The Correlation between Aire and ICOSL Expression in Peripheral Blood CD14+ Cells and Tfh Cells and Disease Activity in RA Patients

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Research article

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Abstract

Background: Recent trans-ethnic genome-wide association study (GWAS) showed that autoimmune regulator (Aire) played a pivotal role in Rheumatoid Arthritis (RA). Our preliminary research showed that Aire can affect T follicular helper (Tfh) cells differentiation by regulating the expression of inducible costimulator molecule ligand (ICOSL) on DCs. The abnormal levels of Tfh cells are related to the pathogenesis of RA, which can promote autoantibody production by helping autoreactive B cells activation.Therefore, the abnormal expression of the Aire in RA patients may lead to increased expression of ICOSL, promoting the differentiation of Tfh cells and the secretion of autoantibodies, consequently resulting in RA. However, to date, changes in the Aire, Tfh cell, and ICOSL levels in the peripheral blood of RA patients have not been reported. This study assessed the expression level of Aire and ICOSL and the number of Tfh cells in the peripheral blood of patients with RA and then explored the relationship between these three factors and RA severity. We are attempting to further explore the pathogenesis of RA to develop targeted treatments.

Methods: Fifteen RA patients were enrolled, basic clinical information of the patients was collected, blood samples were collected to examine the Aire expression, ICOSL expression and $CD4^+CXCR5^+PD1^+$ (Tfh cell) numbers in circulation before treatment. The relationship between these three factors and RA severity was explored.

Results: This study showed that compared with the control group, the levels of circulating Aire in RA group significantly reduced, while the ICOSL expression levels and Tfh cell numbers in the peripheral blood of RA group were increased. Additionally, the Aire expression levels were negatively correlated with the ICOSL expression levels and Tfh cell numbers, and the ICOSL expression levels were positively correlated with Tfh cell numbers. Moreover, the Aire and ICOSL expression levels and Tfh cell numbers were correlated with anti-cyclic citrullinated peptide antibodies (ACPA) levels and disease activity score in 28 joints (DAS28).

Conclusions: These results suggested the abnormal Aire gene expression in RA patients may affect Tfh differentiation by regulating the expression of ICOSL, leading to the autoantibodies production and promote the onset of RA.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by erosive arthritis [1, 2]. As the disease progresses, patients may experience joint deterioration and functional disability as well as pulmonary disease and cardiovascular disease [3–5]. The pathogenesis of RA has not been entirely clarified, and both genetic and environmental factors play crucial roles in the pathophysiology of RA [6]. The latest trans-ethnic genome wide association study (GWAS) identified novel polymorphisms in genes that contribute to the disease. One of the associated genes that seems to play a pivotal role in RA is autoimmune regulator (Aire) [7, 8].

As a transcription factor, Aire can maintain central immune tolerance through the clearance of self-reactive T cells and the induction of the regulatory T cells (Tregs) production by regulating the expression of peripheral tissue-specific antigens (TSAs) in medullary thymic epithelial cells (mTECs) [9]. Apart from that, Aire can also be expressed in monocytes and dendritic cells (DCs) [10]. Our previous research showed that the mouse DC cell line DC2.4 overexpressing Aire could delay the progression of streptozotocin-induced type 1 diabetes, indicating that Aire expressed in peripheral DCs might play role in the maintenance of peripheral immune tolerance [11]. However, the role of Aire in the pathogenesis of RA is not clear.

In addition to genetic and environmental factors, the pathogenesis of RA is also related to immune function disorders in the body. When an antigen enters the human body, it activates helper T lymphocytes to secrete cytokines through molecular simulation, and these cytokines in turn assist in activating autoreactive B lymphocytes to differentiate into plasma cells that secrete autoantibodies, including anti-cyclic citrullinated peptide antibodies (ACPA) and rheumatoid factor (RF), inducing an inflammatory response and leading to disease progression [3]. T follicular helper (Tfh) cells are a subgroup of $CD4^+$ T cells that facilitates the activation of self-reactive B cells and the production of high-affinity antibodies. Tfh cells are localized in the germinal centers of secondary lymphoid organs, and they express characteristic molecules, including C-X-C chemokine receptor type 5 (CXCR5), inducible costimulatory molecule (ICOS); Tfh cells help development of antibody responses via interleukin 21 (IL-21) [12]. Increased numbers of Tfh cells are closely related to the onset of rheumatoid arthritis [13]. However, the reason for the abnormal increase in Tfh cell numbers in RA patients is not fully understood.

Classic Tfh cell differentiation starts with the initial priming of naïve $CD4^+$T cells by DCs in the context of various factors. Inducible costimulator molecule ligand (ICOSL) is mainly expressed on DCs. ICOS, the ligand of ICOSL, is only expressed by activated T cells. In ICOS-deficient mice and humans, Tfh cell development was inhibited [14, 15]. The expression levels of CXCR5 and ICOS in $CD4^+$T cells were reduced after blocking ICOSL on DCs [16]. It is suggested that ICOSL expressed by DCs is an important signaling molecule that affects Tfh differentiation.

Our preliminary research showed that Aire can affect Tfh differentiation by regulating the expression of ICOSL on DCs[17]. Therefore, we hypothesize that the abnormal expression of the Aire gene in RA patients may lead to the increased expression of ICOSL on DCs, promoting the differentiation of Tfh cells and the secretion of large amounts of autoantibodies, consequently resulting in RA. However, to date, changes in the Aire, Tfh cell, and ICOSL levels in the peripheral blood of patients with RA have not been reported, and the relationship between these three factors and the occurrence of the disease is not yet clear. This study assessed the expression level of Aire and ICOSL and the number of Tfh cells in the peripheral blood of patients with RA and then explored the relationship between these three factors and RA severity. We are attempting to further explore the pathogenesis of RA to develop targeted treatments.

Methods

Samples

Fifteen RA patients in the rheumatology department of The First Hospital of Jilin University were enrolled from January 2019 to June 2019 and formed the RA group (11 females and 4 males, average age of 54.5 years). All the patients met the 2010 revised criteria of the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR). Additionally, 15 healthy subjects (10 females and 5 males, average age of 50.7 years) were selected from the Center of Health Examination in the same hospital and formed the control group (CG). Patients were excluded in our study if they had been treated for RA in the past 6 months or if they had other chronic inflammatory and autoimmune diseases. Female patients who were pregnant or breastfeeding were also excluded. Ethical approval was obtained from The First Hospital of Jilin University, and written informed consent was obtained from all the individuals.

Data and sample collection

Basic clinical information of the patients was collected, including name, sex, age, clinical manifestations, physical examinations, final clinical diagnosis, and disease activity score in 28 joints (DAS28). Blood samples were collected from all the individuals to examine the Aire expression, ICOSL expression and $CD4^+CXCR5^+PD1^+$ (Tfh cell) numbers in circulation before treatment.

Sample processing

Eight milliliters of peripheral blood was collected from the included RA and CG individuals and placed in a heparinized tube. Peripheral blood mononuclear cells (PBMCs) were isolated via density-gradient centrifugation with lymphocyte separation medium (Dakewe Bioengineering, Shenzhen,China) at room temperature. The cells were washed twice with pH 7.2 phosphate-buffered saline (PBS).

Flow cytometry

A total of 10^6 nucleated cells were resuspended, divided into tubes A and B, and washed twice with PBS. Then, 100 μl PBS containing FcR blocking reagent (MiltenyiBiotec, Germany) was added to resuspend the cells, followed by incubation for 10 min. Appropriate amounts of CD4-FITC, PD1-APC, and CXCR5-PE-Cy7 (eBioscience, San Diego, CA, USA) were added and incubated for 20 min in tube A. In tube B, the cells were incubated with CD14-FITC and CD275-APC (eBioscience, San Diego, CA, USA) on ice for 20 min and washed with PBS. Subsequently, the cells were incubated in cold, freshly prepared fixation/permeabilization solution (eBioscience) for 45 min, and the cells were stained with Aire-APC (eBioscience, San Diego, CA, USA) on ice for 50 min. The cells were analyzed with a BD FACS Calibur flow cytometer.

RNA isolation and RT-qPCR

Total RNA was extracted from PBMCs using RNAiso™ PLUS (Takara, Japan). cDNA was synthesized from 1.0 μg of total RNA using M-MLV reverse transcriptase and oligo (dT) in a total volume of 20 μL according to the manufacturer's instructions (Takara). The RT-qPCR was performed as following 95 °C for 2 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Primers used for the PCR were: Aire: F: 5'-CTCGGGAACGGGATTCAGAC-3', R: 5'-CTGCCGGAGTCTTCGAACTT-3';GAPDH:F:5'-ATGGGGAAGGTGAAGGTCG-3',R:5'-GGGTCATTGATGGCAACAATATC-3'.

Statistics

Statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software, USA).The data are presented as the mean ± standard deviation. Statistical significance was calculated by Student's t-test or Pearson's correlation coefficient. Values of $P<0.05$ were considered statistically significant.

Results

1. Circulating Aire levels in RA patients

We observed the expression levels of Aire in the PBMCs from RA patients and healthy subjects using RT-qPCR and FACS. The RT-qPCR results showed that the circulating Aire mRNA expression levels in the RA patients were significantly lower than those in the healthy subjects (Fig. 1A). At the protein level, the detection of Aire expression in PBMCs using FACS showed the same trend (Fig. 1B, C).These results suggested that the onset of RA may be related to the decreased expression of Aire.

2. Circulating ICOSL levels in RA patients

Our previous research showed that Aire could decrease the expression of ICOSL to inhibit the differentiation of Tfh cells. Therefore, to observe whether the change in the Aire expression levels in RA patients could affect the expression of ICOSL, we used FACS to detect the expression levels of ICOSL in the PBMCs from RA patients.The results showed that the expression levels of ICOSL in the PBMCs from the RA patients was significantly higher than those in the PBMCs from the healthy subjects (Fig. 2). These results suggested that the decrease in the Aire expression levels in RA patients may lead to an increase in the expression levels of ICOSL, but it was not clear whether this phenomenon affected the differentiation of Tfh cells.

3. Circulating Tfh cell numbers in RA patients

Studies have shown that Tfh cells are crucial for the occurrence and development of RA [18]. Therefore, to understand the change in the numbers of Tfh cells in RA patients, the proportion of Tfh cells in the peripheral blood from RA patients was detected. The FACS results showed that the proportion of Tfh cells in the peripheral blood from the RA patients was significantly higher than that in the peripheral blood from the healthy subjects (Fig. 3). The results indicated that Tfh cells may be involved in the pathogenesis of RA, and the increase in the number of Tfh cells may be related to the decrease in Aire expression by $CD14^+$ PBMCs, which caused the increase in ICOSL expression on the cells.

4. Correlation between the expression levels of Aire and ICOSL in peripheral blood $CD14^+$ cells and Tfh cells

To further elucidate the correlation among the Aire expression level, the ICOSL expression level, and Tfh cell proportion, a pairwise correlation analysis of three indicators was conducted. The results showed that the expression level of Aire was significantly negatively correlated with the expression level of ICOSL and the proportion of Tfh cells in the peripheral blood of the RA patients (Fig. 4A, B), and the ICOSL expression level was significantly positively correlated with the proportion of Tfh cells (Fig. 4C).

5. Correlation among the levels of Aire, ICOSL, Tfh cells and ACPA in the peripheral blood of RA patients

Anticyclic citrullinated peptide antibody (ACPA) is a good indicator for the diagnosis of rheumatoid arthritis. To determine whether circulating Aire levels, ICOSL levels, and Tfh cell numbers were associated with plasma ACPA levels, the correlation between these three indicators and the levels of ACPA in RA patients was examined. The results showed that the expression levels of Aire were significantly negatively correlated with the levels of ACPA in the peripheral blood from the RA patients (Fig. 5A), and the expression levels of ICOSL and the numbers of Tfh cells were significantly positively correlated with the levels of ACPA (Fig. 5B, C).

6. Correlation between the levels of Aire, ICOSL, and Tfh cells in the peripheral blood from RA patients and the disease activity score in 28 joints (DAS28).

To analyze the association between the circulating levels of Aire, ICOSL, and Tfh cells and the DAS28, we compared these three indicators and the DAS28. The results showed that the expression levels of Aire in the peripheral blood of the RA patients was significantly negatively correlated with the DAS28 (Fig. 6A), and the expression levels of ICOSL and the numbers of Tfh cells were significantly positively correlated with the DAS28 (Fig. 6B, C).

Discussion

Rheumatoid arthritis is an autoimmune disease characterized by chronic inflammation of the joint synovium.As the disease progresses, the incidence of disability and functional limitation increases. RA not only causes a decline in a patient's physical function, quality of life and social participation but also causes a substantial economic burden to the patient's family and society [19, 20]. Early diagnosis and active induction of disease remission are the keys to the prevention and treatment of complications; therefore, strengthening the research on the pathogenesis of RA is of great significance to the early diagnosis, treatment, prevention of RA and the development of gene-targeted therapeutic drugs.

As a transcription factor, Aire plays an important role in maintaining central and peripheral immune tolerance. In the periphery, Aire can maintain immune tolerance by inhibiting the production of IL-12 and IL-6 in DCs to further inhibit Th1, Th17 and Tfh cell differentiation [11, 21]. Recent research on RA susceptibility genes found that mutations in the Aire gene increase the risk of RA in Japanese and Hispanic patients [22, 23], and mutations at rs2075876 and rs760426 in the Aire gene are closely related to the pathogenesis of RA in Asian patients [24]. Our findings show that the levels of circulating Aire in RA patients were significantly lower than those in healthy subjects, which was consistent with the research results described above. However, the specific mechanism by which Aire functions in the pathogenesis of RA remains unknown.

Tfh cells are a subset of $CD4^+$ T cells that were discovered in recent years. This population is mainly located in germinal centers of secondary lymphoid organs, expresses high levels of CXCR5, and plays critical roles in the proliferation and differentiation of antigen-specific B cells in germinal centers [25]. Studies have shown that Tfh cells are increased in a variety of autoimmune diseases, such as systemic lupus erythematosus, myasthenia gravis, type 1 diabetes mellitus and Sjogren's syndrome [26-29]. Recent reports suggest that Tfh cells may be responsible for the occurrence and development of RA [30, 31]. Tfh cells can cause B lymphocytes to proliferate in germinal centers and produce a large amount of immunoglobulins, such as ACPAs. Cao et al. and Ma et al. found that RA patients had more Tfh cells in their peripheral blood than healthy controls, and the percentage of Tfh cells was positively correlated with the levels of ACPAs. In addition, higher mRNA expression of Bcl-6 was observed in patients with RA than in healthy controls, and the expression level of IL-21 was higher in RA patients. The results described above indicated that Tfh cells may be involved in the development of RA [18, 32]. Research by Wang et al. also showed that the proportion of Tfh cells in the peripheral blood of patients with RA was significantly higher than that in the peripheral blood of healthy controls [33]. This study showed that the proportion of Tfh cells in the peripheral blood of RA patients was significantly higher than that in the peripheral blood of healthy subjects and was positively correlated with the ACPA levels and disease activity, which was consistent with the research results described above. Increased numbers of Tfh cells can promote the activation of autoreactive B cells in RA patients, which in turn induces the production of large amounts of autoantibodies, such as RF and ACPAs. These autoantibodies combine with autoantigens in the joints to

form immune complexes, inducing inflammatory reactions in local tissues by activating complement and other mechanisms, ultimately exacerbating joint damage and promoting disease progression.

Our study showed that the level of circulating Aire in RA patients was significantly lower than that in healthy subjects, and the proportion of Tfh cells in RA patients was significantly higher than that in healthy subjects. These results prompted us to further investigate the relationship between Aire and Tfh cells. Our previous research showed that Aire could affect Tfh differentiation by regulating the expression of ICOSL on DCs. Therefore, the level of circulating ICOSL in RA patients was assessed, and the results showed that the expression level of ICOSL in RA patients was significantly higher than that in healthy controls. Correlation analysis showed that the expression level of Aire was significantly negatively correlated with the expression level of ICOSL and the proportion of Tfh cells in the peripheral blood of RA patients. The ICOSL expression level was significantly positively correlated with the proportion of Tfh cells, the levels of ACPA and the DAS28. These results suggested that Aire might affect Tfh differentiation by regulating the expression of ICOSL on DCs in RA patients. This may be the mechanism by which Aire affects the pathogenesis of RA, but this conclusion needs to be verified by further in-depth studies. Research in a collagen-induced mouse model of rheumatoid arthritis showed that an anti-ICOSL antibody blocked the ICOS/ICOSL interaction, inhibited T cell proliferation, decreased joint inflammation and delayed and reduced overall disease progression and severity [34, 35]; these results suggested that ICOSL played an important role in the pathogenesis of rheumatoid arthritis, which was consistent with our research results, but the authors did not elucidate whether ICOSL affects the differentiation of Tfh cells. A recent study demonstrated that ICOS expressed by Tfh cells could affect AKT phosphorylation and mTORC1 activation through PDK1, thereby affecting the differentiation of Tfh cells [36]. However, whether the increase in ICOSL expression in RA patients also exerts its effects through this pathway still needs to be explored.

Conclusion

The results of our study showed that compared with the control group, the RA group exhibited significantly reduced levels of circulating Aire, while the ICOSL expression levels and the Tfh cell numbers in the peripheral blood were significantly increased. Additionally, the Aire expression levels were significantly negatively correlated with the ICOSL expression levels and the Tfh cell numbers in the peripheral blood of RA patients, and the ICOSL expression levels were significantly positively correlated with the Tfh cell numbers. Moreover, the Aire and ICOSL expression levels and the Tfh cell numbers were correlated with the ACPA levels and the DAS28. These results suggested that RA patients may have abnormal Aire gene expression, which may affect Tfh cell differentiation by regulating the expression of ICOSL on DCs, leading to the onset of RA. However, the exact role of Aire in the pathogenesis of RA must be confirmed by further research. This study provides new experimental evidence for understanding the pathogenesis of RA. In addition, this study provides a new idea for treating rheumatoid arthritis by regulating Tfh cell differentiation and autoantibody production by targeting Aire.

Declarations

Acknowledgements

Not applicable

Authors’ contributions

All the authors participated in the data collection and writing of the manuscript and also helped in the statistical issues and the revision of the paper. The authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

All patients were fully informed about the study and its aim, and their consent was taken without any financial compensation.

Ethical approval was also taken from The First Hospital of Jilin University.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

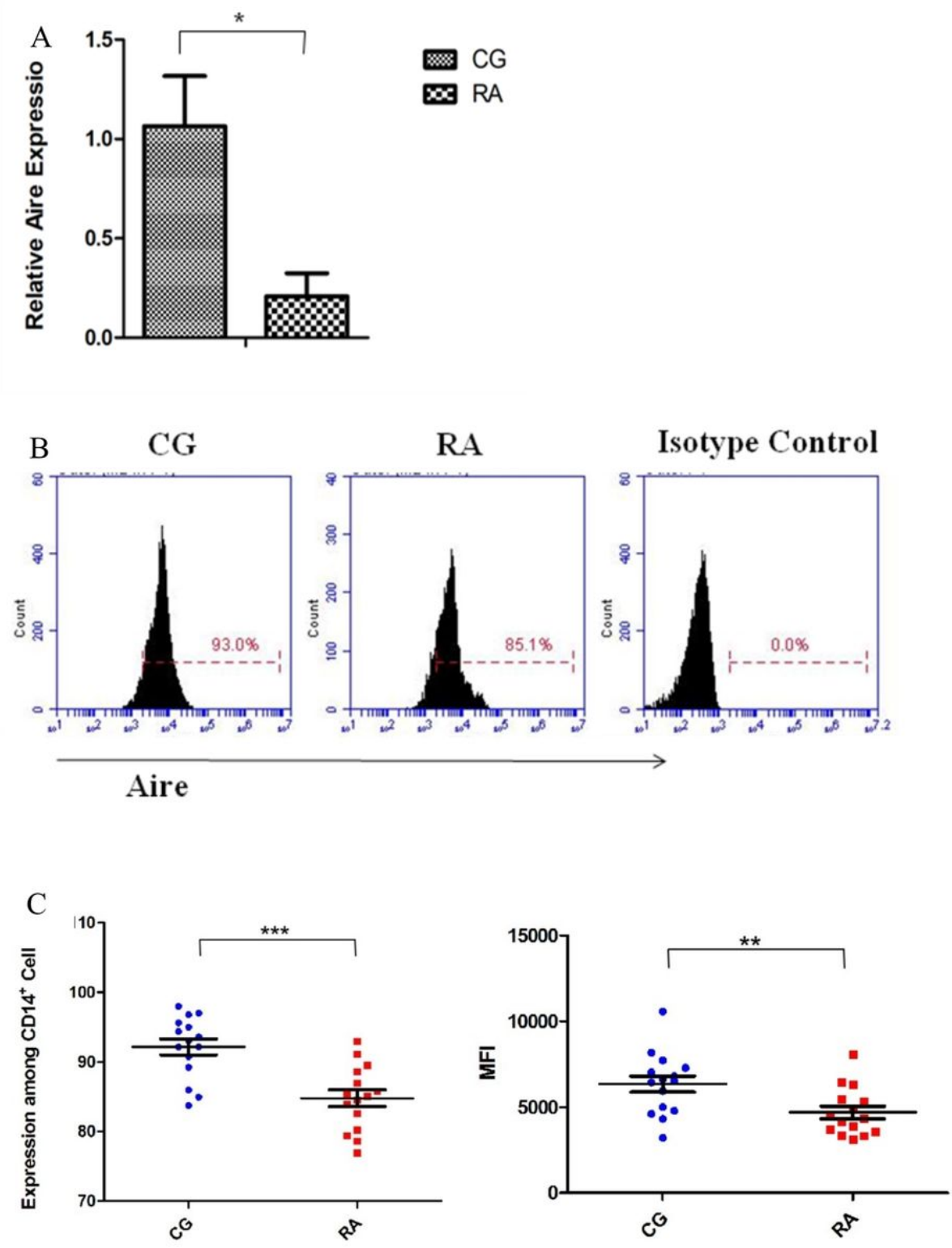

Figure 1

Circulating Aire level in CG and RA patients A. Detection of the expression level of Aire in PBMCs using RT-qPCR; B. Detection of the expression levels of Aire in PBMCs using FACS; C. Statistical analysis results of Aire expression level, the left is the frequency of Aire+ cells among CD14+ PBMCs, the right is the mean fluorescence intensity (MFI) of Aire. CG, control group. RA, Rheumatoid arthritis group.* P<0.05; **P<0.01; *** p<0.001.

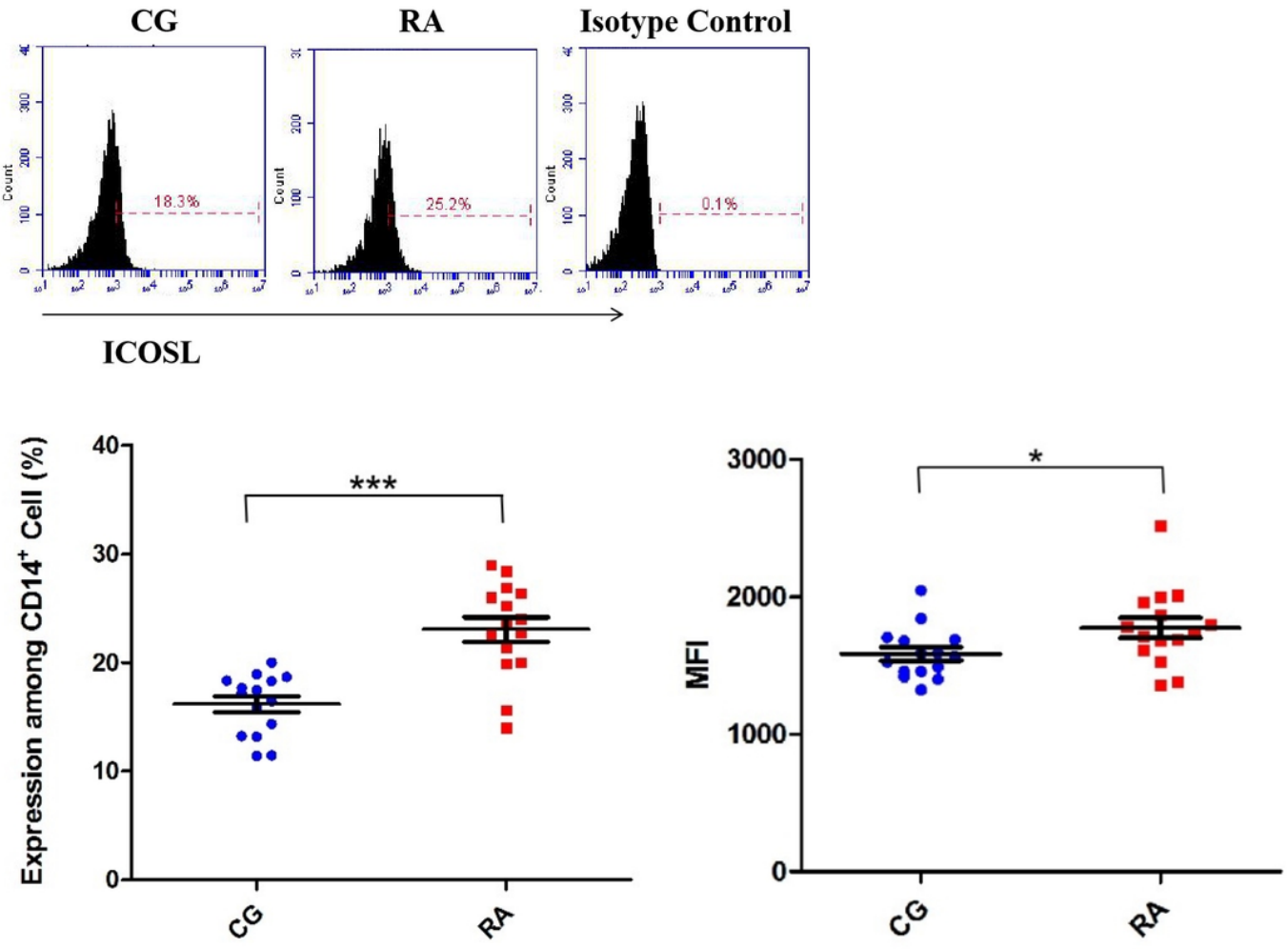

Figure 2

ICOSL expression in PBMCs in CG and RA patients Detection of the expression level of ICOSL in PBMCs using FACS, *P<0.05 , **P<0.01, ***P<0.001.

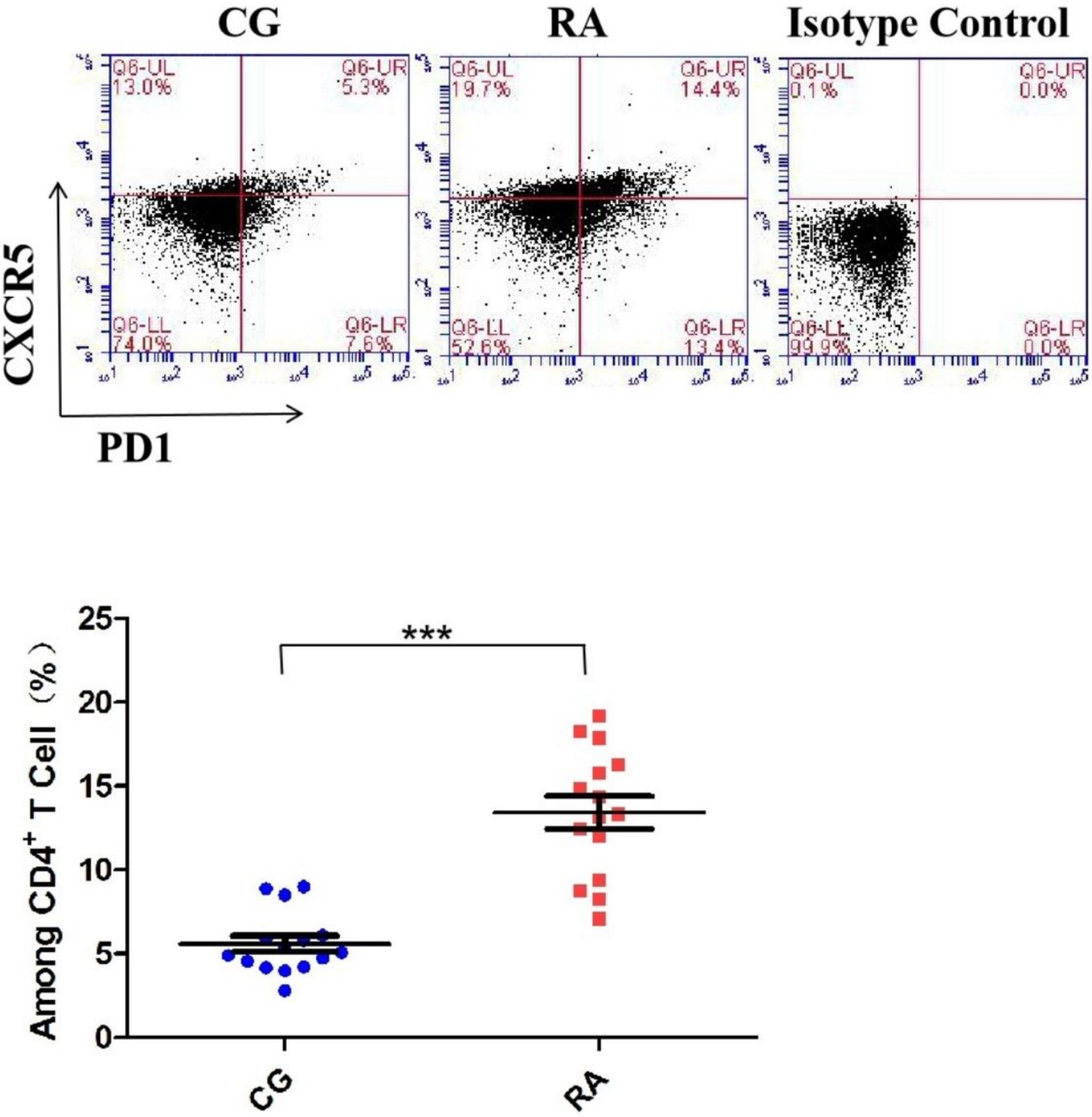

Figure 3

Tfh cells in the peripheral blood of CG and RA patients Detection of Tfh cells in the peripheral blood using FACS, *P<0.05 , **P<0.01, ***P<0.001.

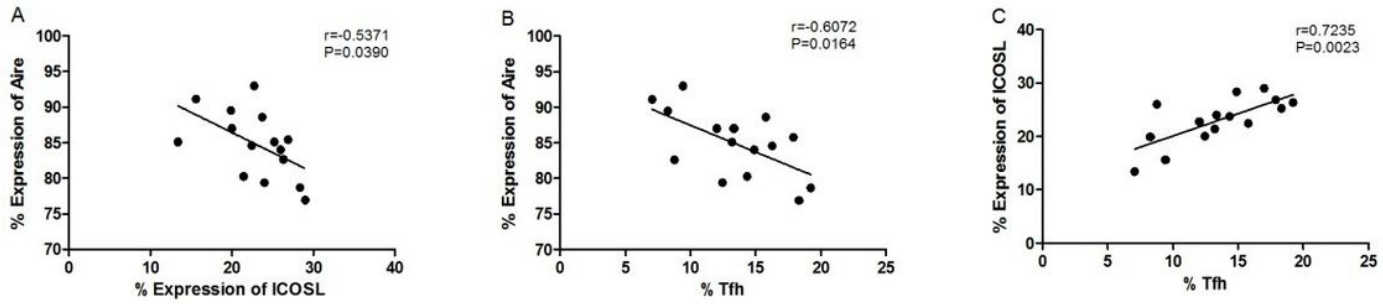

Figure 4

Correlation between Aire, ICOSL and Tfh cells in RA A. Correlation between Aire and ICOSL; B. Correlation between Aire and Tfh; C. Correlation between ICOSL and Tfh.

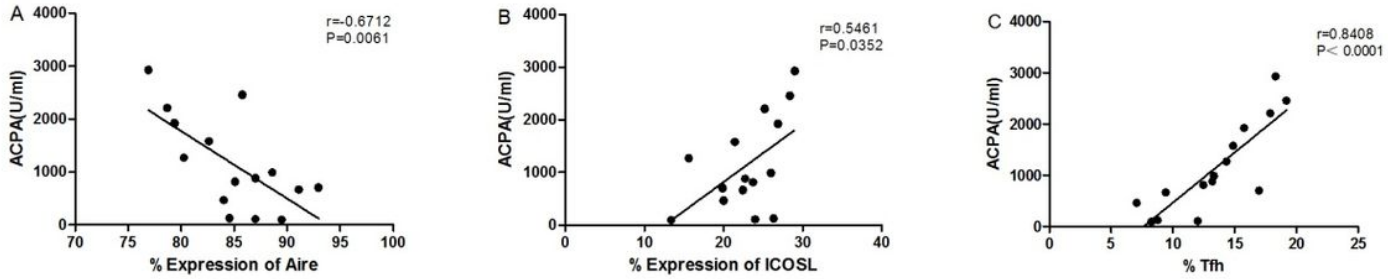

Figure 5

Correlation between Aire, ICOSL, Tfh cells and ACPA in RA A. Correlation between Aire and ACPA; B. Correlation between ICOSL and ACPA; C. Correlation between Tfh and ACPA.

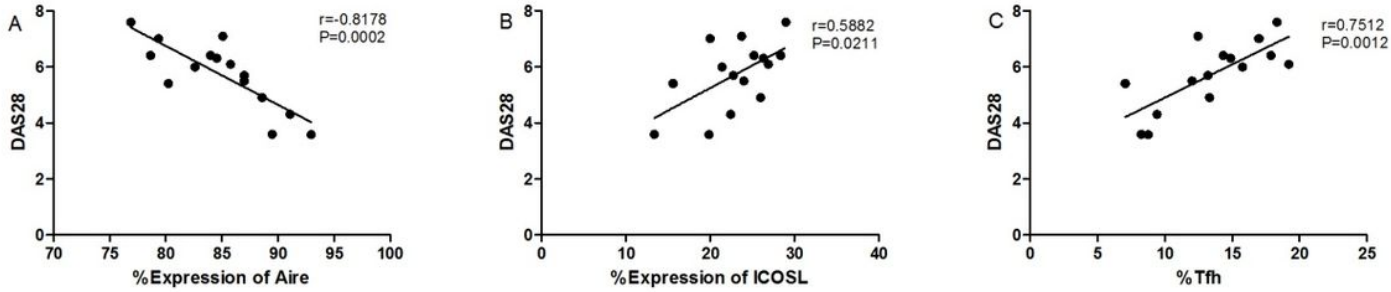

Figure 6

Correlation between Aire, ICOSL, Tfh cells and DAS28 in RA A. Correlation between Aire and DAS28；B. Correlation between ICOSL and DAS28；C. Correlation between Tfh and DAS28.