Original Article

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Increased Expression of TLR8 in Peripheral Blood CD16+ Monocytes Positively Correlates with Disease Activity in Anti-CCP+ Rheumatoid Arthritis Patients

Tsun-Mei Lin[1,3], Jer-Yiing Houng[4,5], Shih-Wei Wang[2,6,]*

Objective: Toll-like receptor (TLR) 8 modulates immune responses in monocytes and contributes to autoimmune inflammation. Nevertheless, the relevance of TLR8 in rheumatoid arthritis (RA) remains unclarified. The aim of our study was to investigate TLR8 expression monocytes in peripheral blood (PB) of RA patients and its association with disease activity.

Methods: We performed reverse transcription-polymerase chain reaction and flow cytometry to detect TLR8 expression in PB from RA patients and healthy controls (HCs). We then analyzed the association between TLR8 expression and disease activity.

Results: The percentage of TLR8 expression in inflammatory PB CD16+ monocytes in RA patients was significantly higher than in HCs ($p < 0.001$). The percentage of TLR8 expression in PB CD16+ monocytes from RA patients was significantly higher than in CD16- subset ($p < 0.001$). Notably, the major significant difference in the percentage of TLR8 expression in PB CD16+ monocytes was found in anti-cyclic citrullinated peptide antibody (anti-CCP) + RA patients ($p < 0.001$). Additionally, the percentage of TLR8 expression in PB CD16+ monocytes positively correlated with disease activity measured by DAS28-CRP in anti-CCP+ RA patients ($r = 0.352$, $p = 0.048$) but showed no significant correlation in the anti-CCP- subset.

Conclusions: TLR8 expression was significantly increased in inflammatory PB CD16+ monocytes from RA patients, especially in the anti-CCP+ subset, and TLR8 expression positively correlated with disease activity. TLR8 might contribute to the pathogenesis of RA, especially in the anti-CCP+ subset.

Key words: TLR8, CD16+ monocytes, anti-CCP, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA), one of the most prevalent autoimmune diseases, is charac-

From the [1]Department of Medical Research and [2]Division of Allergy, Immunology, and Rheumatology, Department of Internal Medicine, E-Da Hospital; [3]Department of Medical Laboratory Science, [4]Department of Nutrition, [5]Graduate Institute of Biotechnology and Chemical Engineering and [6]School of Medicine, College of Medicine, I-Shou University, Kaohsiung, Taiwan.

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* Address reprint request and correspondence to: Shih-Wei Wang, Division of Allergy, Immunology, and Rheumatology, Department of Internal Medicine, E-Da Hospital, No.1, Yida Road, Jiaosu Village, Yanchao District, Kaohsiung City 82445, Taiwan.

Tel: +886-7-615-0011 ext. 5232, Fax: +886-7-615-0940, E-mail: shihwei8888@gmail.com

terized by erosive polyarthritis, joint destruction, and disability. Among those factors that contribute to the pathogenesis of RA, innate immunity is promising. Innate immune cells promote adaptive immunity and result in autoantibody production in the initiation of experimental arthritis models.[1] The innate immune system, including toll-like receptor (TLR), involves autoimmune and inflammatory diseases,[2] and responds to endogenous ligands, which make them promising candidates in the maintenance of RA's inflammation. TLRs contribute to autoimmune arthritis development, resulting from lower incidence and decreased severity of collagen-induced arthritis in TLR deficient DBA mice.[3] Most TLRs are expressed on the cell surface and recognize mainly bacterial products, while TLR3, 7, 8, and 9 respond to self and foreign nucleic acid structures within endosomal compartments.[4] TLRs protect against infection and magnify the inflammatory and destructive process in RA.[5] TLR8 senses bacterial RNA in human monocytes in response to infection and trigger monocytes' activation.[6] Transgenic mice expressing different levels of human TLR8 develop severe inflammation targeting joints, and the severity of the specific phenotypes closely correlates with TLR8 expression levels.[7] TLR8 may play a pathogenic role in human disease because increased TLR8 expression in patients with systemic arthritis correlated with elevated interleukin (IL)-1beta levels and disease status.[7] Also, single-stranded RNA sequences containing defined sequence motifs can preferentially activate human TLR8-mediated immune responses.[8] The inhibition of TLR8 is effective in preventing inflammation and disease development in lupus.[9] The expression of TLR8 increases in RA-mice induced by chicken II collagen.[10] Moreover, TLR8 expression exists in human RA tissue, and inhibitors of TLR8 reduce tumor necrosis factor (TNF) production from human rheumatoid synovial membrane cultures.[11] Moreover, a significant association between the rheumatoid factor (RF) and TLR8 rs5741883 exists in RA patients based on multiplex screening of single-nucleotide polymorphisms in various TLRs.[12] Reduced RA disease severity relates to the M1V variant of TLR8 due to reduced production of inflammatory cytokines, including IL-1beta, IL-6, and TNF-α in response to TLR8 agonists.[13] ODN1411, capable of competitively inhibiting TLR8 signaling, can inhibit cytokine production from human RA synovial membrane cultures.[14] Notably, TLR8 modulates different immune responses in monocytes and is expressed maximally in CD14+ mononuclear cells of PB.[15] Human monocytes express TLR8 mRNA, and TLR8 agonists can induce IL-6, TNF-α, and IL-10 production.[16] The identified TLR8 RNA motifs signal via TLR8 and induce the secretion of T helper 1-like and pro-inflammatory cytokines from TLR8-expressing monocytes.[8] Notably, monocytes participate in the inflammation of RA, and targeting monocyte is a crucial mechanism to inhibit inflammation and erosion in RA.[17] Peripheral blood (PB) monocytes recruited into joints and synovial tissue macrophages with TLRs expression contribute to the persistent joint inflammation of RA.[18] Upregulated TLR8 gene expression in PB mononuclear cells from RA patients correlates with the disease activity.[19]

Collectively, TLR8 expression in PB monocytes is a potential factor to contribute to the pathogenesis of RA. Our present study investigates the association between TLR8 expression in the PB monocytes and clinical indicators, including disease activity and inflammatory biomarkers in RA patients.

Materials and Methods

Study subjects

Forty-nine consecutive patients diagnosed with RA based on the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) criteria

were recruited. Patients with infection, malignancy, or autoimmune diseases other than RA were excluded. The disease activity of each RA patient was determined according to the Disease Activity Score in 28 joints (DAS28). The study was approved by the Human Research Ethics Committee at E-DA Hospital. All subjects provided informed consent before enrollment. Additionally, 43 age- and sex-matched HCs were recruited for comparison.

Laboratory data analysis

Laboratory tests, including anti-cyclic citrullinated peptide antibody (anti-CCP), rheumatoid factors (RA), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) performed at the clinical laboratory of E-DA Hospital. Anti-CCP status was defined as either anti-CCP-positive (≥ 7 U/mL) based on the laboratory's cutoffs.

Determinations of TLR8 mRNA expressions by quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from whole blood using Pharmigeng autoex16 genomic RNA Purification Whole Blood Kit (Pharmigeng, Inc, Taipei, Taiwan) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by reverse transcription with oligo-dT primers. The mRNA expressions of *TLR8* were analyzed by quantitative RT-PCR with Bio-Rad, CFX96 System (Bio-Rad, Hercules, CA).

Intracellular TLR8 protein expression assay on monocytes

The intracellular protein expression of TLR8 in monocytes was determined by flow cytometric analysis. First, 250 μL of freshly collected EDTA-whole blood was stained with 5 μL of PE-conjugated anti-CD14 (clone 340682) or anti-CD16 (clone 555406) antibody (BD Pharmingen, San Jose, CA) for 30 minutes at room temperature. For intracellular staining, the remaining leukocytes were fixed and permeabilized by adding 250 μL of Fixation and Permeabilization Solution (BD Pharmingen, San Jose, CA) for 20 minutes at 4°C, after which cells were incubated with two μL of FITC-conjugated anti-TLR8e (eBioscience™ NBP2-24817) (Thermo Fisher Scientific, Waltham, MA) diluted antibodies for 1

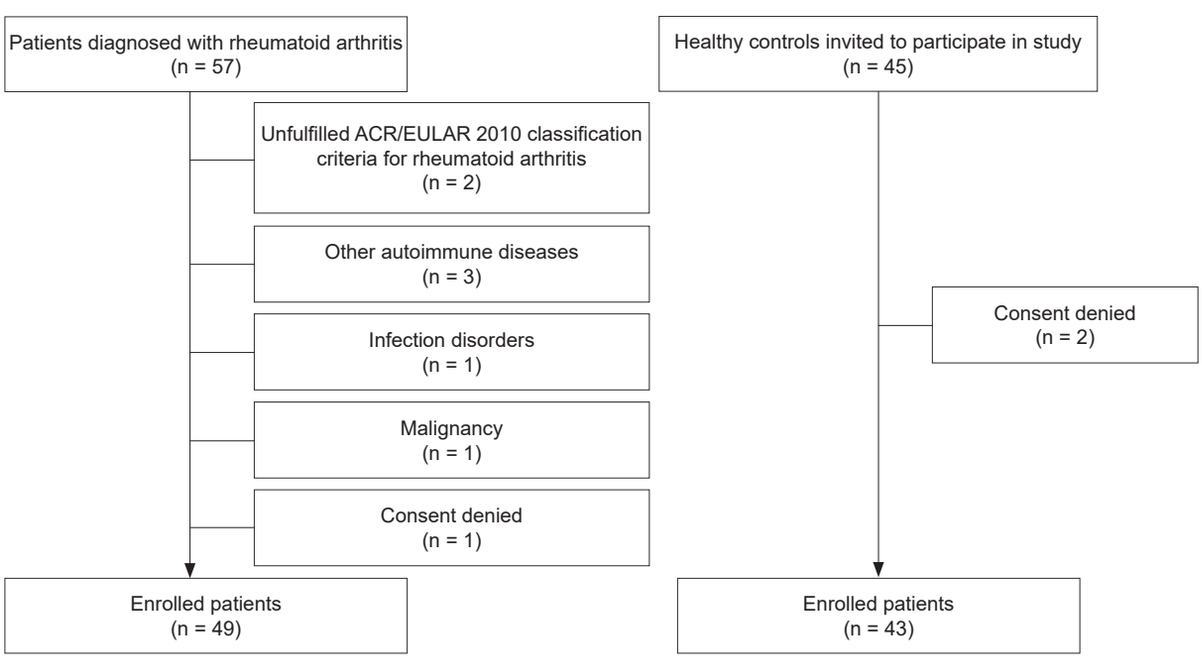

Fig. 1 Flow chart of case enrollment for rheumatoid arthritis (RA) patients and controls.

hour at 4°C. Cells were finally resuspended in PBS and analyzed on a FACSCalibur flow cytometer (BD Pharmingen, San Jose, CA) with WinMDI98 software.

Statistical analysis

Statistical Package for the Social Sciences (SPSS Inc., Chicago, Illinois, USA, version 18.0) was used for statistical analysis. Quantitative results are given as means +/- standard deviation (SD) and assessed with nonparametric Mann-Whitney U test. Spearman's correlation coefficient by rank test was used to assess the correlation between TLR8 expression and clinical indicators, including RA's disease activity. Values of $p < 0.05$ were considered to be significant.

Results

Demographic and clinical characteristics of healthy controls and RA patients

A total of 49 patients with RA (aged 35 to 85 years, mean 62) and 43 controls subjects (aged 41 to 74 years, mean 62) were enrolled. Figure 1 shows the flow chart of case enrollment. Fifty-seven RA patients were invited to participate in this study and 8 patients were excluded due to unfulfilled ACR/EULAR 2010 classification criteria for RA (n = 2); other autoimmune diseases (n = 3); infection disorders (n = 1); malignancy (n = 1); or consent denied (n = 1). Forty-five HCs were invited to participate in our study and 2 controls were excluded due to consent denied. Table 1 shows the detailed clinical characteristics. Age and sex between groups showed no significant differences ($p = 0.96$ and 0.78, respectively). ESR and CRP were significantly higher in RA patients than in control subjects ($p < 0.001$ and 0.001, respectively).

The percentage of TLR8 expression in PB CD16+ monocytes was significantly higher in RA patients

The percentage of TLR8 expression in inflammatory CD16+ monocytes in RA patients was significantly higher than in HCs (53.8 ± 15.1% vs. 39.5 ± 14.8%, $p < 0.001$) (Fig. 2A). The major significant difference in the percentage of TLR8 expression in CD16+ monocytes

Table 1. Clinical characteristics of the study subjects.

Characteristics	Normal controls* (n = 43)	RA patients* (n = 49)	p†
Age (years)	62.1 ± 7.3	62.0 ± 11.9	0.96
Female (%)	31 (72.1)	34 (69.4)	0.78
RF	NA	194.7	NA
Anti-CCP (U/mL)	NA	159.0 ± 148.9	NA
Anti-CCP- positive: n (%)§	NA	39 (79.6)	NA
ESR (mm/hr)	15.2 ± 12.7	35.3 ± 25.3	< 0.001
CRP (mg/L)	2.1 ± 1.8	10.4 ± 15.1	0.001
DAS28-CRP	NA	3.1 ± 0.9	NA

RA: rheumatoid arthritis; RF: rheumatoid factor; Anti-CCP: anti-cyclic citrullinated peptide antibody; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; NA: not applicable; DAS28-CRP: Disease Activity Score of 28 joints using CRP

* Data are expressed as mean and standard deviation (SD).

† $p < 0.05$ was considered statistically significant.

§ Thirty-nine out of 49 RA subjects were anti-CCP-positive.

The data are expressed as n (%), mean ± standard deviation (SD).

was found in anti-CCP+ RA patients (54.3 ± 14.8% vs. 39.5 ± 14.8%, $p < 0.001$) (Fig. 2B).

TLR8 was expressed mainly in the CD16+ subset of PB monocytes in RA subjects

CD16 expression distinguishes a subset of monocytes with highly pro-inflammatory properties from non-CD16 expressing "classical" monocytes. We demonstrated that the percentage of TLR8 expression in CD16+ monocytes in RA patients was significantly higher than in CD16- subset (53.8 ± 15.1% vs. 5.0 ± 2.0%, $p < 0.001$) (Fig. 3A). TLR8 expression (MFI) in CD16+ monocytes was higher than in CD16- subset in RA patients (123.9 ± 53.9 MFI vs. 90.9 ± 18.5 MFI, $p < 0.001$) and in anti-CCP+ RA patients (127.0 ± 58.8 MFI vs. 90.2 ± 18.9 MFI, $p = 0.001$) (Fig. 3B). In contrast, the expression of TLR8 (MFI) in PB monocytes showed no significant difference between CD16+ and CD16- subsets in anti-CCP- RA subjects (115.5 ± 42.6 MFI vs. 91.2 ± 17.7 MFI, $p = 0.093$) (Fig. 3B).

TLR8 expression in PB CD16+ monocytes positively correlated with TLR8 mRNA and disease activity in RA patients

There was a positive correlation between TLR8 expression (MFI) in CD16+ monocytes and TLR8 mRNA (folds) in RA patients ($r = 0.355$, $p = 0.021$) (Fig. 4A).

The association of PB TLR8 expression with CRP and disease activity was analyzed. The percentage of TLR8 expression in PB

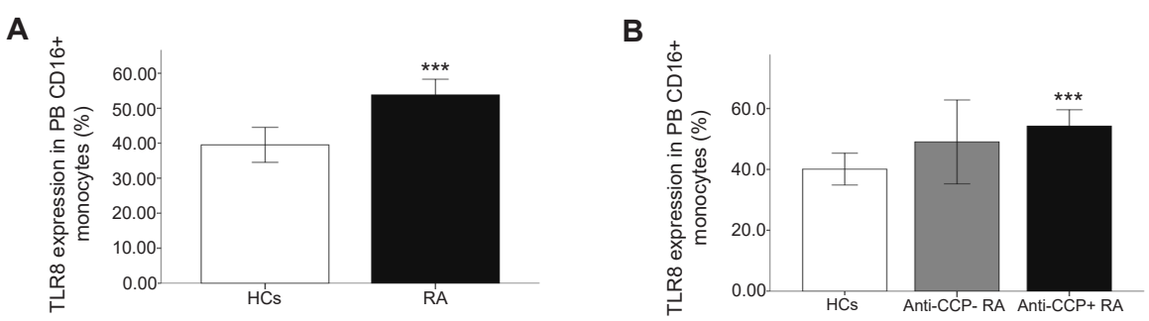

*Fig. 2 The difference of TLR8 expression in CD16+ monocytes between RA patients and HCs. (A) The percentage (%) of TLR8 expressions in CD16+ monocytes in the RA subjects and HCs. (B) The percentage (%) of TLR8 expressions in PB CD16+ monocytes in HCs and the subsets of RA patients. Differences between individual groups were analyzed by the Mann-Whitney test and are described as * (***p < 0.001).*

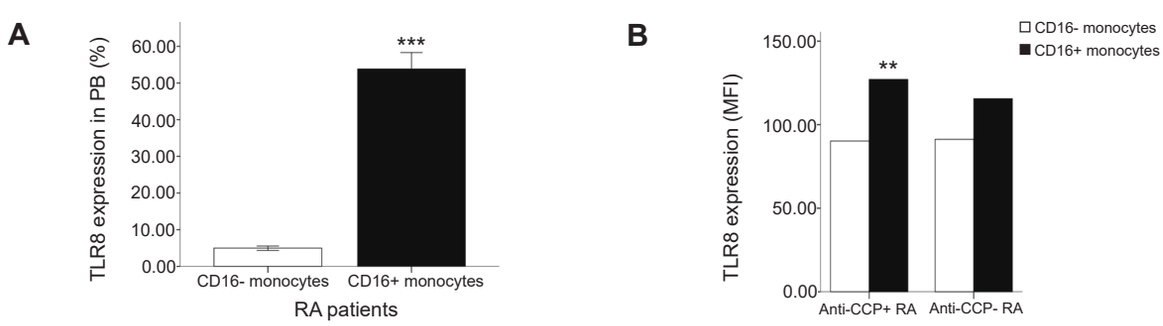

*Fig. 3 TLR8 expression in the subsets of PB monocyte in RA patients. (A) The percentage (%) of the cell-expressing TLR8 in PB monocyte subset in RA subjects. (B) The difference of TLR8 expression (MFI) between PB CD16- monocytes and CD16+ subset in different subsets of RA patients. Differences between individual groups were analyzed by the Mann-Whitney test and are described as * (**p < 0.01 and ***p < 0.001). MFI: mean fluorescence intensity.*

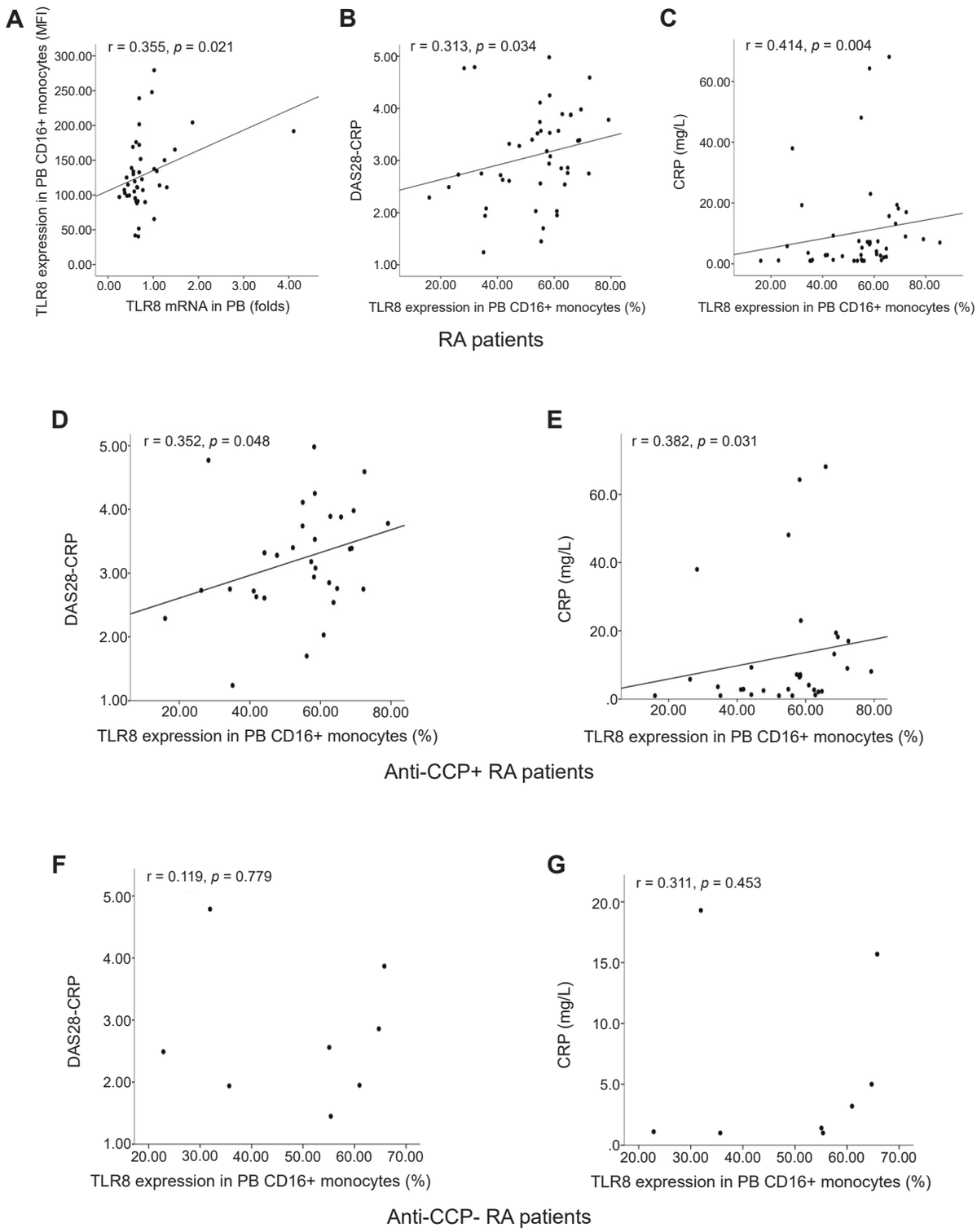

Fig. 4 Relationship between TLR8 expression in PB CD16+ monocytes and clinical indicators in RA patients. (A) The expression of TLR8 (MFI) in PB CD16+ monocytes and its association with TLR8 mRNA (folds) in RA patients. The association between the percentage of TLR8 expression in CD16+ monocytes and (B) disease activity measured by DAS28-CRP and (C) CRP in RA patients. The relationship between the percentage of TLR8 expression in CD16+ monocytes and (D) DAS28-CRP and (E) CRP in anti-CCP+ RA patients. The relationship between the percentage of TLR8 expression in CD16+ monocytes and (F) DAS28-CRP and (G) CRP in anti-CCP- RA patients. Statistical hypothesis testing was performed using Spearman's rank correlation.

CD16+ monocytes showed a positive correlation with disease activity measured by disease activity score of 28 joints using CRP (DAS28-CRP) (r = 0.313, p = 0.034) (Fig. 4B) and CRP (r = 0.414, p = 0.004) (Fig. 4C) in RA patients. The percentage of TLR8 expression (%) in PB CD16+ monocytes in anti-CCP+ RA patients also significantly correlated with DAS28-CRP (r = 0.352, p = 0.048) (Fig. 4D) and CRP (r = 0.382, p = 0.031) (Fig. 4E), while no significant correlation was noted in anti-CCP- RA patients (Fig. 4F & 4G).

Discussion

Our present investigation revealed that the percentage of TLR8 expression in PB CD16+ monocytes from RA patients was significantly higher than that in HCs. We also demonstrated that the TLR8 expression in PB CD16+ monocytes in RA patients was significantly higher than that in the CD16- subset. Most circulating monocytes strongly express CD14, but not CD16 (CD14+CD16-), and a smaller group of monocytes express lower levels of CD14 and CD16 (CD14+/CD16+).[20] CD16+ monocytes, acting in an immediate innate immune response, are more phagocytic and produce higher TNF.[21] CD16+ monocytes are predominant in several active inflammatory bone loss diseases, including RA. The percentage of CD16+ monocytes in RA patients is significantly higher than in controls and correlates with RA disease activity score.[22] Besides, the number of CD16+ monocytes is reduced after anti-TNF-alpha treatment in patients with RA.[23] Notably, our present study showed that the percentage of TLR8 expression in PB CD16+ monocytes significantly correlated with disease activity measured by DAS28-CRP and CRP in RA patients. Those mentioned above implied that the expression of TLR8 in PB CD16+ monocytes may contribute at least partially to RA's pathogenesis.

The present investigation also demonstrated that the major significant difference in the percentage of TLR8 expression in CD16+ monocytes was found in anti-CCP+ RA patients, not in those with anti-CCP- RA. On the other hand, TLR8 expression (MFI) in PB CD16+ monocytes was higher than that in the CD16- subset in anti-CCP+ RA patients, while showing no significant difference in anti-CCP- RA subjects. Anti-citrullinated protein antibodies (ACPAs), including anti-CCP, are a group of autoantibodies targeted against citrullinated proteins/peptides and can predict radiographic joint damage early in the course of RA. RA patients with higher disease activity, more erosions, and more radiological severe damage have high anti-CCP.[24] Anti-CCP antibody concentrations are lower in TLR deficient mice in parallel to the reduced clinical severity.[3] TLR4-dependent cytokine induction is more frequent in ACPA+ compared to ACPA- RA patients and a subset of ACPA bound to their specific autoantigens in immune complexes can activate cells via TLR.[25] Immune complexes formed by the interaction of RA-specific ACPAs with their main target antigen induce TNFα secretion from PB monocytes via TLR.[26] ACPAs can promote IL-1beta production by PBMC-derived macrophages, and levels of IL-1beta elevate in ACPA+ RA patients.[27] ACPAs can bind to surface-expressed citrullinated Grp78 and enhance TNF-alpha production in monocytes.[28] IL-20 levels, triggered by TLR ligands, are independently associated with RA disease activity measured by DAS28 and are significantly higher in anti-CCP+ RA than in anti-CCP- RA.[29] Triggering of TLR via endogenous TLR ligands can synergize with IgG-ACPA-mediated activation and significantly increase cytokine production in RA patients.[30] ACPA+ RA patients have immune complexes containing citrullinated fibrinogen, which can co-stimulate macrophages via dual engagement of TLR-4 and Fc gamma receptor and induce TNF.[26] Our current study demonstrated that TLR8 expression in PB CD16+ monocytes from anti-CCP+ RA patients positively cor-

related with disease activity measured by DAS28-CRP, but failed to reveal significant correlation in patients with anti-CCP- RA. Taken together, our results implied that TLR8 expression in PB CD16+ monocyte might play a pathogenic role in anti-CCP+ RA. There were limitations to this study. (1) Our sample size was relatively small, although the results were significant. Further investigation with a larger sample size is needed to verify our findings. (2) Our research lacked imaging data to reveal the association between TLR8 expression and RA progression.

In conclusion, we found that TLR8 expression was significantly increased in PB CD16+ monocytes in RA patients, especially in the anti-CCP+ subset, and positively correlated with disease activity. TLR8 could be a potential target in treating RA, and the underlying molecular mechanisms deserve further investigation.

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Conflict of Interest

The authors have no financial conflict of interest.

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