

ORIGINAL ARTICLE

IL-10-Expressing Th2 Cells Contribute to the Elevated Antibody Production in Rheumatoid Arthritis

Jinliang Wang,¹ Liheng Ma,² Shufeng Yang,^{3,4} Shaohua Wang,¹ Xuan Wei,^{1,4} and Shuchun Song¹

Abstract—Rheumatoid arthritis (RA) is a common autoimmune disease associated with progressive disability, systemic complications, and early death. Multiple lines of evidence have placed adaptive immune responses in the center of RA pathogenesis. However, the functional roles of T helper cells are insufficiently described. Here, we examined the Th2 cell subsets and their functions in RA patients. A downregulation of IL-4⁺ cells in CD4⁺ T cells were observed in RA patients, indicating a downregulation of Th2 cells, and these results were confirmed by using CXCR3 and CCR6 surface markers. We then found that CXCR3⁺CCR6⁻ Th2 cells can be separated into IL-4⁺ (single positive), IL-10⁺ (single positive), and IL-4⁺IL-10⁺ (double positive) subsets. Further results showed that CXCR5 only expressed on IL-10⁺ Th2 cells. The CXCR5⁺ and CXCR5⁻ Th2 cells each exhibited distinctive features in helping B cell antibody secretion. CXCR5⁺ Th2 cells were more potent at stimulating total Ig and IgM secretion, while CXCR5⁻ Th2 cells were more potent at stimulating IgE. IL-10 was required for helping B cell total Ig, IgM, and IgE production, while IL-4 was required for total Ig and IgE. The frequencies of IL-10⁺ and IL-4⁺IL-10⁺ Th2 cells were positively correlated with rheumatoid factor titer *in vivo*. Together, our study demonstrated distinctive subsets within Th2 cells, each with different impacts on antibody production and RA disease.

KEY WORDS: IL-10; Th2 cells; antibody; rheumatoid arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is a progressive autoimmune disease that can result in cartilage and bone

destruction, cardiovascular and pulmonary complications, and early death [1]. Though it is well established that inflammatory disorders, including synovial inflammation, hyperplasia, and systemic immune disorders, are responsible for disease progression, very little is understood in disease induction. Few biomarkers exist to reliably provide predictions on prognosis and response toward therapies, and sustained remission is rarely achieved [2]. To solve these problems, a more detailed understanding of the molecular and cellular mechanisms in RA is necessary.

The synovial inflammation is characterized by the presence of autoantibodies, including rheumatoid factors (RF) and anticitrullinated protein antibodies (ACPA) [3], and a host of proinflammatory and regulatory cytokines such as TNF- α , IL-1, IL-4, IL-6, IL-8, IL-10, GM-CSF, and TGF- β [4,5], indicating a role of adaptive immune responses [6,7]. Interestingly, inhibitory drugs targeting cytokines that are characteristic of T helper 1 (Th1) and/

Jinliang Wang and Liheng Ma contributed equally to this work.

¹Department of Orthopedics, Zhengzhou Orthopedics Hospital, 58 Longhai Middle Road, Zhengzhou, Henan 450052, China

²Department of radiology, The First Affiliated Hospital of Guangdong Pharmaceutical University, Guangdong, 510080, China

³Department of Orthopedics, The 81st Hospital of PLA, 34 Yanggongjing Road, Nanjing, Jiangsu 210002, China

⁴To whom correspondence should be addressed to Shufeng Yang at Department of Orthopedics, The 81st Hospital of PLA, 34 Yanggongjing Road, Nanjing, Jiangsu 210002, China. E-mail: shufengyangyyy@126.com; and Xuan Wei at Department of Orthopedics, Zhengzhou Orthopedics Hospital, 58 Longhai Middle Road, Zhengzhou, Henan 450052, China. E-mail: xuanwei_zz@sina.com

or Th17 responses have been intensively studied and have been approved as drugs or currently investigated in ongoing phase 1 and phase 2 trials. However, the Th2 and regulatory T (Treg) cell cytokines, including IL-4, IL-10, and TGF- β , have not been intensively examined as drug targets [2]. IL-10 and TGF- β are generally considered as regulatory cytokines, with functions that suppress activated T cell proliferation, inhibit proinflammatory gene transcription, and downregulate antigen presentations [8–10]. Treatment with IL-4 and IL-10 has been seen to decrease TNF- α and IL-1 production [11,12]. But on the other hand, IL-10 also increases antibody production and is associated with RF titer [13]. Whereas IL-4 induces antibody class switching to IgE is a major component of ACPA [14]. The relative contribution of IL-4 and IL-10 in promoting and/or inhibiting RA inflammation is, therefore, a complicated issue and it is yet unknown whether they could act as potential drug targets in RA.

Both IL-4 and IL-10 can be produced by Th2 cells. Loss of Th1/Th2 balance has been reported in RA [15]. But within the Th2 population, it is yet unknown whether all Th2 cells express both IL-4 and IL-10 in RA, or Th2 preferentially expresses one but not the other. Furthermore, if such a preference exists, how the inflammatory process in RA could be affected by the IL-4 and/or IL-10 production in Th2 cells. To elucidate the functions of IL-4 and IL-10 in RA, we examined the Th2 cells in RA patients. Our study demonstrated distinctive subsets within Th2 cells, each with different impacts on antibody production and RA disease.

MATERIALS AND METHODS

Patients and Controls

All RA patients and age- and sex-matched healthy controls were enrolled at Zhengzhou Orthopedics Hospital. The protocols were approved by the Ethics Board of Zhengzhou Orthopedics Hospital. All participants provided written informed consent. The criteria for entry included ages between 19 to 70 years, active RA fulfilling the criteria of the American Rheumatism Association, and ongoing disease for at least 3 months before recruitment. At three of the following must be present for inclusion, including erythrocyte sedimentation rate ≥ 28 mm/h, eight or more tender joints, three or more swollen joints, and morning stiffness lasting 45 min or more. Patients with other serious diseases, such as liver, renal, hematologic, pulmonary, and cardiovascular diseases and tumor, were

excluded. All demographic and clinical information of patients and controls are summarized in Table 1.

Sample Collection

Circulating blood samples were collected from healthy controls and RA patients at the upper arm. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll centrifugation and were cryopreserved at -80°C before use.

Flow Cytometry and Live Sorting

For cytokine and phenotype analyses, PBMCs were first incubated in RPMI1640 complete medium with 10% FBS for 12 h, without or with PMA and ionomycin (1 $\mu\text{g}/\text{mL}$ each) in the presence of brefeldin A and monensin (5 $\mu\text{g}/\text{mL}$ each). Cells were then surface stained with the following mAbs: CXCR5-PerCPCy5.5, CCR6-PECy5, CXCR3-PECy7, CD3-BV605, CD4-BV650, CD8-BV785, and dead cell stain Aqua, followed by intracellular staining of IFN- γ -Pacific Blue, IL-4-PE, IL-17-FITC, and IL-10-APC. The stained cells were analyzed with BD Fortessa. Greater than 2×10^5 events in the lymphocyte gate were collected for each analysis. For live sorting, CXCR3⁻CCR6⁻CD4⁺ T cells were first isolated through magnetic negative selection using Human CD4⁺ T Cell Negative Selection Kit (Stemcell) with anti-CXCR3 and anti-CCR6 monoclonal antibodies

Table 1. Demographic and Clinical Information of RA Patients and Controls

	RA (n = 15)	Control (n = 15)	P
Age (year)	43.4 \pm 15.7	44.8 \pm 17.1	>0.05
Sex (F/M)	6/9	6/9	>0.05
Duration of disease (year)	5.8 \pm 4.0	–	
RF (n)	13	0	
Present	2	15	
Absent			
ACPA (n)	12	0	
Present	3	15	
Absent			
Erythrocyte sedimentation rate (mm/h)	40.0 \pm 21.3	8.1 \pm 4.2	<0.001
Duration of morning stiffness (min)	151 \pm 86	–	
Tender joints (n)	7 \pm 6	–	
Swelling joints (n)	5 \pm 5	–	
C-reactive protein (mg/L)	15.5 \pm 7.8	3.2 \pm 2	<0.001

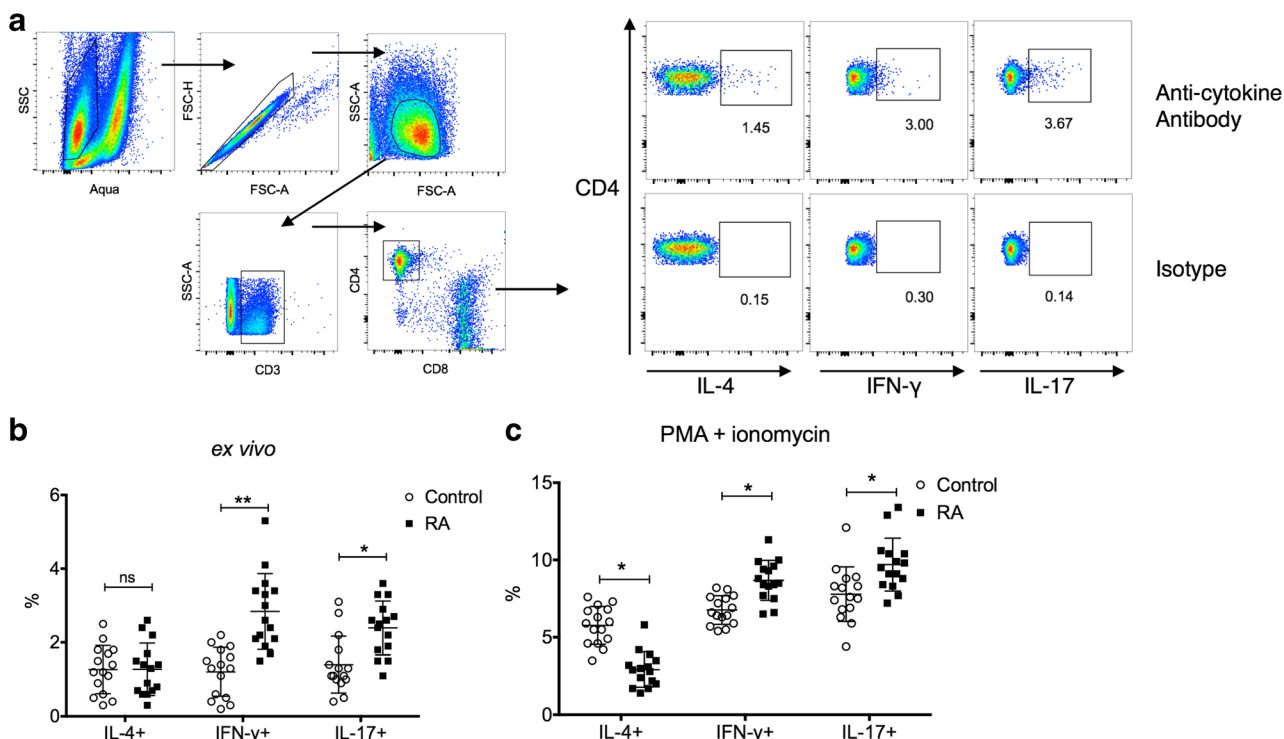


Fig. 1. Identification of Th2 cells by cytokine expression and chemokine receptor expression. **a** Gating strategy of peripheral blood Th subsets directly *ex vivo* without stimulation. The CD4⁺ T cells were gated sequentially by Aqua⁻/singlet/not debris/CD3⁺/CD4⁺CD8⁻. The positive gating for intracellular cytokines, including IL-4, IFN- γ , and IL-17, were then determined by comparing to an irrelevant isotype control. **b** Frequencies of IL-4⁺, IFN- γ ⁺, and IL-17⁺-expressing cells in unstimulated total CD4⁺ T cells from RA patients and healthy controls directly *ex vivo*. **c** Frequencies of IL-4⁺, IFN- γ ⁺ and IL-17⁺-expressing cells in total CD4⁺ T cells after PMA and ionomycin stimulation. Unpaired *t* test with Welch's correction. ***p* < 0.01, **p* < 0.05. *ns* not significant.

added into the antibody cocktail. The purified CXCR3⁻CCR6⁻ CD4⁺ T cells were then surface stained with anti-CXCR5 antibody and sorted into CXCR5⁺ and CXCR5⁻ fractions with BD Aria. Naive CD27⁻ B cells were isolated using the Human B Cell Negative Selection Kit (Stemcell) with anti-CD27 monoclonal antibody added into the antibody cocktail.

T cell-B cell Coculture

Isolated naive B cells were first incubated with endotoxin-reduced SEB (2 μ g/mL) for 12 h and then washed. Sorted CXCR5⁺ or CXCR5⁻ CXCR3⁻CCR6⁻ CD4⁺ T cells were then cocultured with SEB-pulsed naive B cells for 12 days at 10⁵ cells per 200 μ L medium per well at 37°C 5% CO₂. Anti-human IL-4 and IL-10 monoclonal antibodies, as well as irrelevant isotype controls (1 μ g/mL each), were added to some of the wells. All experiments were done in duplicates. One hundred fifty microliter supernatant was obtained at the end of incubation. Ig levels were examined by ELISA.

Statistics

The mean \pm standard deviation was shown for all datasets where applicable. The D'Agostino-Pearson normality test was applied to determine whether parametric or nonparametric tests should be used. *P* < 0.05 was considered significant.

RESULTS

RA Patients Contained Downregulated Frequencies of Th2 Cells than Healthy Controls

We first examined the frequencies of Th2 cells in RA patients. Th2 cells are characterized by IL-4 expression and are negative for CXCR3 and CCR6. Th1 cells are characterized by IFN- γ and CXCR3 expression, whereas Th17 cells by IL-17 and CCR6 expression [16, 17]. We first analyzed the frequencies of IL-4⁺, IFN- γ ⁺, and IL-17⁺-producing cells (Fig. 1a). Comparable frequencies of IL-

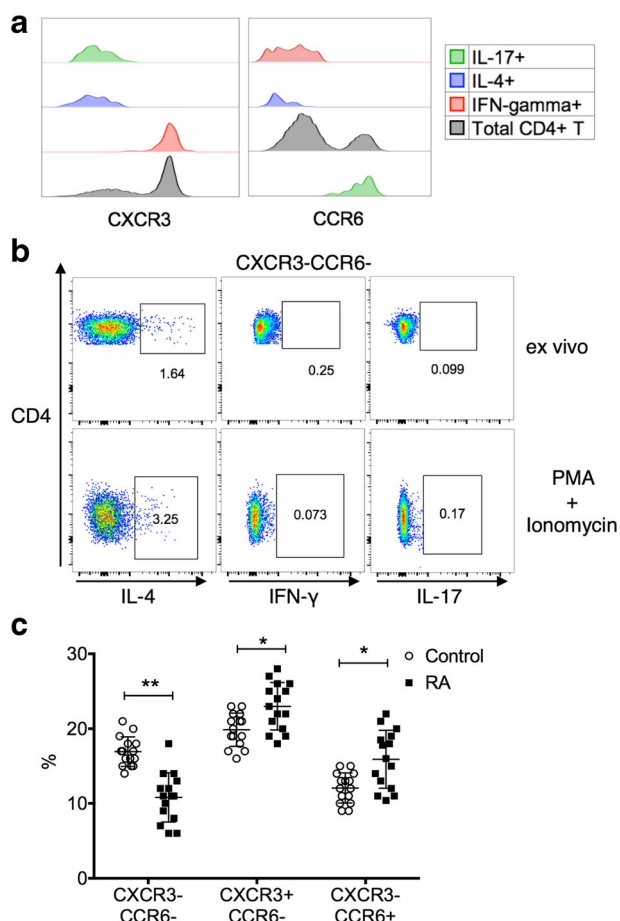


Fig. 2. Surface CXCR3 and CCR6 expression by IL-4⁺, IFN- γ ⁺, and IL-17⁺ expressing CD4⁺ T cells. **a** Surface CXCR3 expression histogram on IL-4⁺ (blue), IFN- γ ⁺ (red), and IL-17⁺ (green) cells overlaid on top of total CD4⁺ T cells (black). **b** Intracellular cytokine IL-4, IFN- γ , and IL-17 expression in CXCR3⁺CCR6⁻ CD4⁺ T cells, directly *ex vivo* without stimulation or after PMA and ionomycin stimulation. **c** Frequencies of CXCR3⁻CCR6⁻ (Th2), CXCR3⁺CCR6⁻ (Th1), and CXCR3⁻CCR6⁺ (Th17) cells under unstimulated condition in controls and RA patients. Unpaired *t* test with Welch's correction. ***p* < 0.01. **p* < 0.05.

4⁺ CD4⁺ T cells were observed in the peripheral blood of RA patients and healthy controls directly *ex vivo*, while elevated frequencies of IFN- γ ⁺ and IL-17⁺ CD4⁺ T cells were observed in RA patients (Fig. 1b). After PMA and ionomycin stimulation, RA subjects contained significantly lower frequencies of IL-4⁺ CD4⁺ T cells and significantly higher IFN- γ ⁺ and IL-17⁺ CD4⁺ T cells than healthy controls (Fig. 1c). Together, these data demonstrated a significant enrichment of Th1 and Th17 cells and a relative lack of Th2 cells in RA subjects.

We also tried to identify Th2 cells by surface chemokine receptor expression to enable purification for further functional analyses. As predicted, IL-4⁺ CD4⁺ T cells were

negative for both CXCR3 and CCR6, while IFN- γ ⁺ and IL-17⁺ CD4⁺ T cells were positive for CXCR3 and CCR6, respectively (Fig. 2a). From another angle, CXCR3⁻CCR6⁻ cells did not express either IFN- γ or IL-17 but expressed IL-4 *ex vivo* or after PMA/ionomycin stimulation (Fig. 2b). Together, these data confirmed that CXCR3⁻CCR6⁻ double-negative expression could serve as a surface phenotype to allow the identification of Th2 cells. We also examined the frequencies of CXCR3⁻CCR6⁻, CXCR3⁺CCR6⁻, and CXCR3⁻CCR6⁺ cells in total CD4⁺ T cells, and found that compared to healthy controls, RA patients contained significantly elevated frequencies of CXCR3⁺CCR6⁻ and CXCR3⁻CCR6⁺ CD4⁺ T cells and reduced frequencies of CXCR3⁻CCR6⁻ CD4⁺ T cells (Fig. 2c), thus demonstrating the reduction of Th2 cells by surface chemokine receptor expression.

IL-4 Single-Positive, IL-10 Single-Positive, and IL-4/IL-10 Double-Positive Th2 Cells Can Be Detected in RA Patients

We then examined whether the Th2 cells are composed of a uniform population of cells or several subsets with distinctive functions. Therefore, we examined the IL-4 and IL-10 expression in total CXCR3⁻CCR6⁻ Th2 cells. IL-4 single-positive (hereafter referred to as IL-4⁺), IL-10 single-positive (hereafter referred to as IL-10⁺), IL-4/IL-10 double-positive (hereafter referred to as IL-4⁺IL-10⁺) subsets could be detected within the CXCR3⁻CCR6⁻ Th2 population (Fig. 3a and b). To allow further examination of Th2 subsets without permeabilizing cells, we examined surface marker expression of IL-4⁺, IL-10⁺, and IL-4⁺IL-10⁺ Th2 cells, and found that CXCR5⁻ Th2 cells were enriched for IL-4⁺IL-10⁺ cells and IL-10⁺ cells, while CXCR5⁺ Th2 cells were enriched for IL-4⁺ cells (Fig. 3a). To confirm this hypothesis, we live-sorted total CXCR3⁻CCR6⁻ Th2 cells into CXCR5⁻ and CXCR5⁺ fractions, which were then incubated for 12 h. The cytokine expression in the supernatant was then measured. We found that the CXCR5⁻ Th2 fraction contained significantly higher amount of IL-4 and very little IL-10, compared to the CXCR5⁺ Th2 fraction, with or without stimulation (Fig. 3c and d). Together, these data demonstrated that the total CXCR3⁻CCR6⁻ Th2 cells can be further separated into two subsets, including the CXCR5⁻ Th2 fraction with IL-4⁺ cells almost exclusively, and the CXCR5⁺ Th2 fraction with both IL-10⁺ cells and IL-4⁺IL-10⁺ cells.

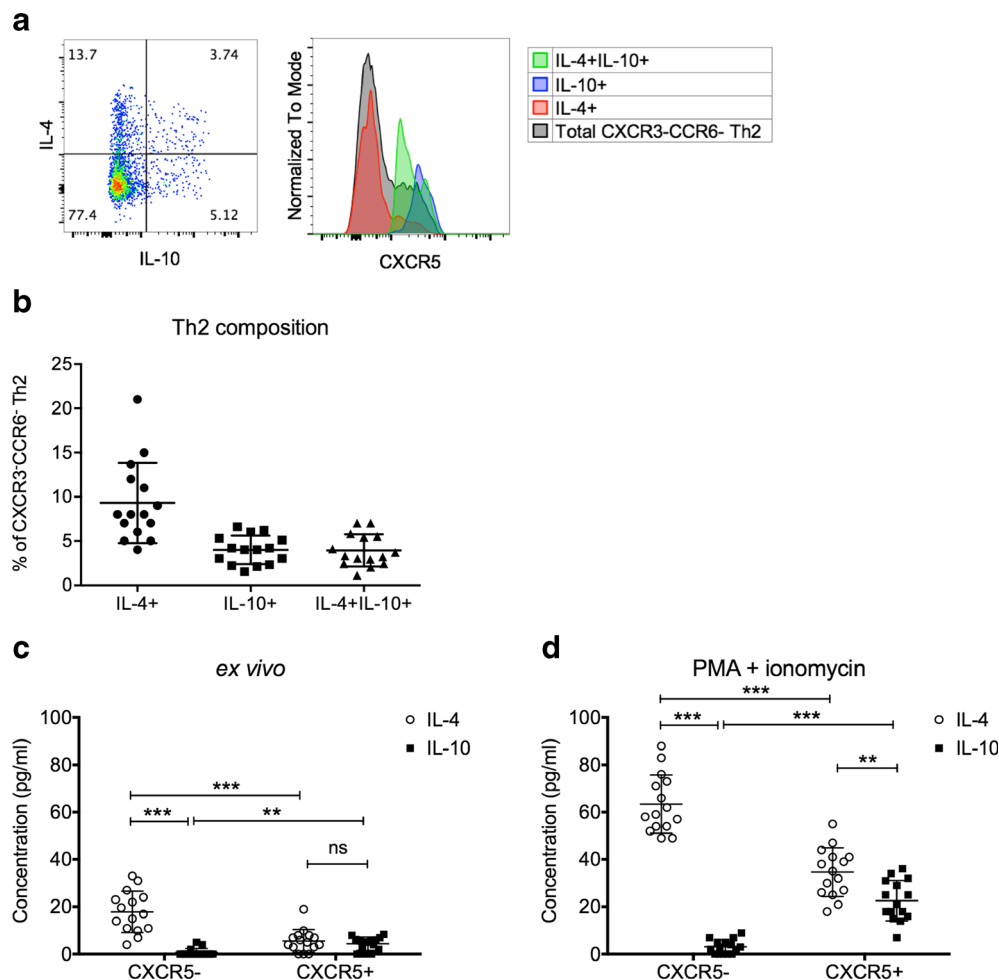


Fig. 3. Characterization of IL-4⁺, IL-10⁺, and IL-4⁺IL-10⁺ Th2 cells in RA patients. **a** Intracellular expression of IL-4 and IL-10 in CXCR3⁺CCR6⁻ Th2 cells from an RA patient, and the surface CXCR5 expression by IL-4⁺ (red), IL-10⁺ (blue), and IL-4⁺IL-10⁺ (green) cells overlaid on top of total CXCR3⁺CCR6⁻ Th2 cells (black). **b** Frequencies of IL-4⁺, IL-10⁺, and IL-4⁺IL-10⁺ cells in total CXCR3⁺CCR6⁻ Th2 cells. **c** IL-4 and IL-10 expression by CXCR5⁺ vs. CXCR5⁻ CXCR3⁺CCR6⁻ Th2 cells after live sorting and culturing directly *ex vivo* for 12 h. **d** IL-4 and IL-10 expression by CXCR5⁺ vs. CXCR5⁻ CXCR3⁺CCR6⁻ Th2 cells after live sorting and culturing for 12 h with PMA and ionomycin stimulation. Two-way ANOVA and Sidak's multiple comparisons test. ****p* < 0.001. ***p* < 0.01. *ns* not significant.

CXCR5⁺ Th2 Were More Potent at Stimulating Autologous B Cell Antibody Production than CXCR5⁻ Th2 Cells

Both IL-4 and IL-10 were known to stimulate antibody production. To examine the effect of IL-4 and IL-10 by Th2 cells on B cell antibody production in RA, CXCR3⁺CCR6⁻ Th2 cells were sorted into CXCR5⁺ and CXCR5⁻ fractions, and were cocultured with autologous naive (CD27⁻) B cells. Superantigen staphylococcal enterotoxin B (SEB) was added to the B cells for 12 h prior to coculture to simulate antigen-specific interaction between T cells and B cells. Excess SEB was washed away. We found that autologous B cells incubated with CXCR5⁺

Th2 cells secreted significantly higher amount of antibodies than those incubated with CXCR5⁻ Th2 cells (Fig. 4a). Depletion of either IL-4 or IL-10 in the coculture with CXCR5⁺ Th2 cell could suppress autologous B cell antibody production, but the depletion of IL-10 yielded a stronger result. In the coculture with CXCR5⁻ Th2 cells, depletion of IL-4 could also suppress autologous B cell antibody production. In terms of antibody isotypes, the CXCR5⁺ Th2 cells were significantly more effective at stimulating IgM and less effective at stimulating IgE, than CXCR5⁻ Th2 cells (Fig. 4b and c). Depletion of IL-10 could suppress the expression of both IgM and IgE, while depletion of IL-4 suppressed the expression of IgE.

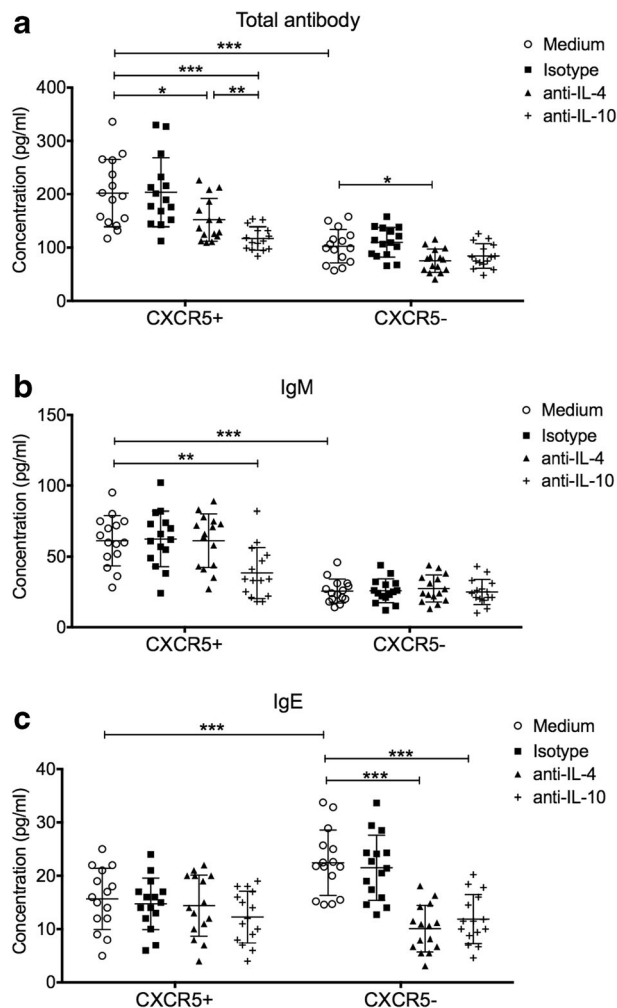


Fig. 4. Potency of CXCR5⁺ vs. CXCR5⁻ CXCR3⁺ CCR6⁻ Th2 cells in helping B cell antibody production. Live-sorted CXCR5⁺ or CXCR5⁻ CXCR3⁺ CCR6⁻ Th2 cells were cocultured with SEB-pulsed naive (CD27⁻) B cells for 12 days. Ig concentration in the supernatant was then measured by ELISA. In a subset of cultures, anti-IL-4 or anti-IL-10 monoclonal antibodies, or irrelevant isotype controls, were added to the culture to assess the effect of cytokine depletion. **a** The total Ig concentration in the supernatant. **b** The IgM concentration in the supernatant. **c** The IgE concentration in the supernatant. All values were averages from duplicate experiments. Two-way ANOVA and Sidak's multiple comparisons test. ****p* < 0.001. ***p* < 0.01. **p* < 0.05.

The Frequencies of IL-10⁺, but not IL-4⁺ nor IL-4⁺IL-10⁺, Th2 Cells Were Correlated with RF Titer in RA Patients

Having observed an *in vitro* effect on antibody production, we then examined the *in vivo* association between IL-4⁺, IL-10⁺, and IL-4⁺IL-10⁺ Th2 cells and RA disease. We found that the frequencies of IL-10⁺ and IL-4⁺IL-10⁺

Th2 cells were positively correlated with RF titer (Fig. 5a). Interestingly, no Th2 subpopulation was correlated with ACPA titer (Fig. 5b).

DISCUSSION

Our data showed the existence of distinctive subsets within the Th2 cells based on IL-4 and IL-10 expression. We first discovered that IL-4⁺ single-positive, IL-10⁺ single-positive, and IL-4⁺IL-10⁺ double-positive Th2 cells exist in RA patients. Also, we found that CXCR5 only expressed on IL-10⁺ Th2 cells. Importantly, CXCR5⁻ Th2 cells, which could express IL-4, cannot be stimulated by PMA and ionomycin to express IL-10, which suggests that CXCR5⁺ and CXCR5⁻ Th2 cells underwent distinctive developmental processes.

CXCR5 is a chemokine receptor that is expressed by B cells and is required for migration and interaction with CXCL13 in germinal center follicles [18]. Expression of CXCR5 on T cells enables T cells to locate to the B cells and provide T cell help during B cell maturation [19, 20]. Therefore, CXCR5⁺ Th2 cells are more likely to interact with B cells than CXCR5⁻ Th2 cells due to different *in vivo* localization patterns. That, combined with the stronger *in vitro* potency in stimulating B cells, suggests that CXCR5⁺ Th2 cells could exacerbate RA disease activity by increasing autoantibody production in an IL-4 and/or IL-10-dependent fashion. Indeed, in patients with higher IL-10⁺ and IL-4⁺IL-10⁺ Th2 cell frequencies, the RF titer was also higher, which demonstrated that the IL-10-expressing Th2 cells contributed to RA disease *in vivo*.

One important question, therefore, is whether these IL-10-expressing Th2 cells contribute to autoantibody maintenance during remission. The RA disease process is characterized by the suppression of inflammation during remissions, which is reactivated during recurrence. It is unclear why sustained remission is rarely achieved with the current treatment schemes, which involve the use of pro-inflammatory cytokine inhibitors, such as TNF- α , IL-1, and IL-6 inhibitors. The discovery that IL-10, an anti-inflammatory cytokine in general, produced by a subset of Th2 cells could elevate antibody production suggests that IL-10 inhibitors might be helpful in restraining RA disease if used with caution. A significant amount of research is needed in this direction. The discovery that IL-10-expression in Th2 cells is restricted to CXCR5-expressing cells could assist selective depletion of CXCR5⁺ Th2 cells in attempt to reduce IL-10 production by Th2 cells safely.

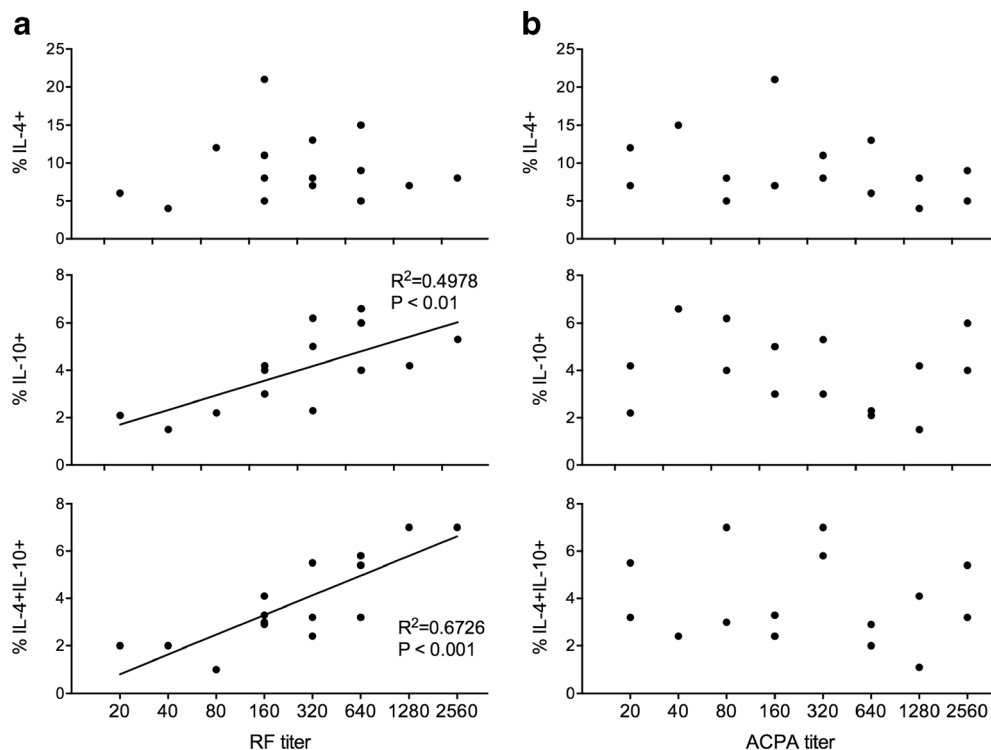


Fig. 5. Correlation of Th2 subsets with RF and ACPA titer in RA patients *in vivo*. **a** The correlation of IL-4⁺, IL-10⁺, and IL-4⁺IL-10⁺ Th2 cells with RF titers in RA patients. **b** The correlation of IL-4⁺, IL-10⁺, and IL-4⁺IL-10⁺ Th2 cells with ACPA titers in RA patients. Spearman correlation. $P < 0.05$ is considered significant.

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COMPLIANCE WITH ETHICAL STANDARDS

The protocols were approved by the Ethics Board of Zhengzhou Orthopedics Hospital. All participants provided written informed consent.

Conflict of Interest. The authors declare that they have no conflict of interest.

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