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ORIGINAL ARTICLE

Elevated circulating T cell subsets and cytokines expression in patients with rheumatoid arthritis

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Abstract

Objective This study aimed to assess the role of different subsets of circulating follicular helper T cells (Tfh), central memory (TCM), effector memory (TEM), Naïve T, chemokines, and cytokines in the pathogenesis of rheumatoid arthritis (RA).

Methods Blood samples from RA patients ($n = 44$) and healthy controls ($n = 37$) were analyzed. The frequencies of circulating Tfh, TCM, TEM, and Naïve T cell subsets were enumerated, and the expression of co-stimulatory molecules, such as inducible co-stimulator (ICOS) and programmed death-1 (PD1), on these cells was evaluated by flow cytometry. The disease state in RA patients was assessed using the DAS28. Concentrations of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), anti-cyclic citrullinated peptide (anti-CCP), and rheumatoid factor (RF) were measured. Cytokines and chemokines, such as IL-1β, TNF-α, IL-4, IL-6, IL-9, IL-17A, MCP-1, IL-10, IL-12p70, and IL-21, were measured by a cytometric beads array assay.

Results The percentages of circulating $PD1^+ICOS^+$ Tfh, $PD1^+ICOS^+$ TEM, and $PD1^+ICOS^+$ TCM of PBMCs from RA patients were higher than those in healthy controls. Furthermore, expression of circulating $PD1^+ICOS^+$ Tfh, $PD1^+ICOS^+$ TEM, and $PD1^+ICOS^+$ TCM showed a positive correlation with DAS28. In addition, increased levels of IL-1β, IL-6, and MCP-1 were detected in the patients with RA compared to healthy controls.

Conclusions Elevated circulating T cell subsets and cytokines expression profile were observed in RA patients. IL-6, MCP-1, and IL-1β were significantly increased in RA, and $PD1^+ICOS^+$ TEM, $PD1^+ICOS^+$ TCM, and $PD1^+ICOS^+$ Tfh cell subsets were positively correlated with disease activity DAS28. Therefore, $PD1^+ICOS^+$ TEM, $PD1^+ICOS^+$ TCM, and $PD1^+ICOS^+$ Tfh cells might serve an important role in the progression of RA.

Keywords Follicular help T cells · Memory T cells · Rheumatoid arthritis

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Background

Rheumatoid arthritis (RA) is a chronic and systematic autoimmune inflammatory disease characterized by inflammation and pain in the joints, the tissue around the joints, and other organs and systemic complications [1, 2]. Although the etiology of RA is not fully understood, both T cells and cytokines are thought to play a critical role in the induction and progression of the inflammatory conditions [3, 4]. Moreover, the aberrant $CD4^+$ T cell activation plays a key role in the initiation and perpetuation of the RA [5].

A large numbers of $CD4^+$ memory T cells infiltrate the inflamed synovium in patients with RA [6]. Memory T cells contain distinct populations of CC chemokine receptor 7^+ ($CCR7^+$), central memory (TCM), and $CCR7^-$ effector memory (TEM) cell subsets characterized by homing to secondary lymphoid organs and displaying immediate effector function [7]. Follicular helper T cells (Tfh) are crucial in the activation of B cells and differentiate to antibody-secreting cells [8]. Previous studies have revealed that distinguishing features of these T cell subsets are the expression of co-stimulatory molecules such as programmed death-1 (PD1) and inducible costimulator (ICOS) [9]. However, whether cell surface molecules expressed by circulating memory cells and Tfh cells play a role in predicting the flares and remission of RA remains unclear.

The aim of our study was to describe the frequency of circulating TCM, TEM, and Tfh cell subsets from RA patients and measure the circulating $PD1^+ICOS^+$ TCM, $PD1^+ICOS^+$ TEM, and $PD1^+ICOS^+$ Tfh cell subsets, as well as their secreting cytokines in RA patients and the correlation with disease activity.

Materials and methods

Patients and healthy controls

This cross-sectional study included 44 patients who were being followed up in the inpatient clinic at the division of Immunology and Rheumatology, the Affiliated Hospital of Guizhou Medical University, China. Patients were hospitalized due to unbearable pain. All patients met the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) 2010 rheumatoid arthritis classification criteria [10]. Patients with other inflammatory diseases or having any history of other chronic diseases such as respiratory disorders, cardiovascular diseases, and kidney impairment as well as those receiving conventional or biologic DMARDs, glucocorticoids, or any other drugs in recent 3 months were excluded. Disease activity was assessed by the Disease Activity Score-28 (DAS28), which was based on erythrocyte sedimentation rates (ESR). The control group consisted of 37 healthy controls without inflammatory or autoimmune diseases, who were unrelated to the patients. Data were collected between March 2017 and January 2018. This study was approved by the Ethics Committee of the Affiliated Hospital of Guizhou Medical University and was carried out in compliance with the Helsinki Declaration. All subjects gave written informed consent.

Plasma samples

A total of 5 ml sample of blood was drawn from both patients and healthy controls using tubes containing EDTA. Then, samples were centrifuged at 3000 rpm for 20 min, and the supernatant was divided into Eppendorf tubes (200 μl per tube). Plasma samples were stored at − 80 °C until cytokines were measured.

Cell surface staining and flow cytometric analysis

Peripheral blood was collected with EDTA as an anticoagulant and analyzed immediately for the molecular phenotypes of lymphocyte, using flow cytometry. The antibodies used for the surface marker analysis include anti-human CD3-APC-H7, CD4-Percp-Cy5.5, CD8-PE-Cy7, PD1-BB515, ICOS-PE, CXCR5-Alexafluor647, CD45RA-BV510, and CCR7-BV421 (BD Biosciences, USA). Briefly, 50 μl of cells was incubated with appropriate antibodies on ice in the dark for 30 min. All the samples were analyzed with a Navios flow cytometry (Beckman Coulter Inc., USA) and KALUZA analysis software programs (Beckman Coulter Inc., USA).

Laboratory tests

Erythrocyte sedimentation rates (ESR) from RA patients were measured. The serum C-reactive protein (CRP), anti-cyclic citrullinated peptide (anti-CCP), and rheumatoid factor (RF) from RA patients were detected by scatter turbidimetry using a Siemens special protein analyzer (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany).

Cytometric beads array

Chemokine and cytokine levels in the plasma were measured with a cytometric bead array (BD Biosciences). The cytometric bead array was simultaneously performed with specific antibodies for IL-1β, TNF-α, IL-4, IL-6, IL-9, IL-17A, MCP-1, IL-10, IL-12p70, and IL-21 in accordance with the manufacturer's instructions. Samples were analyzed on a BD ARIA III flow cytometry, and data analysis was performed with FCAP Array version 3.0 software (Soft Flow).

Statistical analysis

All the data were analyzed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Data were presented as a mean ± standard deviation. The significant differences between groups were determined by the unpaired *t* test or two-way ANOVA. The Pearson method was used for correlation analysis between two variables. A value of $p < 0.05$ was considered statistically significant.

Datas availability The dataset supporting the conclusions of this article will be available to the Editors and Reviewers upon request.

Results

Characteristics of study subjects

A total of 44 patients with RA, comprising 34 females (77.3%) and 10 males (22.7%) with a mean age of 32 ± 14 years and 36 healthy controls, comprising 30 females (83.3%) and 6 males (16.7%) with a mean age of 37 ± 9 years were evaluated. Table 1 shows their demographics and clinical manifestations of these patients. Among RA patients, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), anti-CCP antibody, and rheumatoid factor (RF) were recorded.

Table 1 Demographic, clinical, and laboratory characteristics of the study population

Characteristics	RA ($n = 44$)	HC ($n = 36$)
Age (years)	32 ± 14	37 ± 9
Gender (female/male)	34/10	30/6
Years with disease	5.8 ± 6.7	
Morning stiffness, *n* (%)	28 (64%)	
DAS28 average score	5 ± 2	
ESR (mm/h) (0–15)	53 ± 38	
CRP (mg/l) (0–8)	46.79 ± 54.92	
Anti-CCP (RU/ml)	309.84 ± 344.2	
Positive anti-CCP, *n* (%)	30 (68%)	
RF (IU/ml)	349.72 ± 425.68	
Positive RF, *n* (%)	33 (75%)	
Tender joint counts	13 ± 7	
Swollen joint counts	15 ± 8	

DAS28, disease activity score in 28 joints; *ESR*, erythrocyte sedimentation rate; *CRP*, C-reactive protein; *CCP*, cyclic citrullinated peptide; *RF*, rheumatoid factor

Mean ± standard deviation was used

The distribution of circulating Tfh, Naïve T, TEM, and TCM cell subsets in patients with RA

The circulating Tfh, TEM, and TCM of the PBMCs from the healthy controls and RA patients were analyzed by flow cytometry. We gated the $CD3^+CD4^+CXCR5^+CD45RA^-$ Tfh cells, $CD3^+CD4^+CXCR5^-CD45RA^+$ Naïve T cells, $CD3^+CD4^+CCR7^+CD45RA^-$ TCM, and $CD3^+CD4^+CCR7^-CD45RA^-$ TEM in healthy controls and RA patients (Fig. 1a). The frequencies of $CD3^+CD4^+CXCR5^-CD45RA^+$ Naïve T and $CD3^+CD4^+CCR7^-CD45RA^-$ TEM cell subsets were increased in blood from RA patients compared to healthy controls ($p = 0.034$, $p = 0.0159$), while the frequency of $CD3^+CD4^+CCR7^+CD45RA^-$ TCM cell subsets was declined in the RA group ($p < 0.0001$). The percentages of $CD3^+CD4^+CXCR5^+CD45RA^-$ Tfh did not differ between RA patients and healthy controls (Fig. 1b).

High frequencies of circulating $PD1^+ICOS^+$ TCM, $PD1^+ICOS^+$ TEM, and $PD1^+ICOS^+$ Tfh cell subsets in patients with RA

In order to determine the phenotype of circulating Tfh, TCM, and TEM cell subsets, the circulating Tfh, TEM, and TCM of the PBMCs were analyzed by flow cytometry for the expression of the inducible co-stimulatory molecule (ICOS) and programmed death-1 (PD1). Figure 2b, c, and d showed that the frequencies of $PD1^+ICOS^+$ TCM, $PD1^+ICOS^+$ TEM, and $PD1^+ICOS^+$ Tfh cells in RA patients were higher than those in healthy controls ($p < 0.05$, $p < 0.001$, and $p < 0.05$, resp.). However, no significant difference was detected in a fraction of the $PD1^+ICOS^+$ Naïve T cell subset between the RA patients and healthy controls (Fig. 2e).

The percentage of co-stimulatory molecules on Tfh, TEM, and TCM cell subsets was correlated with DAS28, ESR, RF, and cytokines in patients with RA

We next evaluated whether the percentage of co-stimulatory molecules on Tfh, TEM, and TCM cell subsets in RA was correlated with the severity of disease activity DAS28, ESR, RF, and cytokines. We observed a positive correlation between the percentage of DAS28 and T cell subsets, such as $PD1^+ICOS^+$ Tfh ($r = 0.4318$, $p < 0.0001$, Fig. 3a), $PD1^+ICOS^+$ TEM ($r = 0.3639$, $p < 0.0001$, Fig. 3b), and $PD1^+ICOS^+$ TCM ($r = 0.2199$, $p < 0.005$, Fig. 3c). Also, a positive correlation was also found between the level of ESR and T cell subsets, such as $PD1^+ICOS^+$ Tfh ($r = 0.1171$, $p < 0.05$, Fig. 3d), $PD1^+$ Tfh ($r = 0.4284$, $p < 0.0001$, Fig. 3e), $PD1^+$ TCM ($r = 0.278$, $p < 0.001$, Fig. 3f), and $PD1^+$ TEM ($r = 0.3907$, $p < 0.0001$, Fig. 3g). MCP-1 has a positive correlation with T cell subsets, such as $PD1^+ICOS^+$ TEM ($r =$

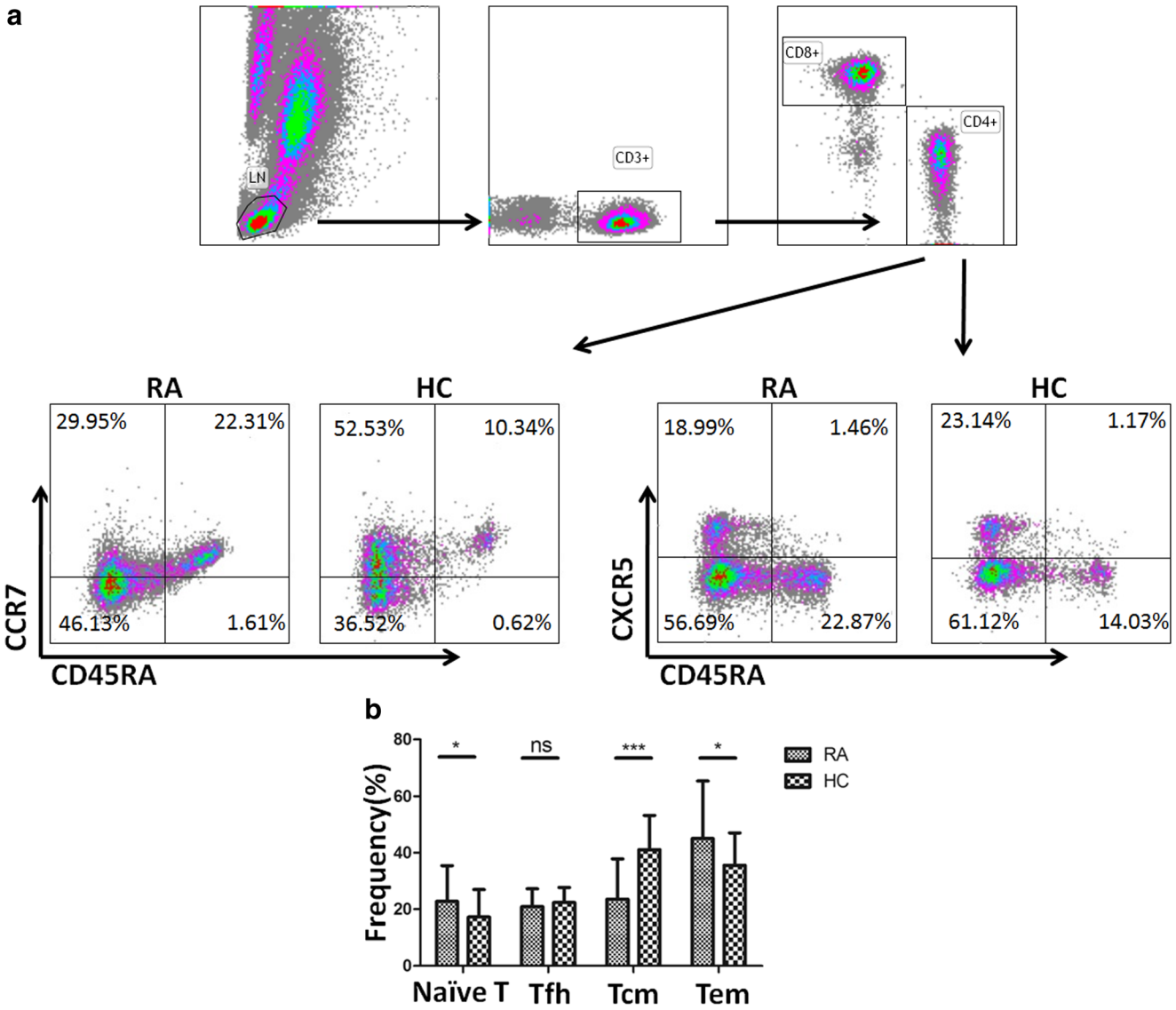

Fig. 1 The distribution of Tfh, TEM, and TCM cell subsets in RA. PBMCs were isolated from blood of RA patients and healthy controls, analyzed by flow cytometry. **a** Representative flow figures of the frequency of circulating TCM, TEM, Tfh, and Naïve T cells ($CD3^+CD4^+CXCR5^+CD45RA^-$ Tfh cells, $CD3^+CD4^+CXCR5^-CD45RA^+$ Naïve T cells, $CD3^+CD4^+CCR7^+CD45RA^-$ TCM, and $CD3^+CD4^+CCR7^-CD45RA^-$ TEM). **b** Statistical graph of Naïve T, Tfh, TEM, and TCM cell subsets in the blood from RA patients ($n = 44$) and healthy control subjects ($n = 37$), expressed as the mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ns, no statistical significance

0.1692, $p < 0.01$, Fig. 3h), $ICOS^+$ TEM ($r = 0.1515$, $p < 0.01$, Fig. 3i), and $ICOS^+$ TCM ($r = 0.174$, $p < 0.01$, Fig. 3j). Moreover, a positive correlation was also found between the level of RF and $PD1^+$ TCM ($r = 0.1054$, $p < 0.05$, Fig. 3k). However, other cytokines did not significantly correlate with T cell subsets (data not shown).

Altered expression of cytokines in patients with RA

We further tried to evaluate the pattern of circulating cytokines and chemokines. The inflammation chemokines and cytokines including IL-1β, TNF-α, IL-4, IL-6, IL-9, IL-17A, MCP-1, IL-10, IL-12p70, and IL-21 were measured by CBA (Fig. 4). The concentration of IL-1β ($p = 0.007$, Fig. 4a), IL-6 ($p < 0.0001$, Fig. 4b), and MCP-1 ($p = 0.0028$, Fig. 4c) in RA patients was significantly higher than that in healthy controls, but there were no obvious changes regarding TNF-α, IL-4, IL-9, IL-17A, IL-10, IL-12p70, and IL-21 (Fig. 4d).

Discussion

Accumulating evidence has determined that disorder regulation of memory T cell differentiation might promote the pathogenesis of RA [11, 12]. However, the detailed distribution of circulating memory T cell subsets and how molecules such as

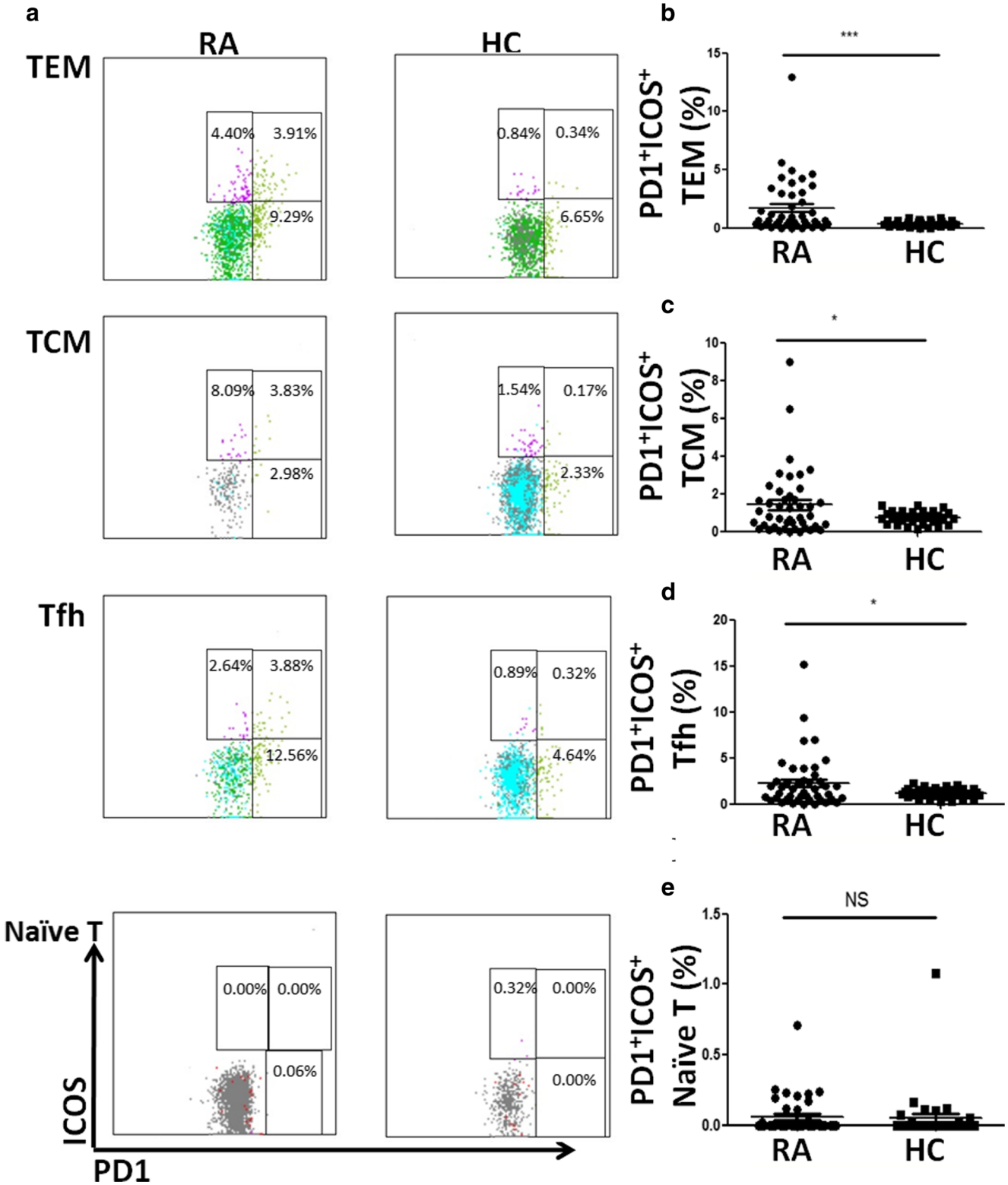

Fig. 2 Frequencies of ICOS$^+$PD1$^+$Tfh, ICOS$^+$PD1$^+$TEM, ICOS$^+$PD1$^+$TCM, and ICOS$^+$PD1$^+$ Naïve T cells in PBMC. Peripheral blood mononuclear cells (PBMC) from RA patients ($n = 44$) and healthy control subjects ($n = 37$) were harvested and stained with appropriate flow antibodies, and the expression of ICOS$^+$PD1$^+$ was detected by flow cytometry. **a** Representative flow figures of ICOS$^+$PD1$^+$TEM, ICOS$^+$PD1$^+$ TCM, ICOS$^+$PD1$^+$ Tfh, and ICOS$^+$PD1$^+$ Naïve T cells. **b**–**e** The statistical graph of ICOS$^+$PD1$^+$TEM (**b**), ICOS$^+$PD1$^+$TCM (**c**), ICOS$^+$PD1$^+$ Tfh (**d**), and ICOS$^+$PD1$^+$ Naïve T (**e**). Each dot in the statistical graph represents an individual subject. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns, no statistical significance

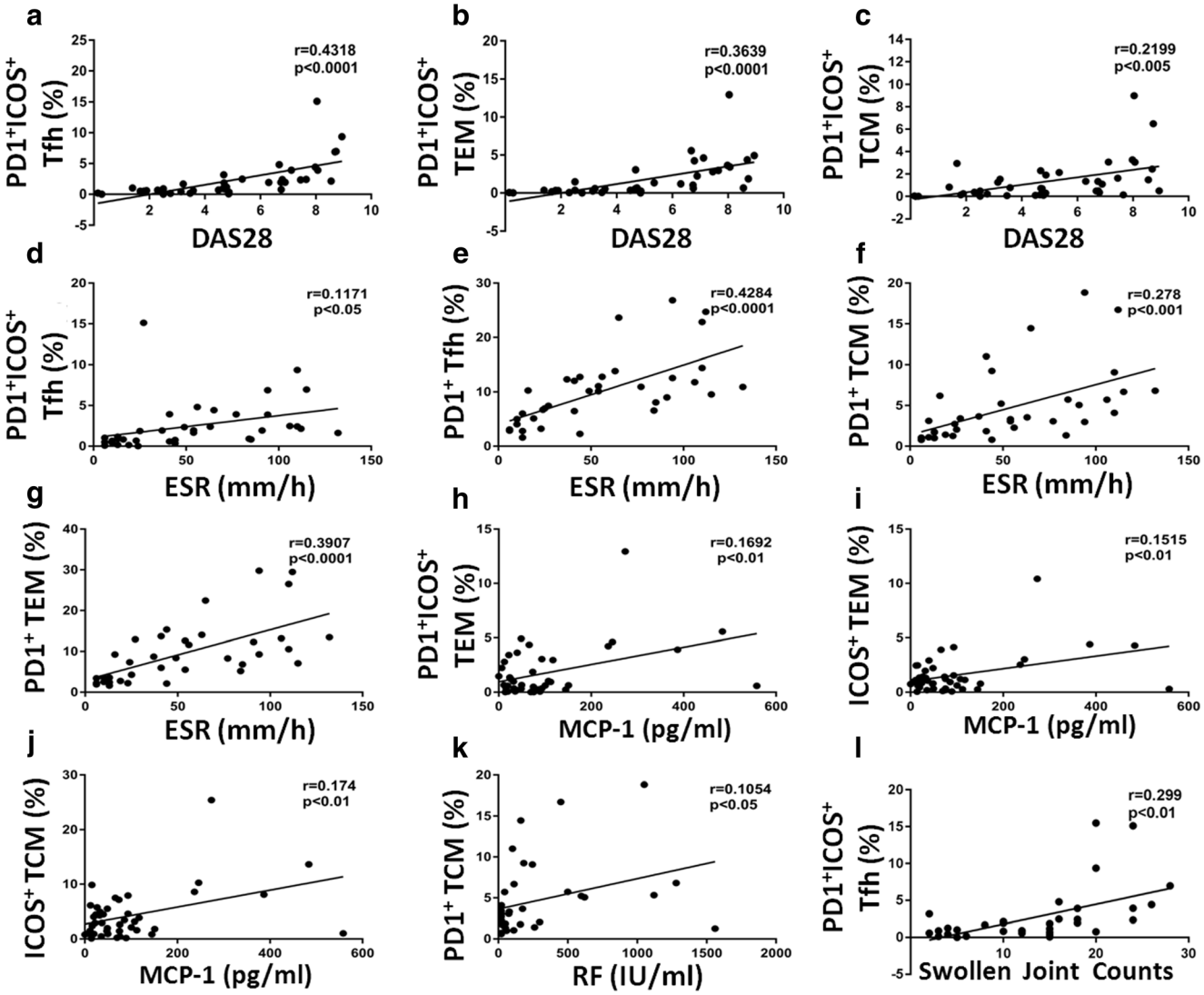

Fig. 3 Correlation of the percentage of co-stimulatory molecules on Tfh, TEM, and TCM cells and DAS28, ESR, RF, and cytokines in RA patients. Frequencies of co-stimulatory molecules on Tfh, TEM, TCM cells, ESR, and RF and the concentration of cytokines were measured, and DAS28 was evaluated for each recruited patient subjects. **a**–**c** The severity of disease activity, DAS 28, correlated significantly with T cell subsets, such as $PD1^+ICOS^+$ Tfh ($r = 0.4318$, $p < 0.0001$), $PD1^+ICOS^+$ TEM ($r = 0.3639$, $p < 0.0001$), and $PD1^+ICOS^+$ TCM ($r = 0.2199$, $p < 0.005$). **d**–**g** The level of ESR correlated significantly with T cell subsets, such as $PD1^+ICOS^+$ Tfh ($r = 0.1171$, $p < 0.05$), $PD1^+$ Tfh ($r = 0.4284$, $p < 0.0001$), $PD1^+$ TCM ($r = 0.278$, $p < 0.001$), and $PD1^+$ TEM ($r = 0.3907$, $p < 0.0001$). **h**–**j** The level of MCP-1 correlated significantly with T cell subsets, such as $PD1^+ICOS^+$ TEM ($r = 0.1692$, $p < 0.01$), $ICOS^+$ TEM ($r = 0.1515$, $p < 0.01$), and $ICOS^+$ TCM ($r = 0.174$, $p < 0.01$). **k** Relationship between the frequency of $PD1^+$ TCM cells and the level of RF ($r = 0.1054$, $p < 0.05$). **l** Relationship between the frequency of $PD1^+$ $ICOS^+$ Tfh cells and swollen joint counts ($r = 0.299$, $p < 0.01$). Each dot represents an individual patient. The correlations were evaluated with Spearman's nonparametric test. $p < 0.05$ represents a significant difference

PD1 and ICOS are behaving on these cells in RA patients has remained mostly unclear. Here, we investigated the distribution of circulating memory T cell in patients with untreated active RA. Terminally circulating effector memory $CD4^+CCR7^-CD45RA^-$ T cells were significantly increased in RA patients, whereas the central memory $CD4^+CCR7^+CD45RA^-$ T cell population was decreased as compared with levels in healthy control individuals (Fig. 1b). Studies have been reported that TEM cells lack CCR7 and migrate to inflamed tissues, while TCM cells express CCR7 and circulate through secondary lymphoid organs [13]. This skewed differentiation was not observed in healthy age-matched control individuals, indicating the inflammatory status in RA.

As chronic autoimmune responses are perpetuated by repeated activated memory T cells, disordered regulation of memory T differentiation might promote the pathogenesis and progression of RA [14]. Previous studies demonstrated that ICOS plays an important pro-inflammatory role in the late effector phase and T memory-dependent B cell response [15]. PD1 is expressed to various degrees on activated T cells to limit the activity of T cells [16]. Our data indicated that the

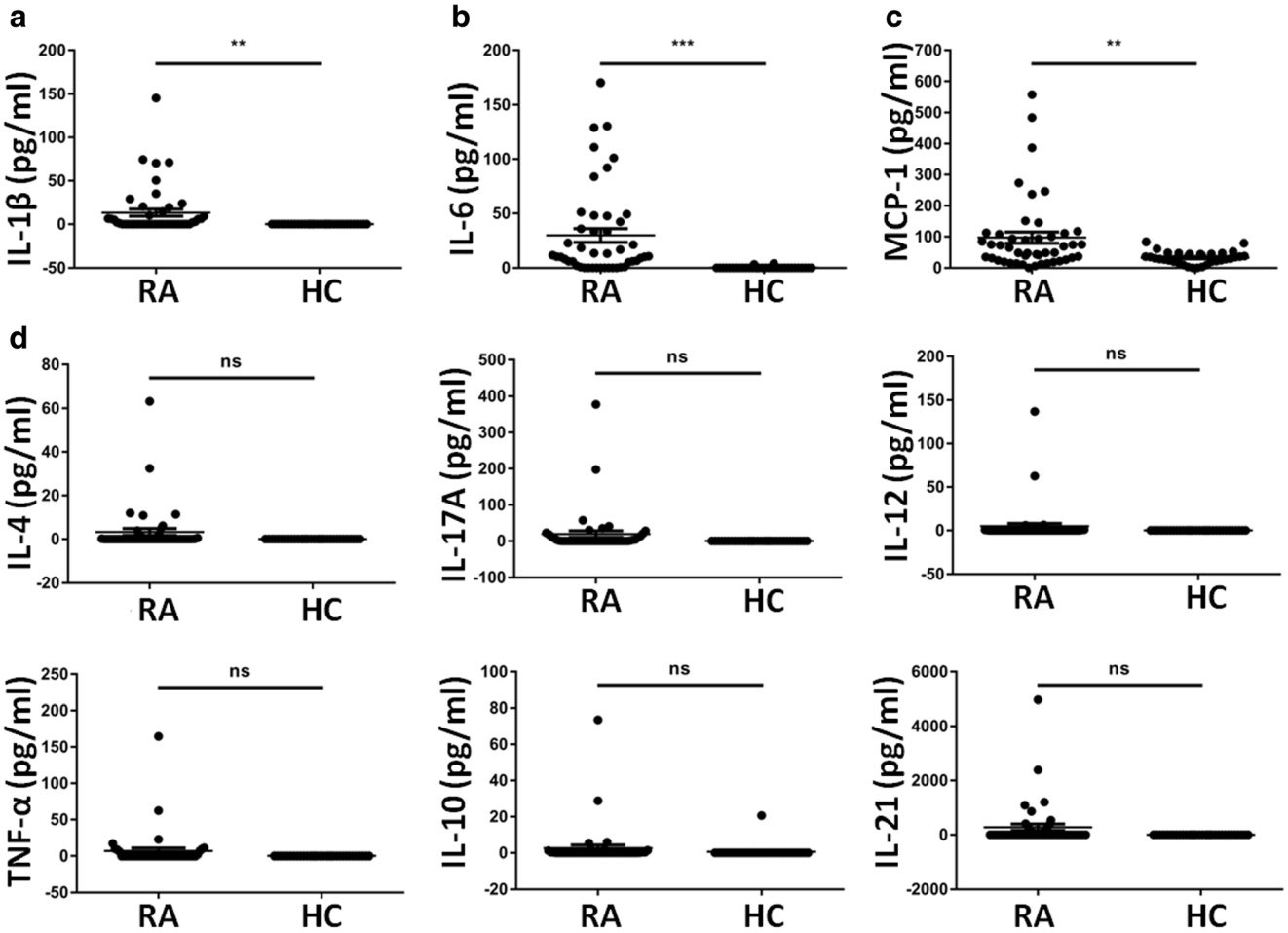

Fig. 4 Analysis of cytokines and chemokines in RA and healthy controls. Plasma from RA patients ($n = 44$) and healthy control subjects ($n = 37$) was harvested for the cytokines and chemokines measurement. The concentrations of IL-1β, IL-6, and MCP-1 in RA patients and healthy controls were assessed by a cytometric beads array (CBA) assay. **a**–**c** The concentrations of IL-1β (**a**), IL-6 (**b**), and MCP-1 (**c**). **d** No distinct differences were detected between the RA and HC regarding IL-4, IL-17A, IL-12, TNF-α, IL-10, and IL-21. Each dot represents an individual subject. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; ns, no statistical significance

circulating $PD1^+ICOS^+$ TEM cells and $PD1^+ICOS^+$ TCM cells were increased in RA patients (Fig. 2b, c). Moreover, the results showed for the first time that the proportions of circulating $PD1^+ICOS^+$ TEM cells and $PD1^+ICOS^+$ TCM cells were correlated with DAS28, which opens a new avenue in the study of RA (Fig. 3b, c). We also found that $PD1^+$ TCM and $PD1^+$ TEM cells were positively correlated with ESR, an inflammation marker in RA. Knowing that DAS28 and ESR are useful indices for estimating the disease activity in RA [17], our results suggest that $PD1^+ICOS^+$ TCM and $PD1^+ICOS^+$ TEM may prove to be potential indices for assessment of disease activity in RA.

Tfh cells, a special $CD4^+$ T cell subset, regulate the antigen-specific B cell immunity, different from other Tfh cells in function [18, 19]. Previous studies demonstrated that the frequency of the circulating Tfh cells was increased significantly in RA patients [20]. However, in our study, no significant difference in the percentage of the circulating Tfh cells was found between RA patients and healthy controls. PD1 and ICOS expressed by circulating Tfh cells are essential for the development and function of Tfh. Our data provide the evidence that the percentage of the circulating $PD1^+ICOS^+$ Tfh cells in RA patients was higher than that in healthy controls (Fig. 2d). Our study showed an association between $PD1^+ICOS^+$ Tfh cells and DAS28 (Fig. 3a). ESR is positively correlated with T cell subsets, such as $PD1^+ICOS^+$ Tfh and $PD1^+$ Tfh. Together, $PD1^+ICOS^+$ Tfh cells were thought to be in relation to pathogenesis and progression of RA. Other studies reported that IL-21, which is produced by Tfh cell subsets, was correlated with DAS28 [21, 22]. Unfortunately, however, this study did not reveal the correlation between IL-21 and DAS28. Moreover, no significant difference in IL-21 concentration was detected between RA patients and healthy controls. The disparities between our data and the results of previous studies may be due to a number of factors, including cohort size, disease duration, and therapy.

Our results also indicated that a distinct cytokine and chemokine imbalance was observed in RA patients. The elevation

of IL-6 (Th2, Th17) could imply breaking the Th1/Th2/Th17/Treg balance in the peripheral blood. IL-1β derived from activated memory T cells can activate antigen-specific T cells at inflammatory sites [23]. Monocyte chemotactic protein-1 (MCP-1), a chemokine, is a mediator of the chronic inflammation [24]. Thus, the elevated IL-1β and MCP-1 imply the chronic inflammatory immune status of the RA patients. Abnormal expression of these cytokines and chemokines might serve a critical role in the development of the disease and increased pro-inflammatory response especially in the active form of RA.

Conclusion

In conclusion, the current results suggest that elevated circulating T cell subsets and cytokines expression profile were observed in RA patients. Cytokines such as IL-6, MCP-1, and IL-1β were significantly increased in RA, and $PD1^+ICOS^+$ TEM, $PD1^+ICOS^+$ TCM, and $PD1^+ICOS^+$ Tfh cell subsets were positively correlated with disease activity DAS28. Therefore, $PD1^+ICOS^+$ TEM, $PD1^+ICOS^+$ TCM, and $PD1^+ICOS^+$ Tfh cells might serve an important role in the progression of RA and become the promising therapeutic targets for the treatment of RA.

Author's contribution ZHY, HBL, and ZZP performed the experiments, analyzed the data, and wrote the manuscript. ZQ, LJ, and FYY were involved in performing the experiments. ZHY and LL conceived the study and assumed overall responsibility for this work.

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Compliance with ethical standards

Ethics approval and consent to participate The study was approved by the Ethics Committee of the Affiliated Hospital of Guizhou Medical University. Informed consent had been obtained from the patients' legal guardians.

Consent for publication Not applicable.

Disclosures None.

Abbreviations RA, rheumatoid arthritis; ICOS, inducible costimulatory molecule; DAS28, Disease Activity Score 28; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; PD1, programmed cell death 1; MCP-1, monocyte chemotactic protein 1; IFN-γ, interferon gamma; IL-1β, interleukin 1 beta; TNF-α, tumor necrosis factor-α; IL-4, interleukin 4; IL-17A, interleukin 17A

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