diego CA, USA), PE-conjugated anti-CD3, anti-Tim3, anti-TIGIT, anti-CD86, and anti-PDL1, FITC-conjugated anti-CD80 (MIH clones, e Bioscience, San Diego, CA, USA). The neutrophils were identified as CD15**CD3** populations [16] and the membranous markers were detected by flow cytometry with triple staining. Briefly, 50 microliters of fresh heparinized whole blood were incubated simultaneously with 5 μL ECD-conjugated anti-CD3, 5 μL PC5-conjugated anti-CD15 and 5 μL fluorescence-conjugated antibodies targeting other membranous molecules on ice in the dark for 30 minutes. Cells incubated with PE- and FITC-conjugated mouse IgG were used as isotype controls. All flow samples were analyzed with a CYTOMICS FC 500 flow cytometer (BECKMAN COULTER) and associated software programs (CXP).

ESR, CRP and autoantibodies measurement

Erythrocyte sedimentation rate (ESR) was determined according to the instructions described by the manufacturer. C-reactive Protein (CRP) and Rheumatoid factor (RF) were measured by nephelometry. Anticitrullinated protein antibodies (ACPA) of IgG in serum were measured by commercially available ELISA kits (kexin, Shanghai, China).

Statistical analysis

Statistical analysis and graphic presentation were carried out with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). A t-test was used where the normality test passed; otherwise, the nonparametric Mann-Whitney test was used to analyze the data. Likewise, the Pearson method or the nonparametric Spearman method was used for correlation analysis. Value of $P < 0.05$ is considered as significant difference.

Results

Characteristics of study subjects

Characteristics of RA patients and healthy controls (HC) enrolled in this study are included in Table 1. There were no significant differences between patients and healthy controls with regard to age or gender. Patients with RA were classified into remission group (DAS28 < 2.6) and active group (DAS28 > 2.6) according to DAS28 [18]. Overall, 71.6% of RA patients are active patients. Among them, 15 patients were new-onset rheumatoid arthritis (< 6 months of disease duration) [26]. All patients received...
therapy with disease modifying antirheumatic drugs (DMARDs).

The frequency of PD-L1-expressing neutrophils is elevated in patients with RA

The neutrophils were identified in peripheral blood as CD15+CD3- populations and analyzed for the expression of costimulatory molecules including PD-1, PD-L1, Tim-3, TIGIT, CD40, CD80 and CD86 by flow cytometry. Data showed that the frequency of PD-L1 positive neutrophils was significantly elevated in RA patients compared to HC (Figure 1B, \(P = 0.0001\)). No significant difference was observed in the frequency of PD1-, CD40-, Tim-3-, TIGIT-, CD80-, or CD86-expressing neutrophils between RA individuals and HC (Figure 1).

Neutrophils are the most abundant immune cells in synovial fluid of the joints of RA patients [8, 9]. And, researchers have reported that the changes in synovial fluid may reflect the development and progression of RA much more directly and clearly [4, 19]. We therefore investigated PD-L1 expression on neutrophils in the synovial fluid of RA patients. As shown in Figure 2, the frequency of PD-L1 positive neutrophils in synovial fluid was significantly increased than that in peripheral blood of HC \((P < 0.0001)\) or that in peripheral blood of cases \((P < 0.0001)\).

The frequency of PD-L1-expressing neutrophils correlated with markers of autoimmune response

Rheumatoid arthritis (RA) is characterized by auto-antibodies overproduction such as RF and ACPA. Thus, the hallmark antibodies of RA including RF and ACPA were determined and analyzed for their correlation with the frequency of PD-L1-expressing neutrophils in this study. Data showed that 41 patients were positive for RF and 37 patients were positive for ACPA in all 49 patients who received auto-antibodies detection. As shown in Figure 3, the frequency of PD-L1-expressing neutrophils was significantly increased in patients with positive RF. And, the frequency of PD-L1-expressing neutrophils trends to elevate in patients with positive ACPA, but a significant difference was not reached. Moreover, the correlations between the frequency of PD-L1-expressing neutrophils in the synovial fluid and the hallmark antibodies in serum of RA were investigated but no obvious correlation was found (data no shown). These results showed that the elevated frequency of PD-L1-expressing neutrophils is correlated with the markers of autoimmune response, suggested that PD-L1-expressing neutrophils may associated with the pathogenesis of RA.

The frequency of PD-L1-expressing neutrophils correlated with markers of inflammation

RA is characterized by synovial hyperplasia and inflammation. RA Patients are frequently accompanied by the elevated levels of inflammatory markers. In order to investigate the correlation between the frequency of PD-L1-expressing neutrophils and inflammatory markers, the markers of inflammation including ESR and CRP were determined and analyzed for their correlation with the frequency of PD-L1-expressing neutrophils in RA patients. As shown in Figure 4, positive correlations between the frequency of PD-L1-expressing neutrophils and ESR, CRP were found. Moreover, we investigated the correlation between the frequency of PD-L1-expressing neutrophils in the synovial fluid and the inflammatory markers in serum of RA. No obvious correlation was found (data no shown). These results indicated that the frequency of PD-L1-expressing neutrophils associated with markers of inflammation.

The frequency of PD-L1-expressing neutrophils correlated with disease activity of RA

Aforementioned results demonstrated that the frequency of PD-L1 expressing neutrophils was correlated with markers of autoimmune response and inflammation. Some of these mark-

Table 1. Baseline characteristics of rheumatoid arthritis patients

<table>
<thead>
<tr>
<th>Categories</th>
<th>RA patients (n = 67)</th>
<th>Healthy controls (n = 52)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females,%</td>
<td>79%</td>
<td>77%</td>
</tr>
<tr>
<td>Age (years, average ± SD)</td>
<td>55 ± 13</td>
<td>50 ± 11</td>
</tr>
<tr>
<td>DAS28 (average ± SD)</td>
<td>3.9 ± 1.8</td>
<td>-</td>
</tr>
<tr>
<td>RF (IU/ml) (49 patients)</td>
<td>447.2 ± 701.1</td>
<td>-</td>
</tr>
<tr>
<td>ACPA (RU/ml) (49 patients)</td>
<td>524.6 ± 967.4</td>
<td>-</td>
</tr>
<tr>
<td>CRP (mg/L) (49 patients)</td>
<td>12.8 ± 17.3</td>
<td>-</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>37.9 ± 31.3</td>
<td>-</td>
</tr>
</tbody>
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Figure 1. Expression of PD-L1, PD1, CD40, TIGIT, CD80 and CD86 on neutrophils in peripheral blood. A. Gates were then set on neutrophils based on CD15 and CD3, neutrophils were defined as CD15⁺CD3⁻ FSC high, SSC high. B. RA patients had elevated frequency of PD-L1-expressing neutrophils compared with HC (P = 0.0001, Mann-Whit-
PD-L1 expression neutrophils elevated in RA

ers, such as RF and ACPA, were reported to correlate with disease activity and the severity of joint destruction of RA [10]. And, ESR and CRP are traditionally valuable for calculating DAS28, a scoring system for assessing the severity in patients with RA. Thus, patients with RA were further classified into active and remission patients according to the DAS28 and analyzed for their relation with the frequency of PD-L1-expressing neutrophils. Data showed that the frequency of PD-L1-expressing neutrophils in patients with active RA was significantly higher compared with patients with remission RA ($P = 0.029$) (Figure 5A). Furthermore, we found that there was a positive correlation between the frequency of PD-L1-expressing neutrophils and the DAS28 score ($r^2 = 0.067, P = 0.034$) (Figure 5B), which demonstrated that the frequency of PD-L1-expressing neutrophils is correlated with disease activity of RA. We also investigated the correlation between the frequency of PD-L1-expressing neutrophils in the synovial fluid and DAS28. No obvious correlation was found (data not shown).

Subsequently, we compared the frequency of PD-L1-expressing neutrophils between new-onset and late-onset patients of RA. Data showed that the frequency of PD-L1-expressing neutrophils trends to elevate in new-onset patients of RA, but a significant difference was not reached (data not shown).

Discussion

Neutrophil is the most abundant leukocyte population and traditionally recognized as one of essential effector cells of the innate immune system in human. In recent years, an increasing interest in the role of neutrophils in interacting with and regulating the adaptive immune response has emerged [12-15, 20, 21]. It is well-known that various RA biomarkers are neutrophils-related and increased frequencies of inflammatory neutrophils are found in RA [10]. However, the immunomodulatory roles and mechanisms of neutrophils in the onset and development of RA are not yet identified. It is well known that the expression of costimulatory molecules plays an important role in determining the activation status and function of immune cells. Some costimulatory molecules, especially some immunosuppressive costimulatory molecule such as PD1, PD-L1, Tim-3 and

Figure 2. Expression of PD-L1 on neutrophils in synovial fluid and peripheral blood. A. The frequency of PD-L1 positive neutrophils in synovial fluid was significantly increased than that in peripheral blood of HC ($P < 0.0001$, Mann-Whitney test). B. The frequency of PD-L1 positive neutrophils in synovial fluid was significantly increased than that in peripheral blood of cases ($P < 0.0001$, Mann-Whitney test).
PD-L1 expression neutrophils elevated in RA

Figure 3. Correlation of frequency of PD-L1-expressing neutrophils with autoantibody. A. The frequency of PD-L1-expressing neutrophils was significantly increased in RA patients with positive RF ($P = 0.0105$, Mann-Whitney test). B. The frequency of PD-L1-expressing neutrophils trends to elevate in patients with positive ACPA, but a significant difference was not reached ($P = 0.197$, t-test).

Figure 4. Correlation of frequency of PD-L1-expressing neutrophils with inflammatory markers. A. The frequency of PD-L1-expressing neutrophils in RA patients correlated significantly with ESR ($r^2 = 0.1105$, $P = 0.0068$, Pearson method). B. The frequency of PD-L1-expressing neutrophils in RA patients correlated significantly with CRP ($r^2 = 0.1183$, $P = 0.0155$, Pearson method).

Figure 5. Correlation of frequency of PD-L1-expressing neutrophils with disease activity. A. The frequency of PD-L1-expressing neutrophils in RA patients was significantly increased in active RA patients compared to remission RA patients ($P = 0.029$, Mann-Whitney test). B. The frequency of PD-L1-expressing neutrophils in RA patients correlated significantly with DAS28 ($r^2 = 0.067$, $P = 0.0341$, Pearson method).
PD-L1 expression neutrophils elevated in RA

TIGIT, have been reported to have abnormal expression on T cells, monocyte or NK cells of RA patients [22-25]. In this study, for the first time, we investigated the expression of CD80, CD86, PD1, PD-L1, Tim-3, CD40 and TIGIT on neutrophils from RA patients, and showed that the frequency of PD-L1-expressing neutrophils was significantly increased in RA patients compared with HC. Moreover, our research revealed that the frequency of PD-L1-expressing neutrophils was associated with disease activity of RA.

RA is associated with the development of autoantibodies, which are often detectable before clinical symptoms of the disease become apparent. In this study, the serous levels of RF and ACPA, the hallmark antibodies of RA were first determined and analyzed for their relation with the frequency of PD-L1-expressing neutrophils. Data showed that the frequency of PD-L1-expressing neutrophils was significantly increased in patients with positive RF, suggested that PD-L1-expressing neutrophils may associated with autoimmune responses of RA. However, although tend to increase in patients with positive ACPA, the frequency of PD-L1-expressing neutrophils showed no statistic correlation with ACPA titer. As ACPA titer often correlate positively with disease activity and the severity of joint destruction and would decrease following therapy with DMARDs, the poor correlation between the frequency of PD-L1-expressing neutrophils and ACPA may be due to the fact that majority of RA patients had received therapy prior to participation in the study.

It is well-known that autoimmune response is a kind of chronic inflammation against self antigens. So the correlation between the frequency of PD-L1-expressing neutrophils and inflammatory markers was analyzed. Our results showed that the frequency of PD-L1-expressing neutrophils was positively related with ESR and CRP. Considering the application of ESR and CRP for calculating DAS28, our research investigated the correlation between the frequency of PD-L1-expressing neutrophils and DAS28. Subsequent results resulted from the DAS28 of RA patients confirmed our speculations. We also compared the frequency of PD-L1-expressing neutrophils between new-onset and late-onset patients of RA. Data showed that the frequency of PD-L1-expressing neutrophils trends to elevate in new-onset patients of RA, but a significant difference was not reached. Thus, we established the correlation between the frequency of PD-L1-expressing neutrophils and disease activity of RA.

In the report, we identified that the frequency of PD-L1-expressing neutrophils from peripheral blood and synovial fluid of RA were up-regulated. Also, the elevation of the frequency of PD-L1-expressing neutrophils from peripheral blood was correlated with RF titre, inflammatory markers and disease activity of RA. But the elevation of the frequency of PD-L1-expressing neutrophils from synovial fluid was not associated with RF titre, inflammatory markers and disease activity of RA. This may be that peripheral blood indicate systemic perspectives and synovial fluid indicate local perspectives in RA.

PD-L1, an immunoregulatory molecule that belongs to the B7 superfamily, was identified as ligands for PD-1, and engagement of PD-1 by PD-L1 induced negative signaling by the recruitment of phosphatases, such as SHP-2, and dephosphorylation of effector molecules involved in downstream TCR signaling [27, 28]. By far, there are only a few papers which reported the expression of PD-L1 on neutrophils. Some researches found that the expression of PD-L1 was up-regulated in infectious diseases and considered that this up-regulation of PD-L1 on neutrophils is involved in immunosuppression [12-15]. It is worth mentioning that our recent researches suggested that the up-regulation of PD-L1 on neutrophils may serves as a negative feedback mechanism [16].

Previously, an immunosuppressive population of neutrophils has been identified in peripheral blood of human in particular events, such as chronic inflammation and tumor [29, 30]. This population of neutrophils displays a remarkable ability to suppress T cell-mediated immune response by multiple mechanisms, including produce reactive oxygen species (ROS) and arginase-1 [12, 31], and was speculated to serve as a negative feedback mechanism to prevent potential tissue damage caused by excessive immune response. Considering the immunosuppressive feature of PD-L1 and the facts that the frequency of PD-L1-expressing neutrophils is associated with disease activity and severity of RA, our research suggests that the increase of the frequency of PD-L1-ex-
pressing neutrophils may serves as a negative feedback mechanism to prevent potential tissue damage caused by excessive autoimmune responses in patients with RA.

Conclusions

To our knowledge, this is the first report on the characteristics of PD-L1-expressing neutrophils in RA. Additionally, our research established a correlation between the frequency of PD-L1-expressing neutrophils and disease activity and severity of RA, which might improve our understanding of neutrophil's role in RA.

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Disclosure of conflict of interest

None.

Authors’ contribution

Conceived and designed the experiments: QL LZ JL. Performed the experiments: QL LZ HM ZH ZL JY XL YG. Analyzed the data: QL LZ JL. Wrote the paper: QL LZ JL. Contributed reagents/materials/analysis tools: HM ZH ZL JY. All authors read and approved the manuscript.

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