ORIGINAL ARTICLE

CD14++CD16+ monocyte subset expansion in rheumatoid arthritis patients: Relation to disease activity and interleukin-17

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Received 5 December 2015; accepted 5 December 2015
Available online 12 January 2016

KEYWORDS
CD14++CD16+ monocytes; CD14+CD16+ monocytes; IL17; Rheumatoid arthritis; Disease activity score (DAS28)

Abstract  Aim of the work: Monocytes are divided into three major subsets based on the expression of the cluster of differentiation CD14 and CD16. The aim of this work was to determine which of the CD16+ monocyte subpopulations is expanded in rheumatoid arthritis (RA) and its association with disease activity and interleukin-17 (IL17) levels.

Patients and methods: Fifty-three RA patients and 20 controls were enrolled in this study. Flow cytometry was performed to detect monocyte subsets and IL17 was measured by ELISA. Disease activity score (DAS28) was assessed.

Results: CD14++CD16+ monocyte percentage was significantly higher in long standing RA patients compared with early patients and controls (p < 0.01, p < 0.001 respectively). It was significantly higher in patients with RA disease activity and remission compared with the controls (p < 0.001, p < 0.01 respectively). It was not significantly associated with resistance to disease modifying antirheumatic drugs (DMARDs), C-reactive protein, rheumatoid factor and anti-CCP positivity (p > 0.05). It significantly correlated with IL17 (p < 0.002). CD14+CD16+ monocyte percentage was not significantly correlated with any of the above parameters. IL17 level was significantly higher in patients with early and long standing RA compared to controls (p < 0.01, p < 0.001 respectively). IL17 was higher in RA patients resistant to DMARDs than in responding patients (p < 0.017).

Conclusion: CD14++CD16+ monocyte subpopulation was expanded in long standing RA and was correlated with IL17 levels indicating its potential pathogenic importance in RA and may represent an attractive target for future therapeutic interventions.

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1. Introduction

Accumulating evidence supports that cluster of differentiation CD4 + CD25 + regulatory T cells play an essential role in controlling rheumatoid arthritis (RA) [1]. T helper-17 cells and interleukin (IL-17) play an important role in the pathogenesis of inflammatory and destructive pattern characteristic [2]. In a previous study, proteinase-activated receptor-2 expression on monocytes was remarkably high in active RA patients and consistent with a pathogenic role while its expression on CD3 + T-cells was not [3].

Cells of the monocyte/macrophage lineage play important roles in RA pathogenesis, the perpetuation of inflammation, and are potential targets for activation by immune complexes (ICs). Activated macrophages are the predominant infiltrating cells found in RA synovium, pannus and nodules [4,5]. Macrophages play a role in phagocytosis, antigen presentation, antibody-dependant cell-mediated cytotoxicity and release of pro-inflammatory cytokines and tissue destructive mediators [6]. The migration of monocytes from blood to synovial tissue and their differentiation into macrophages may be an important step in disease pathogenesis [7]. Macrophages are the major source of pro-inflammatory cytokines in the inflamed RA joint including tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-8 and granulocyte–macrophage colony-stimulating factor (GM-CSF) [4,8]. FcγRIIIa cross-linking has been specifically implicated in cytokine release from adherent human monocytes/macrophages [9,10]. These cytokines are intimately involved in the disease process as demonstrated by the clinical efficacy of TNF or IL-1 blockade in RA [11,12].

Current knowledge defines three major monocyte subsets based on the expression patterns of CD16 and the receptor CD14: classical CD14++CD16−, intermediate CD14++CD16+ and non-classical CD14+CD16− monocytes [13]. These subsets differ essentially in their chemokine receptor expression, phagocytic activity and tissue distribution during inflammation or steady-state conditions [14]. Hence, monocyte subsets are differentially involved in the pathophysiology of inflammation, atherosclerosis and regeneration after injury [15–17]. In particular, the unique features of the intermediate subset (both previously referred as CD16+ monocytes) has become recently more evident and especially in cardiovascular disease this subset was shown to predict independently cardiovascular events at follow-up [18–20]. Thus, human monocyte subsets may represent a novel prognostic marker or therapeutic targets in clinical medicine.

The aim of the current study was to determine which of the two CD16+ monocyte subpopulations is expanded in RA and to investigate their possible association with disease activity and with IL17 levels.

2. Patients and methods

The present study was conducted on 53 RA patients attending out-patient clinic of Physical Medicine and Rehabilitation Department, Menoufia University Hospitals and 20 age and sex matched healthy controls. Patients were diagnosed as RA according to ACR/EULAR classification criteria 2010 [21]. Patients were classified according to RA disease duration into early (≤2 years, n = 19) and long standing RA (>2 years, n = 34). Each group was further subdivided according to the disease activity score in 28 joints (DAS28) [22] into 2 subgroups; activity and remission groups (early RA, activity n = 15, remission n = 4; long standing RA, activity n = 24, remission n = 10). The disease activity was further categorized as low, moderate and high [22]. This study was approved by the ethics committee of the Faculty of Medicine, Menoufia University. All patients provided signed informed consent to provide a blood sample and to review the medical record for research purposes.

2.1. Immunophenotyping of peripheral blood monocytes

100 µl peripheral blood mononuclear cells were incubated with 20 µl FITC-conjugated antihuman CD14 and 20 µl PE-conjugated antihuman CD16 (Immunostep, Spain) for 30 min at 4 °C followed by red blood cells lysis and washing.
once with phosphate buffered saline. Cell acquisition and analysis were done using BD FACSCalibur flow cytometry (BD Biosciences, Franklin Lakes, New Jersey, USA). We gated on monocytes and then analyzed the percentage of CD14++ CD16+ monocytes and CD14+ CD16+ monocytes. The intensity of expression of CD16 on each monocyte subset was measured as mean fluorescent intensity ratio (MFIR). Controls and patient samples were examined using the same settings and conditions for comparison.

2.2. Measurement of serum IL17 level by ELISA
(eBioscience, Inc., San Diego, CA 92121, USA) It was done according to the manufacturer’s instructions.

2.3. Statistical analysis
Analysis was performed with SPSS version 20 statistical software. Quantitative data were expressed as mean ± standard deviation. Two groups of non-normally distributed variables were tested by Mann–Whitney test. Comparisons of three groups of normally distributed variables by ANOVA test and non-normally distributed variables by Kruskal–Wallis test were made. Post hoc test was used after ANOVA (F test) or Kruskal–Wallis test to show any significant difference between the individual groups. Qualitative data were expressed as number and percentage (n and %) and analyzed by Chi-square test. Pearson correlation was used for normally distributed while Spearman correlation was used for non-normally distributed quantitative variables. p value < 0.05 was considered statistically significant.

3. Results
Fifty-three RA patients with a mean age of 44 ± 11.2 years and 20 age and sex matched healthy controls were included in this analysis. The mean disease duration was 7 ± 7.4 years. The demographic and laboratory data of the patients and control and disease duration and activity of the patients are shown in Table 1. The CD14++ CD16+ and CD14+ CD16+ monocytes were assessed in only 12 of the controls while IL17 level was measured in all the controls.

3.1. Regarding disease duration
The percentage of CD14++ CD16+ monocytes was significantly higher in long standing compared with early RA patients and controls (p < 0.01, p < 0.001 respectively) (Table 2, Fig. 1). It was also significantly higher in patients with early RA compared to healthy controls (p = 0.01). The percentage of CD14+ CD16+ monocytes were significantly lower in early RA than healthy controls (p = 0.04). Regarding CD16+ MFIR on CD14++ and CD14+ monocytes; there was no significant difference when comparing between the three groups; long standing RA, early RA and controls (Table 2). IL17 levels were significantly higher in early and long standing RA compared to controls (p < 0.01, p < 0.001 respectively) (Table 2).

3.2. Regarding disease activity
The percentage of CD14++ CD16+ monocytes was significantly higher in patients with RA disease activity and

Table 2: Comparison between early and long standing RA patients, RA patients with active disease and those with remission, healthy controls and different degrees of disease activity regarding IL17 levels, the percentages and MFIR of CD14++ CD16+ and CD14+ CD16+ monocytes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rheumatoid arthritis patients (n = 53) and control (n = 20)</th>
<th>IL17 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD14++ CD16+</td>
<td>CD14+ CD16+</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>MFIR</td>
</tr>
<tr>
<td><strong>Patients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early (19)</td>
<td>12.01 ± 3.2#,@</td>
<td>118.1 ± 41.6</td>
</tr>
<tr>
<td>Late (34)</td>
<td>17.03 ± 6.1#</td>
<td>101.5 ± 35.7</td>
</tr>
<tr>
<td>Controls</td>
<td>8.2 ± 2.4</td>
<td>107.4 ± 33.9</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>0.52</td>
</tr>
<tr>
<td><strong>Patients</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active (39)</td>
<td>15.3 ± 6.01#</td>
<td>112.9 ± 36.7</td>
</tr>
<tr>
<td>In remission (14)</td>
<td>14.9 ± 5.2#</td>
<td>92.2 ± 30.6</td>
</tr>
<tr>
<td>Controls</td>
<td>8.2 ± 2.4</td>
<td>107.4 ± 33.9</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>0.26</td>
</tr>
<tr>
<td><strong>Disease activity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (4)</td>
<td>15.4 ± 6.9</td>
<td>104.7 ± 50.3</td>
</tr>
<tr>
<td>Moderate (27)</td>
<td>13.8 ± 4.8</td>
<td>114.6 ± 37.8</td>
</tr>
<tr>
<td>High (8)</td>
<td>20.6 ± 7.01</td>
<td>111.3 ± 45.9</td>
</tr>
<tr>
<td>p</td>
<td>0.038</td>
<td>0.92</td>
</tr>
</tbody>
</table>

RA: rheumatoid arthritis, MFIR: mean fluorescent intensity ratio, IL17: interleukin 17, CD: cluster of differentiation.
The CD14++ CD16+ and CD14+ CD16+ were assessed in only 12 of the control.
# On post-hoc test; significantly different from corresponding control, at p < 0.05.
@ On post-hoc test; significantly different from long standing patients, at p < 0.05.
# On post-hoc test; significantly different from patients in remission, at p < 0.05.
* On post-hoc test; significantly different from high disease activity at p < 0.05.
remission compared with the healthy controls ($p < 0.001$, $p < 0.01$ respectively). Comparisons according to the degree of disease activity are presented in Table 2. A significant difference was present between patients with moderate and high disease activity, ($p < 0.05$). The CD14$^+$CD16$^+$ monocyte percentage was significantly lower only in RA patients with remission compared with healthy controls (Table 2). However, the CD16$^+$ MFIR on CD14$^+$ monocytes was significantly higher in RA patients with high disease activity compared with those having low and moderate activity ($p < 0.05$). The
Figure 2  CD14^{++}CD16^{+} and CD14^{+}CD16^{+} monocytes (percent and MFIR) and interleukin-17 among patients with early RA with activity or remission as well as control.
percentage of CD14\(^{+}\)CD16\(^{+}\) monocytes, CD16\(^{+}\) MFIR on CD14\(^{+}\) monocytes and serum IL-17 according to the state of disease activity is presented for early RA patients in Fig. 2 and for long standing RA patients in Fig. 3. Significantly higher levels of IL-17 were found in RA patients with disease activity compared to those with disease remission and healthy controls (\(p < 0.01, p < 0.001\) respectively) (Table 2). The level was significantly higher in patients with high disease activity than those with moderate disease activity (\(p < 0.01\)) (Table 2).
Both the percentage of CD14++CD16+ monocytes and CD16+ MFIR on CD14++ monocytes did not significantly associate with resistance to disease modifying antirheumatic drugs (DMARDs), C-reactive protein (CRP), rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP) positivity (p > 0.05, Table 3). The percentage of CD14++CD16+ monocytes significantly correlated with IL17 levels (p < 0.002, Table 4), but not with the disease duration, DAS28, number of swollen and tender joints. CD16+ MFIR on CD14++ monocytes did not significantly correlate with any of the previous mentioned parameters. Both the percentage of CD14+CD16+ monocytes and CD16+ MFIR on CD14+ monocytes did not significantly correlate with the previous mentioned parameters. Both the percentage of CD14+CD16+ monocytes and CD16+ MFIR on CD14+ monocytes did not significantly associate with DMARDs, CRP and RF positivity (p > 0.05). CD16+ MFIR on CD14+ monocytes was significantly higher in patients with positive anti-CCP (p = 0.023, Table 3). Both the percentage of CD14+CD16+ monocytes and CD16+ MFIR on CD14+ monocytes did not correlate with IL17, disease duration, DAS28, number of swollen and tender joints (p > 0.05, Table 4). Significantly higher levels of IL17 were found in RA patients resistant to DMARDs than responding RA patients (p = 0.017) (Table 3).

### 4. Discussion

Monocytes are crucial players in the perpetuation of immune responses and joint damage in RA. Kawanaka et al. reported higher frequency of CD16+ monocytes in the peripheral blood of RA patients [23], but without distinguishing between subpopulations of CD16+ monocytes. The CD14 low monocyte subset has previously been the major focus of attention in RA due to reports of increased numbers in inflammatory diseases [23–26] and following reports suggested that they are the main producers of TNF in controls [27]. However, in our current study we found no significant difference in the percentage of the CD14+CD16+ monocyte subset in RA patients between early and long standing disease, or those in activity and remission; and did not correlate with IL 17 levels, disease...
duration and DAS28 score. CD14+CD16+ monocyte percentage was significantly lower in RA patients with disease remission compared with controls consistent with another study [28]. CD16+MFIR on CD14+ monocytes was significantly higher in RA patients with high disease activity than in patients with low and moderate RA disease activity.

Previous reports showed higher expression of CD16+ on CD14++ monocyte subset in RA compared with controls [23,29–32]. In this study, the percentage of CD14++CD16+ monocytes was significantly higher in long standing compared with early RA patients and controls. Also it was higher in early RA compared to controls. This was consistent with Rossol et al. and Cooper et al., studies who revealed an expansion of the intermediate CD14++CD16+ monocyte population in patients with RA with no growth within the population of nonclassical CD14+CD16+ monocytes [33,34]. Cooper et al. reported significantly higher level of CD14++CD16+ monocytes percentage in patients with long standing disease than in early RA and controls while early RA patients were not significantly different than controls. Klimek et al. found a significantly higher percentage of CD14+CD16+ monocytes in patients with early RA compared to controls [35].

In the present study; the percentage of CD14+CD16+ monocytes was not significantly different between patients with disease activity and remission or according to the degree of activity which is consistent with Klimek et al. [35]. However, a significant difference was present between patients with moderate and high disease activity. CD16+ monocytes have been demonstrated to be the main producers of TNF in response to lipopolysaccharides [36]. It has demonstrated that monocytes from RA patients show an enhanced capacity to produce TNF in response to IgG-containing immune complexes and the extent of TNF-production is correlated with the level of CD16 expression on CD14+ monocytes [33]. It has been demonstrated that magnetic-bead isolated CD16+ monocytes adhere to activated endothelium and migrate into the joint more efficiently than CD16− monocytes due to increased adhesion molecule and chemokine receptor expression [37].

It has been shown that monocytes are important targets for MTX treatment in RA patients [38,39]. In the present study, the percentage of CD14+CD16+ monocytes did not significantly associate with resistance to DMARDs. In contrast to our findings, Cooper et al. demonstrated that increased CD16 expression on CD14+monocytes in RA may be important in determining non-response to MTX therapy [33]. Chara et al. reported that the absolute number of circulating monocytes, and the numbers of CD14+CD16−, CD14+CD16+ and CD14−CD16+ subset cells, are strongly predictive of the clinical response of naive RA patients to MTX treatment [40].

IL-17 – producing CD4+ T cells have emerged as a major pathogenic T cell population that is present at increased frequencies in RA and correlates with disease severity [41]. The underlying mechanisms of this expansion of the Th17 cell population are not fully understood, but in vivo–activated monocytes from rheumatoid synovium have been shown to be able to drive strong Th17 cell differentiation in vitro [8], indicating a central role for direct T cell–monocyte contact in this process. In this study, the percentage of CD14+CD16+ monocytes was significantly correlated with IL17 levels. Rossol et al. reported that CD14+CD16+ monocytes were extremely potent inducers of Th17 cell expansion. Their frequency in the peripheral blood of RA patients correlated closely with Th17 cell frequencies suggesting that the size of the CD14+CD16+ subpopulation is indeed functionally linked to the observed expansion of the Th17 cell population in RA [34]. In this study, IL17 levels were significantly higher in RA patients than healthy controls, RA patients with disease activity than remission, patients with high activity than those with moderate disease activity, RA patients resistant to DMARD than responding patients which is consistent with previous studies [32,42–44].

5. Conclusion

We have demonstrated that CD14+CD16+ monocyte subpopulation expanded in long standing than early RA patients and was higher in those with high disease activity indicating its potential pathogenic importance in driving the inflammatory responses in RA. The positive correlation between CD14+CD16+ monocyte percentage and serum level of IL17 indicated that it may have a role in promotion and maintenance of the pathogenetically relevant Th17 cell compartment in RA. Further studies are needed to determine whether monocyte CD16 expression levels could potentially be used as a prognostic or predictive biomarker of response to methotrexate therapy in RA. Furthermore, blockade of CD16 represents an attractive target for future therapeutic intervention.

Conflict of interest

None.

References


