# Increased TIGIT$^+$PD-1$^+$CXCR5$^-$CD4$^+$T cells are associated with disease activity in rheumatoid arthritis

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**Abstract.** It is well established that increased programmed cell death protein 1 (PD-1)$^+$C-X-C chemokine receptor type 5 (CXCR5)$^-$ CD4$^+$T cells are found in patients with rheumatoid arthritis (RA). T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) is a co-inhibitory receptor that is expressed on T cells. However, the expression patterns and immunomodulatory roles of TIGIT on PD1$^+$CXCR5$^-$CD4$^+$T cells in RA are poorly understood. Patients with RA were recruited and their clinical characteristics were recorded. The expression level of TIGIT on PD-1$^+$CXCR5$^-$CD4$^+$T cells was examined using flow cytometry. Subsequently, the correlation between various parameters of TIGIT$^+$PD-1$^+$CXCR5$^-$CD4$^+$T cells [percentage of the cells, mean fluorescence intensity (MFI) of PD-1 and TIGIT in the cells] in patients with RA and clinical data, including autoantibodies, inflammatory indicators and RA activity, were analyzed. In addition, the risk factors of RA were assessed using univariate and multivariate regression analyses. The percentages of TIGIT$^+$CXCR5$^-$CD4$^+$T, PD-1$^+$CXCR5$^-$CD4$^+$T, TIGIT$^+$PD-1$^+$CXCR5$^-$CD4$^+$T, TIGIT$^-$PD-1$^+$CXCR5$^-$CD4$^+$T cells, the MFI of PD-1 in these cells and the MFI of TIGIT in TIGIT$^+$PD-1$^+$CXCR5$^-$CD4$^+$T cells were revealed to be significantly increased in patients with RA compared with those in healthy individuals. The parameters of TIGIT$^+$PD-1$^+$CXCR5$^-$CD4$^+$T cells (percentage and/or MFI of PD-1) in patients with RA were found to be associated with the levels of anti-cyclic citrullinated peptide antibodies, rheumatoid factor and inflammatory markers, such as lymphocyte count, lymphocyte percentage, neutrophil percentage, lymphocyte-to-monocyte ratio, neutrophil-to-lymphocyte ratio, systemic immune inflammation index, derived neutrophil-to-lymphocyte ratio, erythrocyte sedimentation rate and C-reactive protein. In addition, the MFI of PD-1 in TIGIT$^+$PD-1$^+$CXCR5$^-$CD4$^+$T cells was associated with a disease activity score of 28 and the patient visual analogue scale. Multivariate logistic regression analysis demonstrated that the percentage of TIGIT$^+$PD-1$^+$CXCR5$^-$CD4$^+$T cells and the MFI of PD-1 in TIGIT$^+$PD-1$^+$CXCR5$^-$CD4$^+$T cells were risk factors for RA. The present study suggests that the increase in TIGIT$^+$PD-1$^+$CXCR5$^-$CD4$^+$T cells is associated with the production of autoantibodies and RA activity and may serve a role in RA pathogenesis.

## Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune rheumatic disease that can cause bone erosion and significant disability, decreasing the quality of life of patients (1). The prevalence rate of this disease is 0.5-1%, with an increasing prevalence among women compared with men worldwide (1). Although the pathogenesis of RA is not completely understood, considerable insight into the role of CD4$^+$T cells in the development of RA has been gained over the past decade (2-4). In general, patients with RA exhibit increased numbers of cytotoxic CD4$^+$T cells in the peripheral blood, hyperactive CD4$^+$T cell function with CD80/CD86-CD28 interaction, which may be associated with changes in the activation of B lymphocytes and antibody production (2-4).

Antibody production by B cells is strongly dependent on T helper cells, particularly C-X-C chemokine receptor type 5 (CXCR5)$^+$ follicular T helper cells (5,6). There is considerable evidence that circulating the proportion of CXCR5$^+$ follicular T helper cells is increased in patients with RA (7-9). Previously, a novel population of CD4$^+$ T cells with B cell-potentiating capacity, named peripheral T helper cells, has been characterized in the synovium and peripheral blood of individuals

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with seropositive RA (10). Peripheral T helper cells are characterized by the lack of Bcl-6 and CXCR5 expression but do express programmed cell death protein 1 (PD-1), they are called PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells. These PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells appear to be phenotypically similar to CXCR5$^+$ follicular T helper cells, since they express factors associated with B cell activation, including IL-21 and inducible T cell costimulator, which were shown to serve an important role in RA development (10).

T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) is a co-inhibitory receptor that is expressed on T and natural killer cells (11). Several studies have previously shown that TIGIT expression can be detected on PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells (10,12,13). In addition, Godefroy *et al* (14) previously reported that TIGIT-positive circulating follicular helper T cells exhibit potent B cell activation properties. However, the effect of TIGIT as a surface marker of human peripheral PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells and the role of peripheral TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells in RA remains poorly understood.

In the present study, TIGIT and PD-1 were used to characterize the phenotype and role of circulating CXCR5$^-$ CD4$^+$ T subsets in patients with RA, whilst also testing the hypothesis that TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells are associated with RA activity.

## Patients and methods

*Patients*. A total of 82 patients with RA (female, 56; mean ± SEM of age, 57.65±12.38 years) that were admitted to the Department of Rheumatology, First Affiliated Hospital of Nanchang University (Nanchang, China) were enrolled into the present study and designated the RA group from July 2017 to September 2019. All RA cases fulfilled the revised American College of Rheumatology criteria for RA (15). Patients with RA with other autoimmune, inflammatory, or hormonal diseases, cancers or mental disorders were excluded. Information on tender joint count (TJC), swollen joint count (SJC), patient visual analogue scale (VAS), disease activity score 28 (DAS28), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), anti-cyclic citrullinated peptide antibodies (anti-CCP), white blood cell count (WBC), red blood cell count, platelet count (PLT), hematocrit, hemoglobin, lymphocyte count (L) and percentage (L%), monocyte count (M) and percentage (M%), neutrophil count (N) and percentage (N%), lymphocyte-to-monocyte ratio (LMR), neutrophil-to-lymphocyte ratio (NLR), derived NLR (dNLR), platelet-to-lymphocyte ratio (PLR) and systemic immune inflammation index (SII), were all collected. The VAS was used for pain assessment, asking the patient to locate on a 100 mm line the point that best identified the intensity of pain in the previous week (where 0 represents no pain and 100 mm represents the maximal pain perceived) (16). DAS28 values were calculated as follows: DAS28 (CRP)=0.56x$\sqrt{}$(TJC28) +0.28x$\sqrt{}$(SJC28)+0.014xVAS+0.36xln(CRP+1)+0.96; DAS28 (ESR)=0.56x$\sqrt{}$(TJC28)+0.28x$\sqrt{}$(SJC28)+0.014xVAS+0.70xln (ESR) (17). In the same period, 46 age- and sex-matched healthy individuals (female, 28; mean ± SEM of age, 53.46±14.22 years) without a clinical diagnosis of any autoimmune diseases and free of other inflammatory conditions were recruited as the healthy controls (HC). The patient characteristics of the two groups are shown in Table I. The research protocol complied with the principles outlined in the Declaration of Helsinki and was approved by the Medical Ethical Committees of the First Affiliated Hospital of Nanchang University [approval no. (2022) CDYFYYLK (06-004)]. Written informed consent was obtained from the patients.

*Flow cytometry*. Peripheral blood mononuclear cells (PBMC) were freshly isolated from the blood samples collected from patients with RA and the HC using the human peripheral blood lymphocyte separation medium (Sigma-Aldrich; Merck KGaA) as follow: First, FicollPaque gradient, a lymphocyte isolation solution (Sigma-Aldrich; Merck KGaA), was added to the centrifuge tube. After the anticoagulant peripheral blood and sterile phosphate buffer solution (PBS) were thoroughly mixed in accordance with 1:1, the mixture was used to slowly superposition on the FicollPaque gradient surface along the tube wall by pipette (peripheral blood:PBS:FicollPaque gradient=1:1:1). Then, centrifuge horizontally at 400 g x for 30 min, at 20˚C. After centrifugation, it could be seen that the mixture was divided into three layers, the upper layer consisted of plasma and PBS, the lower layer consisted mainly of red blood cells and granulocytes, and the middle layer consisted of FicollPaque gradient. At the upper and middle interface there is a narrow band of white cloud layer mainly dominated by PBMC. Subsequently, PBMC was sucked up from white cloud layer by pipette and added to a new centrifuge tube. PBS (Solarbio; Life Sciences) was added to the PBMC in new centrifuge tube, and the mixture was centrifuged horizontally at 200 g x for 10 min, at 20˚C. The supernatant was decanted after centrifugation, and PBMC was washed using PBS twice. The phenotypes of TIGIT and PD-1 in CXCR5$^-$CD4$^+$T cells were detected immediately using flow cytometry. The following conjugated monoclonal antibodies (mAbs) were used for flow cytometry: Phycoerythrin (PE)-Texas red-conjugated anti-CD4 (PE-Texas red/ECD; cat. no. 6604727; Beckman Coulter, Inc.), PE and FITC-IgG control mAbs (cat. nos. A07796 and A07795; Beckman Coulter, Inc.), PE-Cy7-conjugated anti-CXCR5 (cat. no. 25-9185-42; MU5UBEE clone), PE-conjugated anti-TIGIT (cat. no. 12-9500-42; MBSA43 clone), FITC-conjugated anti-PD-1 (cat. no. 11-9969-42; MIH4 clone; eBioscience; Thermo Fisher Scientific, Inc.). Cells incubated with IgG control mAbs were used as isotype controls. All the cell suspensions (5x10$^5$/ml per aliquot of antibody) with antibodies (each antibody 5 $\mu$l) were incubated for 30 min on ice. Samples were detected on a CYTOMICS FC 500 flow cytometer (Beckman Coulter, Inc.) and data were analyzed using the in-built software (CXP 2.0; Beckman Coulter, Inc.).

*CRP, autoantibody, ESR, urine and routine blood measurements*. CRP and RF content in the serum of patients with RA were acquired using nephelometry, according to the manufacturer's instructions (IMMAGE® 800 protein chemistry analyser; Beckman Coulter, Inc.). Anti-CCP of IgG class in the serum was detected using ELISA kit for anti-CCP antibody (cat. no. KX-E-CCP01096; Shanghai Kexin Biotech Co., Ltd.). ESR was determined using an automatic measuring instrument (eSr Xc-40B; Beijing PuliSheng Instrument Co., Ltd.)

Table I. Clinical and routine laboratory characteristics of patients with RA and HC.

| Categories | Patients with RA (n=82) | HC (n=46) | Z or $X^2$ value | P-value |
|---|---|---|---|---|
| Females, n (%) | 56 (68.29) | 28 (60.87) | 0.72 | 0.40 |
| Age, years | 57.65±12.38 | 53.46±14.22 | 1.74 | 0.08 |
| DAS28-ESR | 5.5±1.23 | - | - | - |
| DAS28-CRP | 4.88±1.15 | - | - | - |
| Swollen joint count | 9.90±6.18 | - | - | - |
| Tender joint count | 13.10±6.64 | - | - | - |
| Visual analogue scale | 5.06±1.29 | - | - | - |
| ESR | 63.75±42.71 | - | - | - |
| CRP | 38.81±46.77 | - | - | - |
| Anti-cyclic citrullinated peptide antibodies | 703.06±800.29 | - | - | - |
| Rheumatoid factor | 520.09±831.42 | - | - | - |
| White blood cell count | 7.20±2.60 | 5.72±1.22 | 1201.00 | <0.01 |
| Red blood cell count | 3.99±0.76 | 4.68±0.74 | 755.00 | <0.01 |
| Hemoglobin | 113.51±22.78 | 140.50±11.99 | 511.50 | <0.01 |
| Hematocrit | 0.36±0.07 | 0.47±0.03 | 582.0 | <0.01 |
| Platelet count | 277.49±99.88 | 233.85±53.64 | 1321.00 | <0.01 |
| L | 1.57±0.59 | 1.94±0.48 | 3.63 | <0.01 |
| L% | 23.82±9.68 | 34.33±6.18 | 661.50 | <0.01 |
| M | 0.49±0.22 | 0.37±0.12 | 1263.00 | <0.01 |
| M% | 7.18±3.57 | 6.45±1.62 | 1714.00 | 0.39 |
| N | 5.03±2.47 | 3.26±0.86 | 946.50 | <0.01 |
| N% | 66.86±13.21 | 56.77±6.06 | 829.00 | <0.01 |
| NLR | 3.73±2.58 | 1.74±0.52 | 699.00 | <0.01 |
| Platelet-to-lymphocyte ratio | 204.14±124.13 | 126.06±37.17 | 1069.00 | <0.01 |
| Lymphocyte-to-monocyte ratio | 3.91±2.31 | 5.69±1.87 | 4.47 | <0.01 |
| Systemic immune inflammation index | 1102.93±970.69 | 400.45±128.62 | 786.00 | <0.01 |
| dNLR | 2.65±1.83 | 1.29±0.55 | 754.50 | <0.01 |

Unless otherwise shown, data are presented as mean ± standard deviation. CRP, C-reactive protein; DAS28, disease activity score 28; NLR, neutrophil to-lymphocyte ratio; dNLR, derived Neutrophil to lymphocyte ratio; ESR, erythrocyte sedimentation rate; HC, healthy controls; L, lymphocyte count; L%, lymphocyte percentage; LMR, lymphocyte-to-monocyte ratio; M, monocyte count; M%, monocyte percentage; N, neutrophil count; N%, neutrophil percentage; RA, rheumatoid Arthritis.

whereas routine blood measurements were collected using a Sysmex Xe-2100 analyzer (Sysmex Corporation), according to the manufacturer's protocols. According to the routine blood measurement results, indicators of inflammation, including LMR, NLR, PLR, SII, dNLR, were calculated using the previously described formula [LMR=L/M, NLR=N/L, PLR=PLT/L, SII=PLT x N/L, dNLR=N/(WBC-N)] (18).

*Effect of patient treatment on the measured parameters*. To determine whether changes in RA status could be reflected by changes in $TIGIT^+$ $PD\text{-}1^+$ $CXCR5^-$ $CD4^+$ T and $TIGIT^-$ $PD\text{-}1^+$ $CXCR5^-$ $CD4^+$ T cell parameters, including the percentage of $TIGIT^+$ $PD\text{-}1^+$ $CXCR5^-$ $CD4^+$ T cells, the percentage of $TIGIT^-$ $PD\text{-}1^+$ $CXCR5^-$ $CD4^+$ T cells, the mean fluorescence intensity (MFI) of PD-1 in $TIGIT^+$ $PD\text{-}1^+$ $CXCR5^-$ $CD4^+$ T, the MFI of PD-1 in $TIGIT^-$ $PD\text{-}1^+$ $CXCR5^-$ $CD4^+$ T cells and the MFI of TIGIT in $TIGIT^+$ $PD\text{-}1^+$ $CXCR5^-$ $CD4^+$ T cells, were all measured. A total of four patients with new-onset RA underwent therapeutic regimens with cortico-steroids/immunosuppressive drugs. Each patient with RA received 15 mg prednisone (once a day), 200 mg hydroxychloroquine sulfate (twice a day) and 12.5 mg methotrexate (once a week) for ≥1 week, following normal protocols for the treatment of this condition at The First Affiliated Hospital of Nanchang University. After the final treatment, blood was re-sampled for $TIGIT^+$ $PD\text{-}1^+$ $CXCR5^-$ $CD4^+$ T and $TIGIT^-$ $PD\text{-}1^+$ $CXCR5^-$ $CD4^+$ T cell levels. Post-treatment values for each endpoint were then compared with the day 0 (before treatment) values.

*Statistical analysis*. The data are presented as the mean ± standard deviation (unless otherwise stated). Statistical

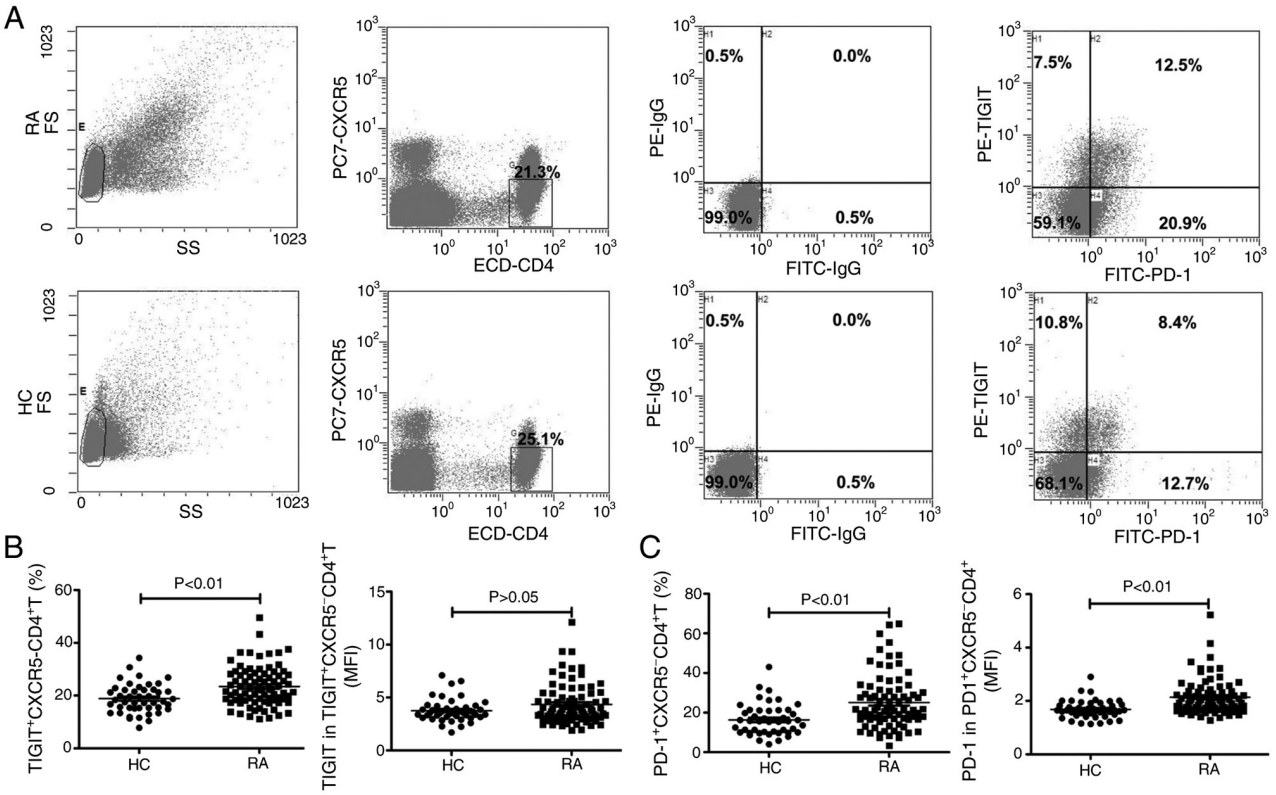


Figure 1. TIGIT and PD-1 expression in CXCR5- CD4+ T cells in patients with RA and the HC. (A) Representative dot plots of population gating. (B) Percentage of TIGIT+CXCR5-CD4+T cells and the MFI of TIGIT in CXCR5- CD4+ T cells between patients with RA and the HC. (C) Percentage of PD-1+ CXCR5- CD4+ T cells and the MFI of PD-1 in CXCR5- CD4+ T cells between patients with RA and the HC. HC, healthy controls; MFI, mean fluorescence intensity; PD-1, programmed cell death protein 1; RA, rheumatoid arthritis; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domain; SS, side scatter; PE, phycoerythrin; ECD, PE-Texas red.

analysis was performed using SPSS (version 16.0; SPSS, Inc.) and GraphPad Prism (version 5.0; GraphPad Software, Inc.). Differences in PD-1 and TIGIT expression between groups were analyzed using an independent-sample unpaired t-test or a non-parametric Mann-Whitney U test (GraphPad Prism; version 5.0). Paired t-tests were used to evaluate changes in TIGIT+PD-1+CXCR5-CD4+T and TIGIT-PD-1+CXCR5-CD4+T cell levels after treatment in the four new-onset patients with RA (GraphPad Prism; version 5.0). Correlation analysis was performed using a Spearman's correlation test (GraphPad Prism; version 5.0). Univariate analysis and multivariate regression analysis (logistic regression) were used to analyze the risk factors (SPSS; version 16.0). P<0.05 was considered to indicate a statistically significant difference.

## Results

*Expression levels of TIGIT and PD-1 in CXCR5- CD4+ T cells in patients with RA and HC*. The expression levels of TIGIT and PD-1 in CXCR5- CD4+ T were first evaluated in patients with RA and the HC using flow cytometry (Fig. 1). A significantly higher percentage of TIGIT+ CXCR5- CD4+ T cells, significantly higher percentage of PD-1+ CXCR5- CD4+ T cells and significantly higher MFI of PD-1 in CXCR5- CD4+ T cells, were all observed in patients with RA compared with those in the HC group (all P<0.05; Fig. 1B and C). However, no significant differences could be identified in the MFI of TIGIT among the CXCR5- CD4+ T cell population between patients with RA and the HC (Fig. 1B).

*Increased TIGIT+ PD-1+ CXCR5- CD4+ T and TIGIT- PD-1+ CXCR5- CD4+ T cells in patients with RA*. CXCR5- CD4+ T cells were divided into three groups, TIGIT+ PD-1+, TIGIT- PD-1+ and TIGIT+PD-1-, based on the TIGIT and PD-1 expression levels. The percentage of both TIGIT+ PD-1+ CXCR5- CD4+ and TIGIT- PD-1+ CXCR5- CD4+ T cells in patients with RA were significantly higher compared with those in the HC group (both P<0.05; Fig. 2A and B). However, no significant differences could be identified in the percentage of TIGIT+ PD-1- CXCR5- CD4+ T cells between patients with RA and the HC (Fig. 2C). The MFI of TIGIT in TIGIT+ PD-1+ CXCR5- CD4+ T cells, the MFI of PD-1 in TIGIT+ PD-1+ CXCR5- CD4+T cells and the MFI of PD-1 in TIGIT- PD-1+ CXCR5- CD4+ T cells in patients with RA were also significantly higher compared with those in the HC group (all P<0.05; Fig. 2D-F). However, no significant differences were identified in the MFI of TIGIT in TIGIT+ PD-1- CXCR5- CD4+ T cells between patients with RA and the HC group (Fig. 2G). In addition, the MFI of TIGIT in TIGIT+ PD-1+ CXCR5- CD4+ T cells was significantly higher compared with that in TIGIT+ PD-1- CXCR5- CD4+ T cells in patients with RA (P<0.01; Fig. 2H). The MFI of PD-1 in TIGIT+ PD-1+ CXCR5- CD4+ T cells was also significantly higher compared with that in the TIGIT- PD-1+ CXCR5- CD4+ T cells in patients with RA (P<0.01; Fig. 2I).

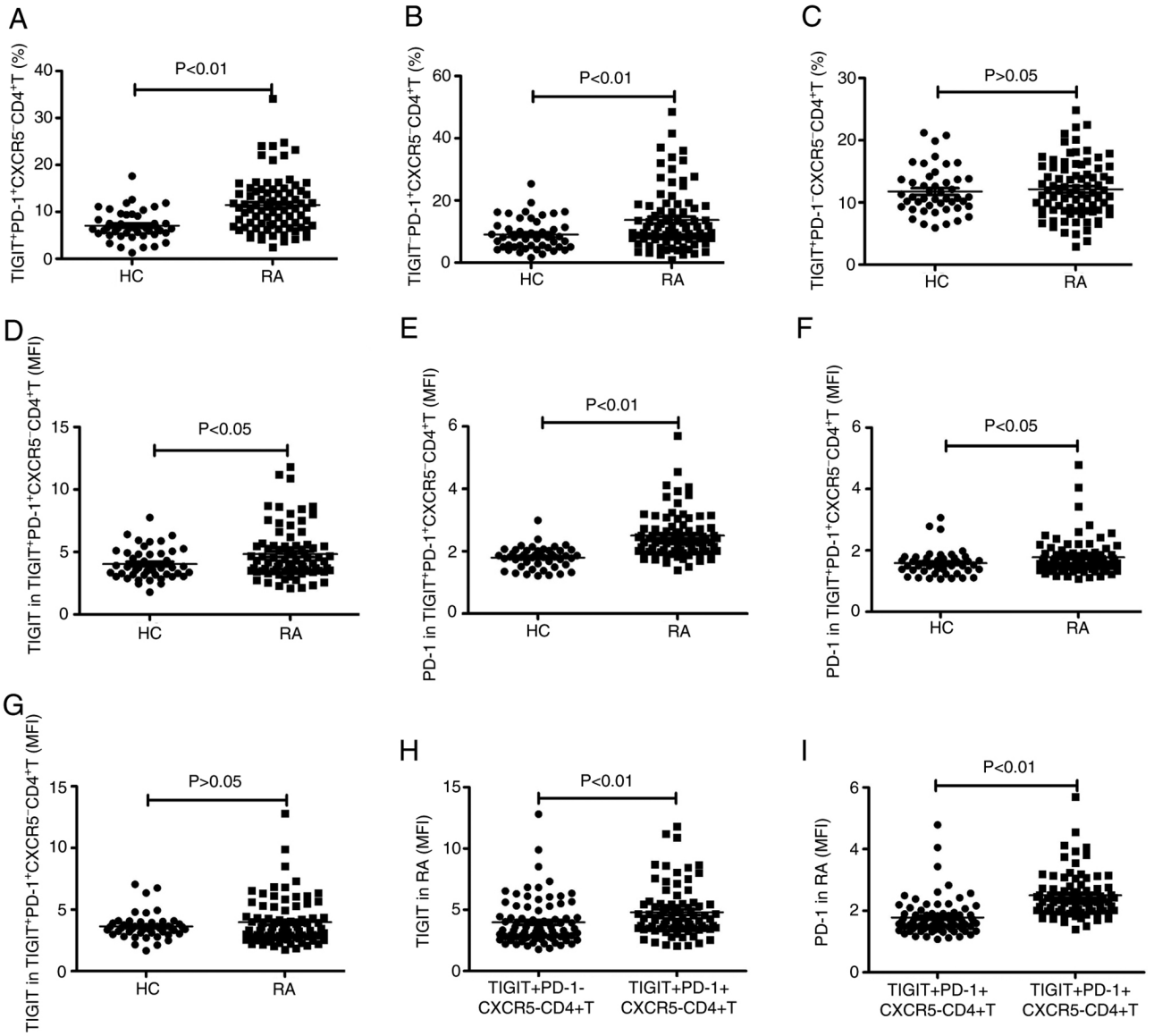


Figure 2. Expression levels of TIGIT and PD-1 in TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T, TIGIT$^-$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T and TIGIT$^+$ PD-1$^-$ CXCR5$^-$ CD4$^+$ T cells in patients with RA and the HC. (A) Percentage of TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells, (B) TIGIT$^-$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells, (C) TIGIT$^-$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells, the MFI of (D) TIGIT and (E) PD-1 in TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells, (F) the MFI of PD-1 in TIGIT$^-$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells, (G) the MFI of TIGIT in TIGIT$^+$ PD-1$^-$ CXCR5$^-$ CD4$^+$ T cells between patients with RA and the HC. (H) The MFI of TIGIT in TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells and TIGIT$^+$ PD-1$^-$ CXCR5$^-$ CD4$^+$ T cells from patients with RA. (I) The MFI of PD-1 in TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells and TIGIT$^-$PD-1$^+$CXCR5$^-$CD4$^+$T cells from patients with RA. HC, healthy controls; MFI, mean fluorescence intensity; PD-1, programmed cell death protein 1; RA, rheumatoid arthritis; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domain; Ig, immunoglobulin; CXCR5, C-X-C chemokine receptor type 5.

*TIGIT$^+$PD-1$^+$CXCR5$^-$CD4$^+$T and TIGIT$^-$PD-1$^+$CXCR5$^-$CD4$^+$T levels are correlated with the presence of auto-antibodies.* Anti-CCP and RF are hallmarks of autoantibodies in RA (19). The association between the levels of these autoantibodies and various parameters of TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T and TIGIT$^-$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cell levels, including the percentage of these cells and the MFI of TIGIT and PD-1 in these cells, were assessed. As presented in Fig. 3, a significant correlation between the percentage of TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells and anti-CCP levels ($r_s$=0.293; P<0.05), a positive correlation between the percentage of TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells and increased anti-CCP levels (anti-CCP>25 RU/ml; $r_s$=0.351; P<0.01), as well as increased RF levels (RF>20 IU/ml) ($r_s$=0.310; P<0.05), were observed in the RA group. In addition, a significant correlation between the MFI of PD-1 in the TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells and increased RF(RF>20 IU/ml) ($r_s$=0.279, P<0.05; Fig. 3D) were observed in the RA group. A positive correlation between the percentage of TIGIT$^-$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells ($r_s$=0.363; P<0.05), MFI of PD-1 in TIGIT$^-$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells ($r_s$=0.305; P<0.05) and increased RF (RF>20 IU/ml) were also observed in the RA group (Fig. 3E and F).

*Increased TIGIT$^+$PD-1$^+$CXCR5$^-$CD4$^+$T and TIGIT$^-$PD-1$^+$CXCR5$^-$CD4$^+$T cell levels are correlated with markers of inflammation.* WBC, L, L%, M, M%, LMR, N, N%, NLR, PLR, SII, dNLR, ESR and CRP are common predictors of inflammation that were investigated in the present study in patients with RA. The relationship between these predictors of inflammation and the percentage of TIGIT$^+$ PD-1$^+$

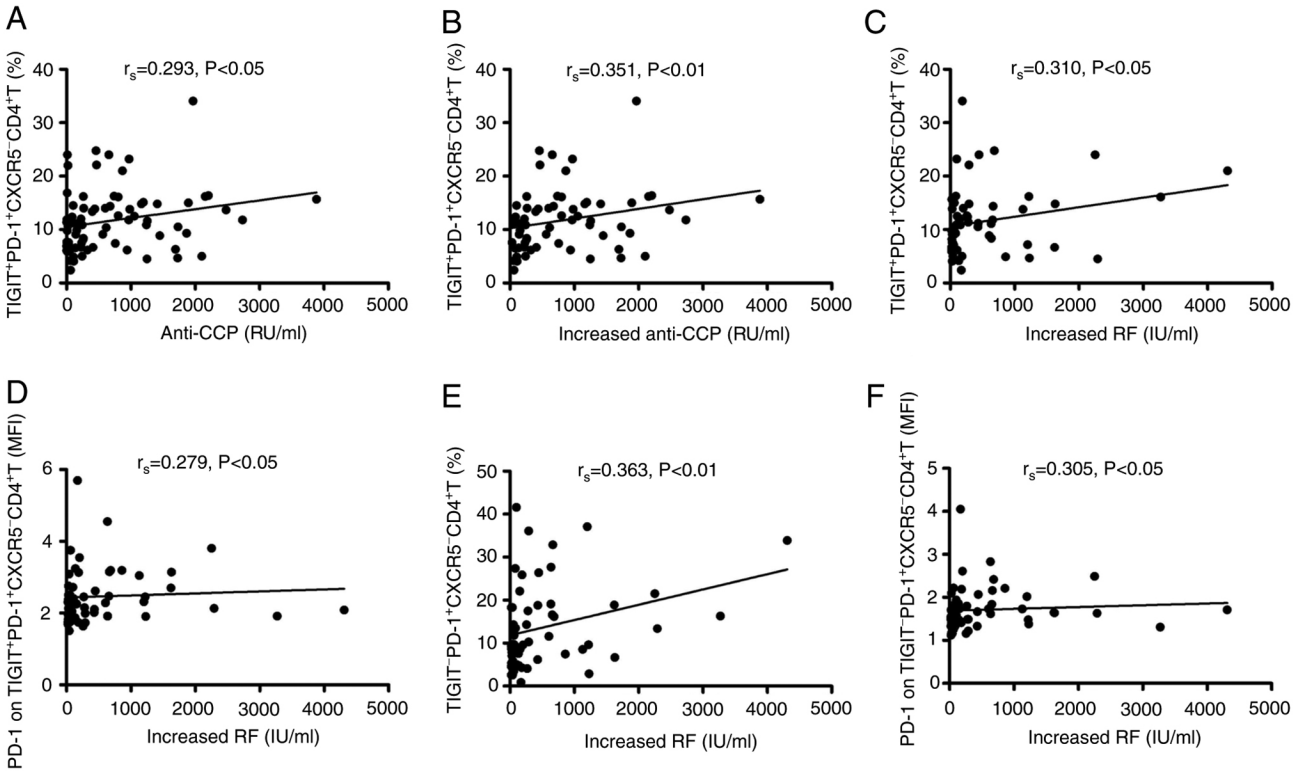


Figure 3. TIGIT+ PD-1+ CXCR5- CD4+ T and TIGIT- PD-1+ CXCR5- CD4+ T cell levels are correlated with those of anti-CCP and RF in patients with RA. The percentage of TIGIT+ PD-1+ CXCR5- CD4+ T cells was positively correlated with (A) anti-CCP, (B) increased anti-CCP (anti-CCP>25 RU/ml) and (C) increased RF (RF>20 IU/ml). The correlation between (D) MFI of PD-1 in TIGIT+ PD-1+ CXCR5- CD4+ T cells, (E) Percentage of TIGIT- PD-1+ CXCR5- CD4+ T cells, (F) MFI of PD-1 in TIGIT- PD-1+ CXCR5- CD4+ T cells and increased RF (RF>20 IU/ml). Anti-CCP, anti-cyclic citrullinated peptide antibodies; MFI, mean fluorescence intensity; PD-1, programmed cell death protein 1; RA, rheumatoid arthritis; RF, rheumatoid factor; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domain; CXCR5, C-X-C chemokine receptor type 5.

CXCR5- CD4+ T cells, MFI of TIGIT in TIGIT+ PD-1+ CXCR5- CD4+ T cells, MFI of PD-1 in TIGIT+ PD-1+ CXCR5- CD4+ T cells, percentage of TIGIT- PD-1+ CXCR5- CD4+ T cells and MFI of PD-1 in TIGIT- PD-1+ CXCR5- CD4+ T cells, were investigated. As presented in Fig. 4, the MFI of PD-1 in TIGIT+ PD-1+ CXCR5- CD4+ T cells was negatively correlated with L ($r_s$=-0.329; P<0.01), L% ($r_s$=-0.352; P<0.01), LMR ($r_s$=-0.309; P<0.01). By contrast, the MFI of PD-1 in TIGIT+ PD-1+ CXCR5- CD4+ T cells was significantly correlated with N% ($r_s$=0.293; P<0.01), SII ($r_s$=0.278; P<0.05), CRP ($r_s$=0.244; P<0.05) and positively correlated with NLR ($r_s$=0.338; P<0.01), dNLR ($r_s$=0.306; P<0.01), ESR ($r_s$=0.323; P<0.01). The MFI of PD-1 in TIGIT- PD-1+ CXCR5- CD4+ T cells was negatively correlated with L ($r_s$=-0.310; P<0.01), L% ($r_s$=-0.364; P<0.01), whilst being positively correlated with N% ($r_s$=0.343; P<0.01), NLR ($r_s$=0.360; P<0.01), dNLR ($r_s$=0.349; P<0.01) and significantly correlated with N ($r_s$=0.221; P<0.01), SII ($r_s$=0.288; P<0.01). However, no correlation could be observed between the percentage of TIGIT+ PD-1+ CXCR5- CD4+ T and TIGIT- PD-1+ CXCR5- CD4+ T cells, MFI of TIGIT in TIGIT+ PD-1+ CXCR5- CD4+ T cells and the markers of inflammation (data not shown).

*Increased TIGIT+PD-1+CXCR5-CD4+T cell levels are correlated with RA disease activity.* To investigate whether the increased TIGIT+ PD-1+ CXCR5- CD4+ T and TIGIT- PD-1+ CXCR5- CD4+ T cell levels could be used as biomarkers for RA activity, correlation analysis was performed to explore the association between the increased TIGIT+ PD-1+ CXCR5- CD4+ T and TIGIT- PD-1+ CXCR5- CD4+ T cell levels in patients with RA with disease activity, namely DAS28-ESR, DAS28-CRP, TJC and VAS. As shown in Fig. 5, the MFI of PD-1 in TIGIT+ PD-1+ CXCR5- CD4+ T cells was correlated with DAS28-ESR ($r_s$=0.249; P<0.05) and VAS ($r_s$=0.245; P<0.05). However, no correlation could be observed between the percentage of TIGIT+ PD-1+ CXCR5- CD4+ T and TIGIT- PD-1+ CXCR5- CD4+ T cells, MFI of TIGIT in TIGIT+ PD-1+ CXCR5- CD4+ T cells and the MFI of PD-1 in TIGIT- PD-1+ CXCR5- CD4+ T cells and disease activity (data not shown).

TIGIT+ PD-1+ CXCR5- CD4+ T and TIGIT- PD-1+ CXCR5- CD4+ T cell levels were then compared in four new-onset RA cases before and after corticosteroid/immunosuppressant therapy. Compared with the pre-treatment values, the MFI of PD-1 in TIGIT+ PD-1+ CXCR5- CD4+ T cells was significantly decreased as a result of the treatment regimen for ≥1 week (P<0.05; Fig. 5C). By contrast, there was no difference between pre-treatment and post treatment in the percentage of TIGIT+ PD-1+ CXCR5- CD4+ T cells, percentage of TIGIT- PD-1+ CXCR5- CD4+ T cells, MFI of TIGIT in TIGIT+ PD-1+ CXCR5- CD4+ T cells and the MFI of PD-1 in TIGIT- PD-1+ CXCR5- CD4+ T cells (data not shown).

*Increased TIGIT+ PD-1+ CXCR5- CD4+ T cell levels are a risk factor for RA.* The aforementioned results suggest that the increased TIGIT+ PD-1+ CXCR5- CD4+ T and TIGIT- PD-1+ CXCR5- CD4+ T cell levels were associated with RA

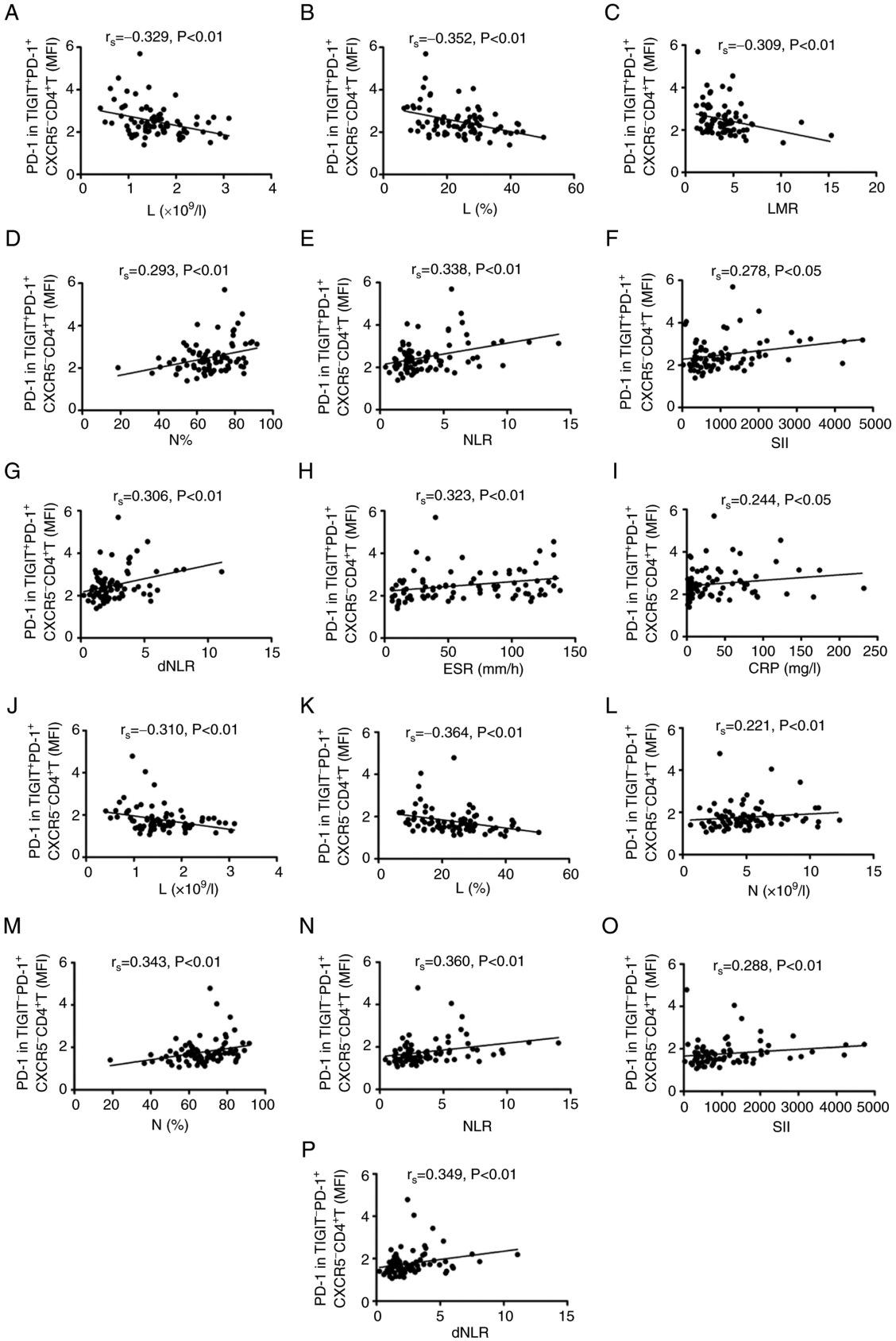


Figure 4. TIGIT+ PD-1+ CXCR5- CD4+ T and TIGIT- PD-1+ CXCR5- CD4+ T cell levels are correlated with those of the markers of inflammation in patients with RA. The MFI of PD-1 in TIGIT+ PD-1+ CXCR5- CD4+ T cells was negatively correlated with (A) L and (B) L%, (C) LMR, whilst it was positively correlated with (D) N%, (E) NLR, (F) SII, (G) dNLR, (H) ESR and (I) CRP. The MFI of PD-1 in TIGIT- PD-1+ CXCR5- CD4+ T cells was negatively correlated with (J) L and (K) L%, whereas it was positively correlated with (L) N, (M) N%, (N) NLR, (O) SII and (P) dNLR. CRP, C-reactive protein; dNLR, derived neutrophil-to-lymphocyte ratio; ESR, erythrocyte sedimentation rate; L, lymphocyte count; L%, lymphocyte percentage; LMR, lymphocyte-to-monocyte ratio; MFI, mean fluorescence intensity; N, neutrophil count; N%, neutrophil percentage; NLR, neutrophil-to-lymphocyte ratio; PD-1, programmed cell death protein 1; RA, rheumatoid arthritis; RF, rheumatoid factor; SII, systemic immune inflammation index; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domain; CXCR5, C-X-C chemokine receptor type 5.

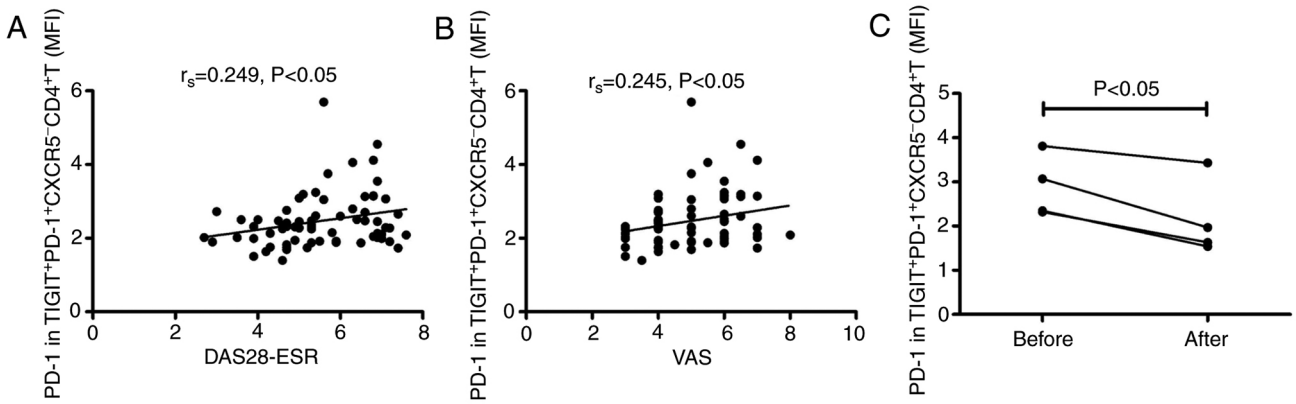


Figure 5. TIGIT+ PD-1+ CXCR5- CD4+ T cell levels are correlated with rheumatoid arthritis activity. The MFI of PD-1 in TIGIT+ PD-1+ CXCR5- CD4+ T cells was positively correlated with (A) DAS28-ESR and (B) VAS. (C) The MFI of PD-1 in TIGIT+ PD-1+ CXCR5- CD4+ T cells was decreased following treatment. DAS28, disease activity score 28; ESR, erythrocyte sedimentation rate; MFI, mean fluorescence intensity; PD-1, programmed cell death protein 1; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domain; CXCR5, C-X-C chemokine receptor type 5; VAS, visual analogue scale.

pathogenesis. Therefore, to investigate whether the increased TIGIT+ PD-1+ CXCR5- CD4+ T and TIGIT- PD-1+ CXCR5- CD4+ T cell levels are risk factors for RA, univariate and multivariate analyses were performed. As shown in Table II, after univariate and multivariate logistic regression analysis, the percentage of TIGIT+ PD-1+ CXCR5- CD4+ T cells (OR=1.19; 95% CI=1.02-1.38; P=0.02) and the MFI of PD-1 in TIGIT+ PD-1+ CXCR5- CD4+ T cells (OR=19.53; 95% CI=4.33-88.05; P<0.01) were considered to be risk factors for RA.

## Discussion

CD4+ T cells are the central mediators of specific autoimmune diseases, such as RA (1-5). CD4+ T cells can be divided into the CXCR5- CD4+ T peripheral helper cell and CXCR5+ CD4+ T follicular T helper cell subtypes according to CXCR5 expression, which can promote B cell responses and antibody production (20,21). It has been previously found that inhibitory molecules TIGIT and PD-1 can serve pivotal roles in adjusting the function and activation conditions of CD4+ T cells (22-24). TIGIT/PD-1-expressing circulating follicular T helper cells are novel subsets of T cells that exhibit heightened B-cell activation functions (14,25,26). However, the immunomodulatory effects of TIGIT-expressing peripheral T helper cells on the development of RA remains to be fully elucidated. In the present study, the level of PD-1 in CXCR5- CD4+ peripheral helper T cells from patients with RA was measured, where it was revealed to be significantly upregulated in patients with RA compared with that in the HC group, which is consistent with findings from previous studies (3,5). In addition, the present study explored the levels of TIGIT in CXCR5- CD4+ T cells from patients with RA and HC. The level of TIGIT in CXCR5- CD4+ T cells was significantly increased in patients with RA compared with that in the HC group, which is consistent with a previous report also showing a significantly increased TIGIT protein level (10).

The co-expression of TIGIT and PD-1 in immune cells has been increasingly attracting attention (14,27,28). This is because their co-expression in T cells has been shown to be associated with dysfunctional behaviors (such as proliferation, cytokine production and degranulation) and clinical outcomes. In the present study, based on the TIGIT and PD-1 levels, CXCR5- CD4+ T cells were divided into TIGIT+ PD-1+ CXCR5- CD4+ T, TIGIT- PD-1+ CXCR5- CD4+ T and TIGIT+ PD-1- CXCR5- CD4+ T cells. The results indicated that the percentage of TIGIT+ PD-1+ and TIGIT- PD-1+ CXCR5- CD4+ T cells are significantly elevated in patients with RA compared with that in the HC group, whereas no differences in the percentage of TIGIT+PD-1-CXCR5-CD4+T cells could be observed. Further analysis of the expression levels of PD-1 and TIGIT in these CXCR5- CD4+ T cells demonstrated that the MFI of TIGIT and PD-1 in TIGIT+ PD-1+ CXCR5- CD4+ T and TIGIT- PD-1+ CXCR5- CD4+ T cells were significantly increased in patients with RA compared with those in the HC group. These data suggest that the TIGIT+ PD-1+ CXCR5- CD4+ T cell levels were increased, including the percentage of these cells, in addition to the MFI of TIGIT and PD-1 in these cells in patients with RA. Furthermore, the present study revealed that increased TIGIT+ PD-1+ CXCR5- CD4+ T cell levels were associated with DAS28-ESR, VAS and treatment, which reflected RA activity and severity.

RA is a type of chronic inflammation (1). Inflammatory markers, such as ESR, CRP, WBC, L, L%, M, M%, LMR, N, N%, NLR, dNLR, PLR and SII may reflect the presence of chronic inflammation (29-31). The data indicated that the MFI of PD-1 in TIGIT+ PD-1+ CXCR5- CD4+ T cells was correlated with L, L%, N%, LMR, NLR, SII, dNLR, CRP and ESR. These data suggest that upregulated TIGIT+ PD-1+ CXCR5- CD4+ T cells may indicate higher severity of chronic inflammation in RA.

RA is characterized by high levels of serum autoantibodies containing RF and anti-CCP (19). Considering that peripheral helper cells can promote antibody production (5-10), the correlation between TIGIT+ PD-1+ CXCR5- CD4+ T cells and RF and anti-CCP was explored in the present study. The results indicated that the percentage of TIGIT+ PD-1+ CXCR5- CD4+ T cells was positively correlated with anti-CCP and RF. In addition, the MFI of PD-1 in TIGIT+ PD-1+ CXCR5- CD4+ T cells was positively correlated with RF, suggesting that increased TIGIT+ PD-1+ CXCR5- CD4+ T cell levels may be involved in the autoimmune response in RA.

These aforementioned results suggest that increased TIGIT+ PD-1+ CXCR5- CD4+ T cell levels were associated

Table II. Univariate and multivariate analyses of risk factors associated with RA.

| | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| Parameter | OR | 95% CI | P-value | OR | 95% CI | P-value |
| Frequency of TIGIT$^+$PD1$^+$CXCR5-CD4$^+$T cells | 1.29 | 1.14-1.45 | <0.01 | 1.19 | 1.02-1.38 | 0.02 |
| MFI of TIGIT on TIGIT$^+$PD1$^+$CXCR5-CD4$^+$T cells | 1.33 | 1.04-1.71 | 0.02 | - | - | - |
| MFI of PD1 on TIGIT$^+$PD1$^+$CXCR5-CD4$^+$T cells | 36.87 | 8.64-157.32 | <0.01 | 19.53 | 4.33-88.05 | <0.01 |
| Frequency of TIGIT-PD1$^+$CXCR5-CD4$^+$T cells | 1.09 | 1.03-1.16 | <0.01 | - | - | - |
| MFI of PD1 on TIGIT$^+$PD1$^+$CXCR5-CD4$^+$T cells | 2.34 | 0.94-5.81 | 0.07 | - | - | - |

MFI, mean fluorescence intensity; PD-1, programmed cell death protein 1; RA, rheumatoid arthritis; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domain; CXCR5, C-X-C chemokine receptor type 5.

with the levels of inflammatory markers, autoantibodies, RA activity and severity. Furthermore, the present study indicated that increased TIGIT$^-$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cell levels in patients with RA are associated with the levels of inflammatory markers, including L, L%, N, N%, NLR, SII, dNLR and autoantibody RF. However, no association was identified between increased TIGIT$^-$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cell levels and RA activity and severity. These results suggest that TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells may serve a more pathogenic effect compared with TIGIT$^-$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells in patients with RA. Notably, univariate and multivariate logistic regression analysis results demonstrated that the percentage of TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells and the MFI of PD-1 in TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells were considered to be risk factors for RA, suggesting that increased TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cell levels are involved in RA pathogenesis.

Furthermore, no difference in TIGIT$^+$ PD-1$^-$ CXCR5$^-$ CD4$^+$ T cell levels was observed between patients with RA and those in the HC group, suggesting that TIGIT$^+$ PD-1$^-$ CXCR5$^-$ CD4$^+$ T cells may not serve a role in RA pathogenesis. This result was consistent with the findings by Akiyama *et al* (25), which demonstrated that TIGIT$^+$ CXCR5$^-$ CD4$^+$ T cells produces less IL-21 compared with TIGIT$^-$ CXCR5$^-$ CD4$^+$ T cells in IgG4-related disease. It is known that PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells can participate in the occurrence and development of RA (8-9). Considering that there was no difference in the level of TIGIT$^+$ PD-1$^-$ CXCR5$^-$ CD4$^+$ T cells and that TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells may serve a more important role compared with TIGIT$^-$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells in patients with RA, the present study suggested that the co-expression of TIGIT and PD-1 in CXCR5$^-$CD4$^+$T cells has a synergistic effect. However, the mechanism of their synergy should be studied further.

Yang *et al* previously demonstrated elevated serum levels of IgG4 in patients with systemic autoimmune rheumatic diseases, such as antineutrophil cytoplasmic antibody-associated vasculitis, systemic lupus erythematosus and RA (32). In addition, another previous study reported that PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells are associated with IgG4-related diseases Responder Index, number of involved organs and serum level of soluble IL-2 receptor (33). In the present study, TIGIT$^{+/-}$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells were demonstrated as being associated with autoantibodies containing RF and anti-CCP. Therefore, the level of antibody-IgG4 in patients with RA and the association between TIGIT$^{+/-}$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T and IgG4 in patients with RA were explored. The serum levels of IgG4 in 37 patients with RA were detected using chemiluminescent immunoassay and the normal ranges for serum IgG4 level was 36-2,090 $\mu$g/ml. Only one RA patient was revealed to be positive (5,416 $\mu$g/ml). The positive rate of IgG4 in RA patients was only 2.7%. Yang *et al* (32) demonstrated the percentage of patients with RA who also had elevated IgG4 is only 2.7%. The present study results demonstrated that the positive rate of IgG4 in patients with RA was low and was consistent with the report of Yang *et al* (32), which led to the relationship between positive IgG4 and TIGIT$^{+/-}$PD-1$^+$CXCR5$^-$CD4$^+$T cells not being investigated.

The present study has a number of limitations. The samples were collected from only one institution, which may lead to bias. Therefore, multicenter clinical studies are necessary. In addition, the samples, including the treated samples were too small, which may lead to weak correlation ($r<\pm0.3$). Thus, a study with a larger sample sizes is required to verify these findings.

In conclusion, the present study revealed an association between the increased TIGIT$^+$ PD- 1$^+$ CXCR5$^-$ CD4$^+$ T cell levels and autoantibody production, RA activity and RA severity. The findings of the present study suggested that TIGIT may be a potential marker for circulating PD-1$^+$ CXCR5$^-$ CD4$^+$ T subsets and TIGIT$^+$ PD-1$^+$ CXCR5$^-$ CD4$^+$ T cells as a potential therapeutic target.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

QL, PF and YonG performed the experiments. BF, YanG, QH, ZH and JL analyzed and interpreted the data. QL and JL made substantial contributions to the design supervision of the present study and wrote the manuscript. All authors reviewed the results and read and approved the final version of the manuscript. QL and JL confirm the authenticity of all the raw data.

## Ethics approval and consent to participate

This study was authorized by the Ethics Committee of the First Affiliated Hospital of Nanchang University (Nanchang, China). All participants provided written informed consent prior to the initiation of the present study.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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