Increased circulating CD14^{bright}CD16^{+} intermediate monocytes are regulated by TNF-α and IL-6 axis in accordance with disease activity in patients with rheumatoid arthritis

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Abstract

Objective

Although circulating CD14^{bright}CD16+ monocyte subsets are increased in inflammatory disease, the pathogenesis of the increase in the inflammatory condition of the cells is still unclear and the relationship to cytokines is unknown particularly in rheumatoid arthritis (RA). The purpose of this study was to investigate the influence anti-cytokine treatment has on CD14^{bright}CD16+ monocytes in patients with RA.

Methods

Thirty-two RA patients and 14 healthy volunteers (HV) were enrolled in this study. All the patients had never been treated with methotrexate (MTX) or biological agents. Peripheral blood samples and clinical information of the patients were obtained at the time of 0, 12 and 24 weeks of treatment. Peripheral blood samples were also obtained from the HV. The expression levels of CD14 and CD16 on monocytes were measured by flow cytometry (FCM).

Results

Eight patients received anti-interleukin (IL)-6 receptor antibody, tocilizumab (TCZ) treatment alone, 12 patients received anti-tumour necrosis factor (TNF)-α antibody, adalimumab (ADA) with MTX treatment and the others received only MTX treatment. FCM analysis revealed that the proportion of CD14^{bright}CD16+ monocytes significantly increased in patients at baseline compared with HV. The proportion of CD14^{bright}CD16+ monocytes significantly decreased after TCZ, and ADA with MTX treatment. The proportion of intermediate monocytes was significantly and positively correlated with disease activity and it improved in accordance with the proportion of CD14^{bright}CD16+ monocytes after inhibition of signal transduction of inflammatory cytokines.

Conclusion

We showed that the population of CD14^{bright}CD16+ monocytes significantly decreased with the change of disease activity by key cytokines, IL-6 or TNF-α signal blockade in RA. This result indicates that the proportion of those monocytes is important for reflecting disease activity in RA.

Key words

rheumatoid arthritis, monocytes, interleukin-6, tumour necrosis factor-α, CD16

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Received on August 15, 2017; accepted in revised form on October 19, 2017.

Funding: this work was supported by an institutional research grant from Keio University.

Competing interests: M. Tsukamoto has no conflicts of interest to declare. K. Suzuki has received research grants from Eisai Co., Ltd., and Bristol-Myers Squibb. N. Seta has received research grants from Chugai Pharmaceutical Co, Ltd., Mitsubishi Tanabe Pharma Co., Ono Pharmaceutical Co., Ltd. and Nippon Kayaku Co., Ltd. T. Takeuchi has received lecture fees or research grants from Abbott Japan Co., Ltd., Astellas Pharma, Bristol-Myers K.K., Chugai Pharmaceutical Co, Ltd., Daiichi Sankyo Co., Ltd., Eisai Co., Ltd., Janssen Pharmaceutical K.K., Mitsubishi Tanabe Pharma Co., Pfizer Japan Inc., Sanofi-Aventis K.K., Santen Pharmaceutical Co., Ltd., Teijin Pharma Ltd., Asahikasei Pharma Corp., Taisho Toyama Pharmaceutical Co., Ltd. Janssen Pharmaceutical K.K., Astra Zeneca K.K., Eli Lilly Japan K.K., Novartis Pharma K.K., Abbvie GK.

Introduction

Rheumatoid arthritis (RA) is characterised by polyarthritis, which is caused by chronic inflammation in the synovial membranes leading to cartilage and joint destruction. Monocytes play important roles in the pathogenesis of RA (1, 2). Peripheral blood monocytes are derived from precursors in the bone marrow. Circulating monocytes migrate into synovial tissue and differentiate into macrophages and osteoclasts (3). In RA, activated macrophages are enriched in the rheumatoid synovium and produce large amounts of proinflammatory cytokines (4). Bone destruction is mainly attributed to osteoclasts.

Human blood monocytes were divided into CD14^{+}CD16^{+} and CD14^{+}CD16^{-} subsets by CD16 expression 20 years ago (4). Recently, monocytes have been divided into three subsets; CD14^{bright}CD16^{-} subset, CD14^{bright}CD16^{+} subset and CD14^{dim}CD16^{+} subset (6). A new third monocyte, CD14^{bright}CD16^{+} intermediate subpopulation was defined by levels of CD14 expression in CD14^{+}CD16^{+} monocytes and circulating CD14^{bright}CD16^{+} subset that increased in coronary artery disease and inflammatory disease such as infection and auto-inflammatory disease (7). It has been reported that the population of CD14^{bright}CD16^{+} monocytes increases in RA (8). We demonstrated that the proportion of CD14^{bright}CD16^{+} monocytes correlated with RA disease activity in peripheral blood and decreased after methotrexate (MTX) treatment (9), but the response of anti-cytokine treatment to monocyte subsets is still unclear.

Therefore, the purpose of this study is to investigate the influence anti-cytokine treatment has on CD14^{bright}CD16^{+} monocytes in patients with RA.

Materials and methods

Subjects and study design

The study enrolled 32 RA patients (mean age ± SD, 59.4±11.4 years, 23 females) who met the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria, and 14 healthy volunteers (HV) (mean age 49.2±10.8 [range, 30–72], 12 females). All patients visited Keio University Hospital between January 2013 and May 2014 and were untreated with MTX and biological agents. They were considered to have moderate or high disease activity (scoring ≥3.2 on the 28-joint disease activity score based on erythrocyte sedimentation rate [DAS28-ESR]). All participants gave written informed consent in accordance with the Declaration of Helsinki. Eight patients received the humanised anti-interleukin (IL)-6 receptor monoclonal antibody, tocilizumab (TCZ) alone for 12 weeks, while the remaining 24 patients received MTX alone for 12 weeks. After 12 weeks, 12 patients additionally received the humanised anti-tumour necrosis factor (TNF)-α monoclonal antibody, adalimumab (ADA), for inadequate response to MTX, while the other 12 received only MTX treatment. TCZ, ADA and/or MTX treatment was decided by a physician. MTX was initiated at an oral dose of 4–16 mg weekly. Peripheral blood samples were taken from the patients with MTX treatment at baseline and following 12 and 24 weeks, and were taken from the patients with TCZ treatment at baseline and after 12 weeks. Peripheral blood samples were also obtained from the HV. Clinical parameters including C-reactive protein (CRP), ESR, matrix metalloproteinase-3 (MMP-3), anti-cyclic citrullinated peptide antibody (ACPA) and rheumatoid factor (RF) titers were obtained by routine clinical laboratory methods. DAS28-ESR score, DAS28-CRP score, clinical disease activity index (CDAI) and simplified disease activity index (SDAI) were also determined. The clinical characteristics of the patients were retrospectively collected from their medical records.

Monocyte subset determination

Heparinised whole blood was stained with Phycoerythrin-Cy7 (PE-Cy7)-conjugated anti-CD14 (clone M5E2, BD Pharmingen, San Diego CA, USA) and V450-conjugated anti-CD16 antibodies (clone 3G8, BD Horizon, San Jose CA, USA), and were analysed using a flow cytometer with built-in software (MACSQuant Analyzer® and MACSQuantify® software, Miltenyi Biotec, Bergisch Gladbach, Germany). Monocyte subsets were identified on

the basis of forward scatter/side scatter characteristics and CD14 positive gating. Subpopulations of CD14^{bright}CD16^{-}, CD14^{bright}CD16^{+}, and CD14^{dim}CD16^{+} monocytes were distinguished by their surface expression pattern of CD14 and CD16 according to a previous report (7) and the proportion of each monocyte subset was determined.

Statistical analysis

We used commercial statistical software (JMP 12 system®, SAS Institute Inc., Cary NC, USA). The Wilcoxon rank sum test was used to assess the statistical significance of differences between groups. Correlation of two continuous variables was analysed using Spearman's rank correlation coefficient. A p-value <0.05 was considered statistically significant.

Results

Clinical characteristics of the RA patients

Baseline characteristics of the 32 patients are shown in Table I. A total of 68.7% were RF-positive and 61.2% were ACPA-positive. The mean DAS28-ESR score of the patients was 4.97±1.16. The mean MTX dose was 11.1 (8–16) mg/week at 12 weeks. CRP and MMP-3 are higher in the patients who received TCZ than in those who received MTX. Other parameters did not differ between patients treated with TCZ and MTX. There was no significant difference between the patients received MTX with and without ADA treatment. Between 10 and 16 weeks of treatment is included in 12 weeks.

Proportions of each monocyte subset

Figure 1 shows a comparison of the three monocyte subsets between RA patients at baseline and HV. The proportion of CD14^{bright}CD16^{+} monocytes in RA patients was significantly higher than that in healthy volunteers (mean 14.0±7.8% *vs*. 7.4±2.1%) and the CD14^{bright}CD16^{-} population was significantly decreased in RA patients (mean 71.4±10.6% *vs*. 79.7±5.7%) compared to the HV. On the other hand, there was no significant difference in the proportion of CD14^{dim}CD16^{+} monocytes between two groups (mean 8.5±4.7% *vs*. 7.3±4.4%).

Table I. Baseline characteristics of RA patients.

	Total (n=32)	TCZ (n=8)	MTX (n=24)	*p*
Age (years)	59.4 ± 11.4	62.1 ± 8.44	58.5 ± 12.3	0.11
Female, no. (%)	23 (71.8)	4 (50.0)	19 (79.1)	0.61
Duration (months)	43.2 ± 70.2	37.0 ± 47.2	45.3 ± 77.2	0.82
DAS28-ESR	4.97 ± 1.16	5.32 ± 1.81	4.85 ± 0.87	0.84
DAS28-CRP	4.22 ± 1.11	4.80 ± 1.58	4.03 ± 0.86	0.32
CDAI	20.0 ± 12.1	25.4 ± 19.0	18.2 ± 8.7	0.63
SDAI	21.1 ± 13.0	27.8 ± 20.7	18.8 ± 8.8	0.27
CRP (mg/dl)	1.06 ± 1.38	2.35 ± 2.20	0.63 ± 0.58	0.021
ESR (mm/h)	40.1 ± 30.7	57.1 ± 52.7	34.5 ± 17.1	0.71
MMP-3 (ng/ml)	160.8 ± 200.4	336.5 ± 320.4	102.3 ± 92.7	0.038
RF positive, no. (%)	22 (68.7)	44 (100.0)	16 (66.6)	0.65
ACPA positive, no. (%)	19 (61.2)	6 (75.0)	15 (65.2)	0.44

DAS-28: disease activity score in 28-joint count; CDAI: clinical disease activity index; SDAI: simplified disease activity index; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; MMP-3: matrix metalloproteinase-3; RF: rheumatoid factor; ACPA: anti-cyclic citrullinated peptide antibody. Presented as mean and standard deviation (SD).

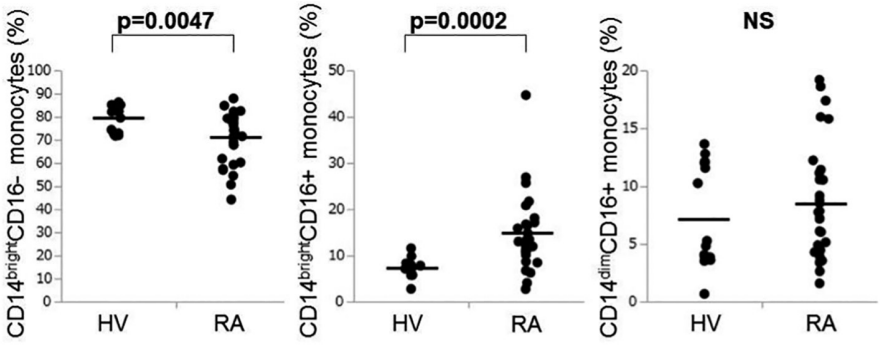

Fig. 1. Proportions of CD14^{bright}CD16^{-}, CD14^{bright}CD16^{+} and CD14^{dim}CD16^{+} monocytes in peripheral blood from RA patients and healthy volunteers at baseline CD14^{bright}CD16^{-}, CD14^{bright}CD16^{+} and CD14^{dim}CD16^{+} monocytes were identified by flow cytometry. The proportion of the three subsets of monocytes in the RA patients at baseline (n=32) was compared with that in the healthy volunteers (HV) (n=14). Statistics: Wilcoxon rank sum test.

After 12 weeks of TCZ treatment, the proportion of the CD14^{bright}CD16^{+} population had significantly decreased from 17.0% to 7.2% and that of CD14^{bright}CD16^{-} monocytes had significantly increased from 67.8% to 80.1% in the RA patients, while there was no significant difference in the proportion of CD14^{dim}CD16^{+} monocytes between baseline and 12 weeks (Fig. 2A). In patients with MTX alone, the CD14^{bright}CD16^{+} population had significantly decreased from 12.8% to 10.1% and that of CD14^{bright}CD16^{-} monocytes had significantly increased from 73.2% to 79.3% at 12 weeks (Fig. 2B). In patients with MTX and ADA, no change was detected in the proportion of CD14^{bright}CD16^{-} and CD14^{bright}CD16^{+} monocytes at 12 weeks of MTX treatment alone (71.8% to 72.8%, 13.3% to 11.6%, retrospectively). The proportion of CD14^{bright}CD16^{-} monocytes significantly increased and that of CD14^{bright}CD16^{+} monocytes significantly decreased at 12 weeks after ADA was added to the treatment (11.6% to 7.8%, 72.8% to 78.7%, retrospectively) (Fig. 2C). The proportion of CD14^{dim}CD16^{+} monocytes did not change after 12 or 24 weeks in all treatment.

Change in RA disease activity after treatment

The change in disease activity after treatment is shown in Figure 3. We utilised DAS28-ESR as a representative indicator for the following analysis. The DAS28-ESR score decreased after treatment with TCZ (A) and MTX (B and C) in a manner which correlated with CD14^{bright}CD16^{+} monocytes.

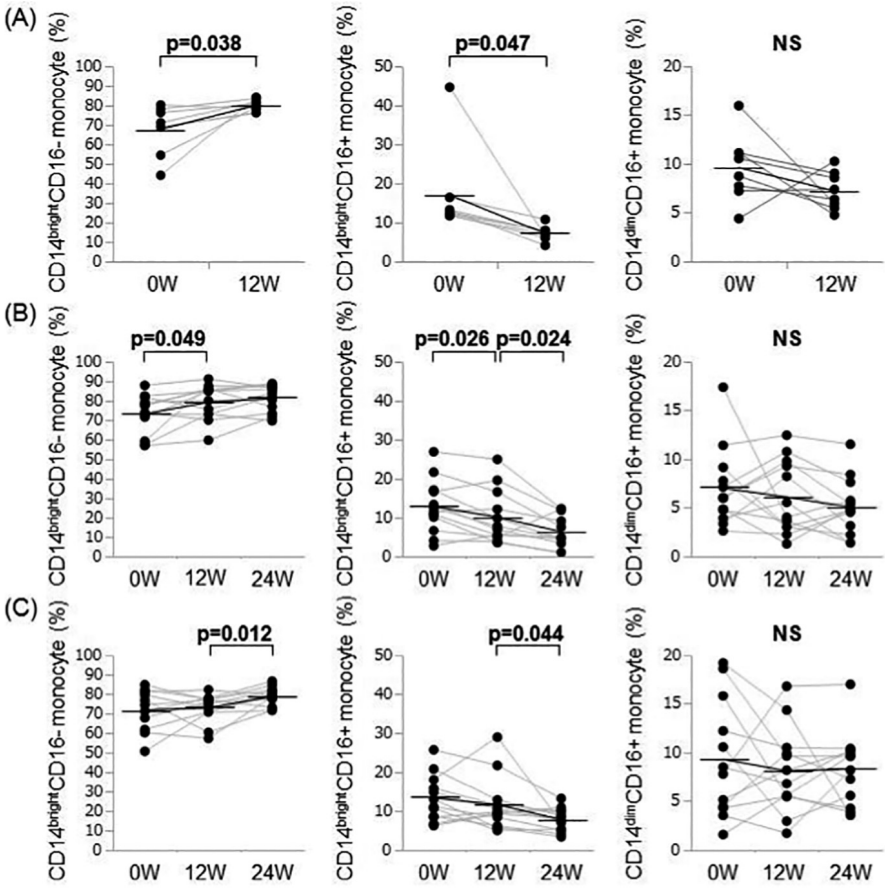

Fig. 2. Change in monocyte subsets after treatment CD14^{bright}CD16^{-}, CD14^{bright}CD16^{+} and CD14^{dim}CD16+ monocytes were identified by flow cytometry. **A**: The proportion of the three subsets of monocytes in RA patients at baseline and 12 weeks after TCZ treatment (n=8). **B**: The proportion of the three subsets of monocytes at baseline, 12 and 24 weeks of MTX treatment in RA patients with MTX alone (n=12). **C**: The proportion of the three subsets of monocytes at baseline, 12 and 24 weeks in the RA patients who received MTX alone for 12 weeks and additionally received ADA because of inadequate response to MTX (n=12). Statistics: Wilcoxon rank sum test. Black bars: mean.

However, at 12 weeks, the DAS28-ESR score in the patients received MTX+ADA treatment (C) was significantly higher than that received MTX alone (B) (p=0.016). The DAS28-ESR score decreased during 12 weeks of ADA treatment added. The DAS28-ESR score in MTX+ADA treatment did not differ from that of MTX alone at 24 weeks.

Association between clinical parameters and CD14^{bright}CD16^{+} monocyte subset

We showed that the relationship between monocyte subsets and DAS28-ESR at baseline (Fig. 3D). The proportion of CD14^{bright}CD16^{+} monocytes was significantly and positively correlated with DAS28-ESR at baseline (p=0.0021, r=0.4), while the proportion of CD14^{bright}CD16^{-} monocytes tended to correlate with DAS28-ESR negatively. Change in CD14^{bright}CD16^{+} monocytes was significantly associated with change in DAS28-ESR after 12 weeks in all the RA patients (p=0.017) (Fig. 3E).

Discussion

In this study, we demonstrated that circulating CD14^{bright}CD16^{+} monocytes are increased in RA patients and decreased after anti-IL-6 treatment or anti-TNF-α treatment in a manner which correlates with decreasing disease activity. In contrast, CD14^{bright}CD16^{-} monocytes are increased after treatment in a manner which correlates with decreasing disease activity.

The role of the CD14^{bright}CD16^{+} subset in RA has not been fully clarified. It has been shown that the CD14^{bright}CD16^{+} monocyte population increases in inflammatory or infectious conditions and, upon lipopolysaccharide stimulation, produces TNF-α, IL-1β, IL-6, and IL-10 (7, 10). Although an increase in CD14^{bright}CD16^{+} monocytes in RA patients has also been reported (8), no report has yet investigated the possibility of correlations between CD14^{bright}CD16^{+} monocytes and anti-cytokine treatment in active RA patients. When circulating human monocytes were divided into two subsets by CD16 expression, CD14^{+}CD16^{+} monocytes correlated with disease activity and bone destruction (12, 13). The proportion of CD14^{+}CD16^{-} monocytes significantly increased (11), while that of CD14^{+}CD16^{+} monocytes significantly decreased after eighty-four days of anti-TNF-α antibody, infliximab treatment in five RA patients (12). The proportion of CD14^{+}CD16^{+} monocytes and disease activity decreased after biological treatments in the 14 RA patients who received MTX with ADA, TCZ or other biological agents (certolizumab pegol, etanercept or abatacept) (13). Monocytes were divided into three subsets in our study. Our results supported previous observations due to larger numbers that were enrolled and again showed a correlation between monocyte subsets and anti-cytokine treatment. We previously showed that the proportion of CD14^{bright}CD16^{+} monocytes decreased after MTX treatment (9) and again showed that they decreased after TCZ or ADA treatment in a manner which correlated with reduced disease activity, DAS28-ESR.

It was reported at that time that CD14^{bright}CD16^{+} monocytes may migrate into the synovium from peripheral blood and differentiate into M1 or M2 macrophages in the tissue (14). CD14^{bright}CD16^{+} monocytes increased under Crohn's disease and bacterial infection (7) and decreased after infliximab (IFX), anti-TNF-α antibody treatment (15). IFX treatment increased the TNF production of macrophages from patients with Crohn's disease in response to bacteria (15). In addition, IFX treatment increased IL-10 production from M2 macrophages induced by IFX in response to bacteria. In RA, immunoregulation of M2 macrophages may also

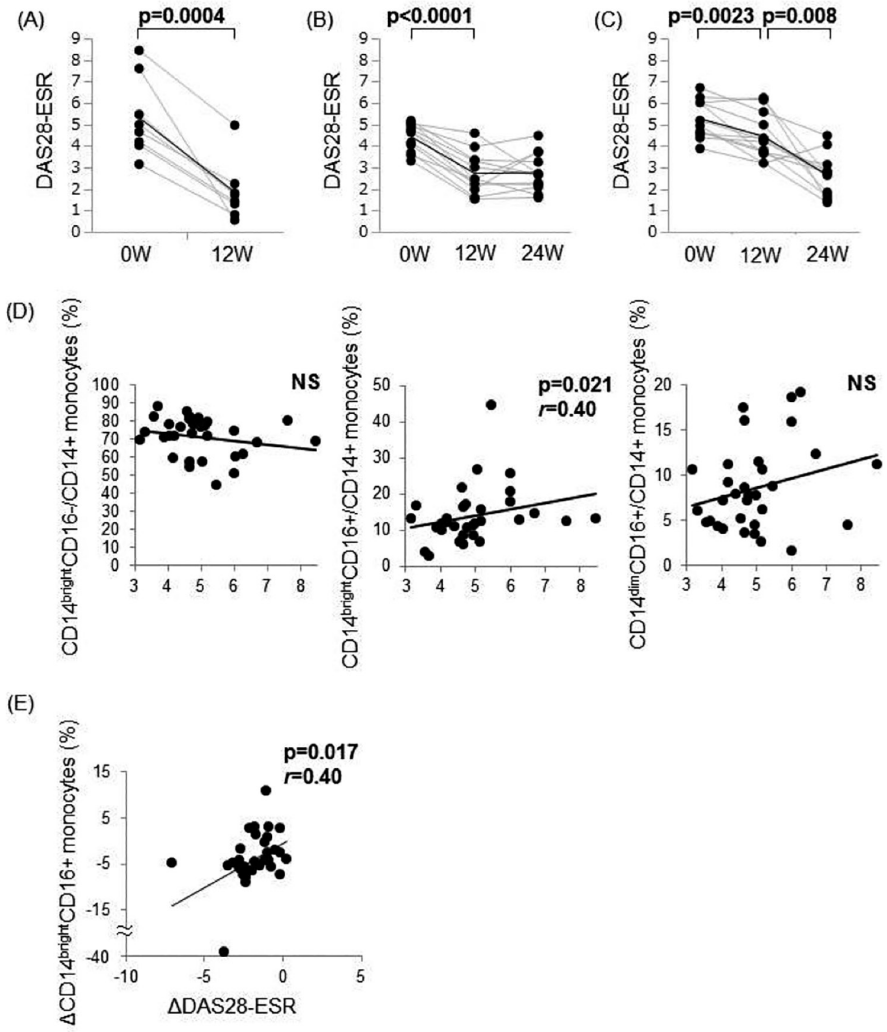

Fig. 3. Change in RA disease activity after treatment.
The DAS28-ESR score was evaluated at baseline, 12 and/or 24 weeks of TCZ (**A**), MTX (**B**) or MTX+ADA (**C**) treatment. **A**: DAS28-ESR in RA patients at baseline and 12 weeks of TCZ treatment (n=8). **B**: DAS28-ESR at baseline, 12 and 24 weeks of MTX treatment in RA patients with MTX alone (n=12). **C**: DAS28-ESR at baseline, 12 and 24 weeks in the RA patients who received MTX alone for 12 weeks and additionally received ADA for clinical inadequate response to methotrexate (n=12). The DAS28-ESR score in C was significantly higher than that in B at 12 weeks of treatment of MTX (p=0.016). **D**: Correlation between the proportion of $CD14^{bright}CD16^{-}$, $CD14^{bright}CD16^{+}$ and $CD14^{dim}CD16^{+}$ monocytes and DAS28-ESR at baseline in patients with RA (n=32). **E**: Change in $CD14^{bright}CD16^{+}$ monocytes was significantly associated with change in DAS28-ESR after 12 weeks in all RA patients. Statistics: Wilcoxon rank sum test (A, B, C) or Spearman's rank correlation coefficient (D, E). Black bars: mean.

be induced by anti-cytokine blockade that may control inflammation.

We note two limitations of the study. First, the number of patients was relatively still small, albeit large enough to provide statistically significant data. Second, we could not show that $CD14^{bright}CD16^{+}$ monocytes are directly associated with cytokines in RA. The functions of these monocytes in RA will need to be clarified in future studies.

In conclusion, CD14brightCD16+ intermediate monocytes significantly decreased with the change of disease activity by key cytokines, IL-6 or TNF-α signal blockade in RA. This result indicates that the proportion of circulating monocytes may be important for reflecting disease activity in RA.

Acknowledgments

The authors sincerely thank Ms Keiko Yoshimoto, Ms Harumi Kondo and Ms Mayumi Ota for helping with this research and the acquisition of clinical information.

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