

Original Article

The abnormal expression of CCR4 and CCR6 on Tregs in rheumatoid arthritis

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Abstract: The study aims to investigate the frequency of CD4⁺CD25⁺Foxp3⁺CD127⁻ T regulatory cells (Tregs) and the expression of CCR4, CCR6 and/or other chemokine receptors on Tregs in peripheral blood (PB) in patients with rheumatoid arthritis, as well as in PB, draining lymph nodes (dLNs), lungs and spleens in collagen-induced arthritis (CIA) mice. We also study the possible role of CCR4 and CCR6 abnormal expression on Tregs in RA patients and the underlying mechanisms. The numbers of Tregs and chemokine receptors expression profile on Tregs in PB from RA patients and healthy controls were investigated by flow cytometry (FACS) using three- or four-color intracellular staining. DBA/1 Foxp3^{flp} reporter mice were immunized with collagen II (CII) emulsified with CFA. At day 60 after CII immunization, mice were sacrificed and Foxp3 (GFP) expression in PB, dLNs, Lungs and spleens was examined by FACS. The numbers of Tregs in PB were significantly lower in RA patients than in healthy controls (1.21±0.43% vs 3.50±0.98%, P<0.05). The levels of chemokine receptor CCR4 or CCR6 expression on Tregs in PB were higher in active RA patients than in healthy controls (91.13±2.98% vs 79.45±4.72%, P<0.05; or 67.33±7.53% vs 42.73±5.60%, P<0.05). The levels of CCR4 or CCR6 expression on Tregs in active RA patients were positively correlated to DAS28 scores (r=0.42, P<0.03; or r=0.58, P<0.02). Similarly, the numbers of CCR6 expression on GFP⁺ cells in the spleens, dLNs, lungs and blood of CIA were all increased than those of normal mice (P<0.01). Frequency of CCR4 expression on GFP⁺ cells in dLNs of CIA was somehow higher but slightly lower in the spleens of CIA compared to normal mice without significant differences (P>0.05). Frequency of CCR5 expression on GFP⁺ cells in the spleens and dLNs of CIA were both increased than those of normal mice, but there were no significant differences (P>0.05). CCR7 or CCR9 expression on Tregs from spleen and dLN of either normal or CIA mice was undetectable. Although the frequency of CD4⁺Foxp3⁺Tregs in peripheral blood was decreased in active rheumatoid arthritis patients, the levels of chemokine receptors such as CCR4 and CCR6 among the Tregs were increased, implicating that Tregs in active RA have obtained the ability migrating to inflammatory joints and may reflect the feedback regulation of the body to local inflammation. Furthermore, CCR4 and CCR6 expressed on Tregs may be related to the activity and severity of RA.

Keywords: T regulatory cells, chemokine receptor, rheumatoid arthritis, collagen induced arthritis

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease that primarily affects multiple joints. Impairment or imbalance in the regulatory functions of the immune system and the pathogenic role of Th1 and/or Th17 cells appear to be important mechanisms in the onset of RA [1]. Active suppression by T regulatory (Treg) cells plays a key role in the control of

self-antigen reactive T-cells and the induction of peripheral tolerance in vivo. Over the last decade, increasing attention has been focused on naturally occurring CD4⁺CD25^{high}Tregs (nTregs) [2]. In RA, as well as in other autoimmune disorders, nTregs exhibit reduced immunosuppressive properties. nTregs are characterized by the presence of Foxp3, an intracellular transcription factor that controls their development and function. nTregs arise spontane-

ously in the thymus and control responses to tissue-specific autoantigens that are presented to the immune system locally in the periphery [3]. Important questions remain unanswered regarding where this inhibition takes place and the molecular mechanism underlying Treg cells trafficking in vivo.

nTreg cells are thought to develop or differentiate in the thymus and migrate to the periphery. Emerging evidence suggests that Tregs compartmentalization and trafficking maybe tissue and/or organ specific and that distinct chemokine receptor and integrin expression may contribute to selective retention and trafficking of Treg cells at sites where regulation is required [4, 5]. Cao et al reported that RA patients displayed an enrichment of CD4⁺CD25^{high} T cells in synovial fluid (SF) as compared to peripheral blood (PB) [6]. In general, Treg cells entry into the inflamed tissues are thought to be indispensable for an efficient suppression in vivo [7]. These findings imply that organ specific targeting of Tregs by the modulation of their homing profile might increase the in vivo suppressive efficacy of Tregs devised for therapeutic application.

Treg cells specifically express the C-C chemokine receptor CCR4 and respond to the chemokines thymus and activation-regulated chemokine (TARC/CCL17) and macrophage derived chemokine (MDC/CCL22), which are agonistic ligands of this receptor. Mature dendritic cells (DCs) producing CCL17 and CCL22 were found to preferentially attract Treg cells, suggesting the involvement of these chemokines in Tregs function [8]. Treg cells are capable of preventing the development of colitis in a mouse model of inflammatory bowel disease (IBD), in which CCR4 plays an important role in Treg cell trafficking to draining lymph nodes (LNs) and that this is critical for Treg cell suppressive function in vivo [9].

Previous studies with mice have shown that CCR6 is mainly expressed on memory T cells, some natural CD4⁺CD25⁺ regulatory T cells, B cells, some DCs, Langerhans cells, and Th17 cells, implicating that CCR6 may be required for the trafficking of these cells via CCL20 since CCL20 is CCR6 ligand [10, 11].

To further analyze the roles of chemokine receptors on Treg cells in patients with RA, we

have searched for cell-surface molecules that are specifically expressed in Treg cells and are crucial for their functions to investigate their expressions in RA. In the current study, we examined the frequency of CD4⁺CD25^{high}Foxp3⁺Tregs in peripheral blood mononuclear cells (PBMCs) from active RA patients by using flow cytometry at the single-cell level. We also investigated the expression of trafficking chemokine receptors on CD4⁺CD25^{high}Foxp3⁺cells, including CCR4, CCR5, CCR6, CCR7, and CCR9, which are known to play important roles in regulatory T cell migration to inflammatory tissues [12].

Materials and methods

Patients and healthy controls

Forty-five patients with active RA, fulfilling the American College of Rheumatology 1987 revised criteria for RA [13] and thirty healthy controls (HC) were enrolled in this study. Clinical indices and biological markers in this study included age, sex, disease duration, rheumatoid factor (RF), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and number of Disease Modifying Anti-Rheumatic Drugs. Data were collected into a predesigned form. Standardized joint counts, including tender joints and swollen joints, were recorded. The 3-variable DAS28 (DAS28-3) was computed using the formula [14]:

$$\text{DAS28} = [0.56 \times \sqrt{T_{28}} + 0.28 \times \sqrt{SW_{28}} + 0.70 \times \text{Ln}(\text{ESR})] \times 1.08 + 0.16$$

where TJC = tender joint count, SJC = swollen joint count and ESR = erythrocyte sedimentation rate. Patients and healthy controls were matched for age and sex. All the participants gave their written informed consent. The protocol was approved by the ethics committee of Tongji University.

Mice

Foxp3^{gfp} knockin mice on the DBA/1 background were developed by backcrossing of Foxp3^{gfp} knockin mice on the C57BL/6 background to DBA/1 mice for 13 generations in Zheng's lab at the University of Southern California. DBA/1 Foxp3^{gfp} reporter mice were immunized with collagen II (CII) emulsified with CFA. At day 60 after CII immunization, mice

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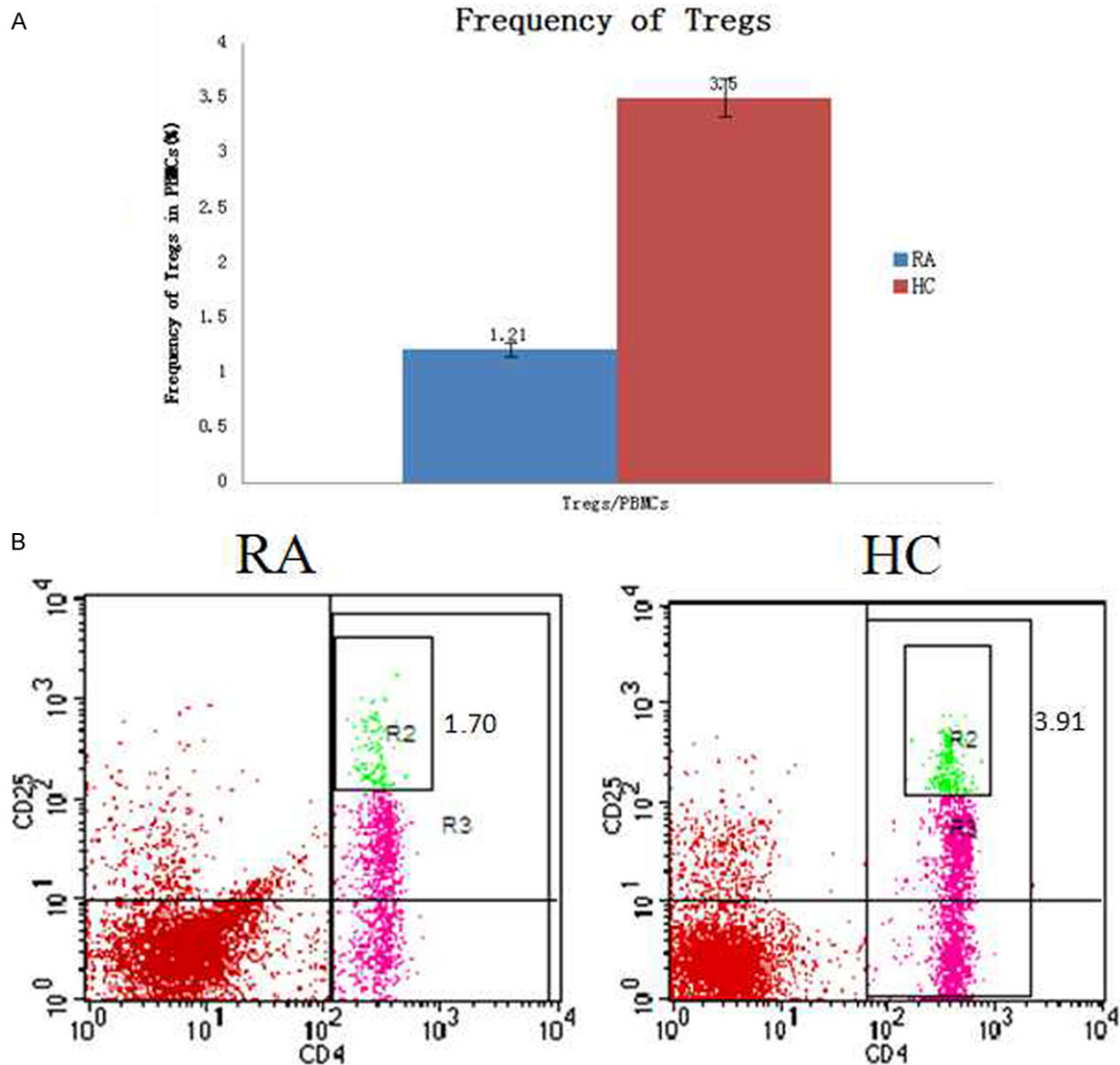


Figure 1. Frequency of Tregs in PB of RA patients was significantly decreased compared with that of the HC ($P < 0.05$, A), and one of the representative figures of the flow cytometry was shown in (B).

were sacrificed and GFP expression in spleen, draining lymph nodes (LNs), blood and lungs (for each group, $n=6$) was examined by FACS. All experiments were performed with age- and sex-matched mice and were repeated at least twice in accordance with Institutional Animal Care and Utilization Committee- approved protocols.

Cell isolation

PBMCs were isolated from heparin anti-coagulated whole blood samples, using Ficoll-Hypaque (Fresenius Kabi, Norway) density gradient centrifugation. The centrifugation was

performed at 840 g for 20 min at 20°C. Trypan blue staining revealed the viability of the freshly isolated cells to be greater than 95%.

Cell staining and flow cytometric analysis

Four-color flow cytometry was used to analyze the expression of chemokine receptors on $CD4^+CD25^{\text{high}}FoxP3^+$ T cells. In brief, PBMCs were washed three times in phosphate buffered saline (PBS) supplemented with 0.5% newborn calf serum (NCS) (Invitrogen Corporation, Scotland, UK), and then stained (30 min at 4°C) with allophycocyanin (APC)-labeled monoclonal antibody (mAb) against

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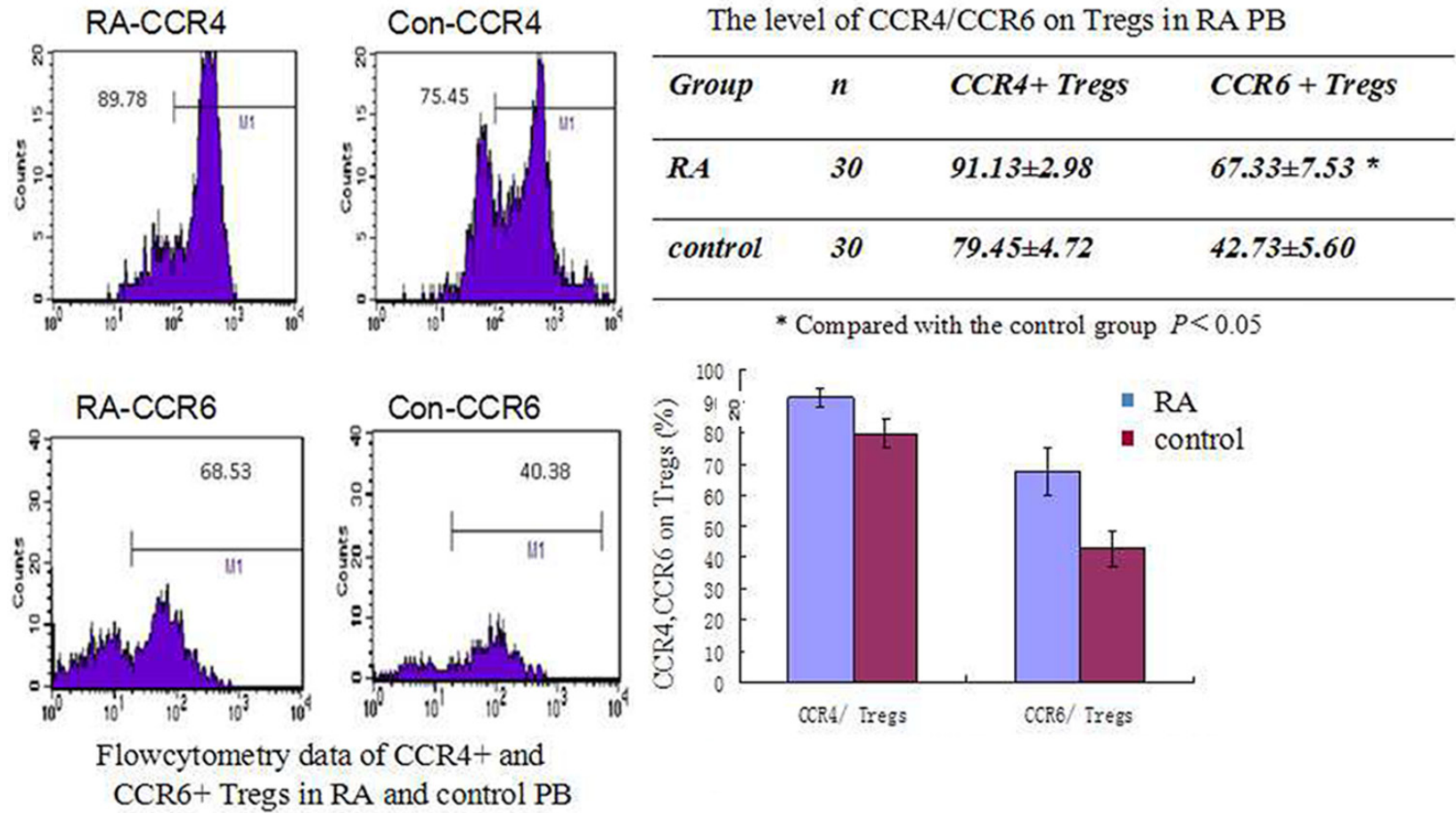
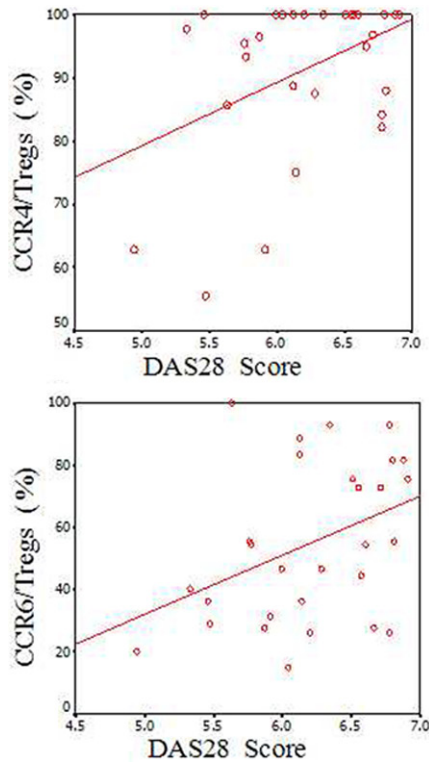


Figure 2. Expression of CCR4⁺ and CCR6⁺Tregs increased in RA PB compared with HC PB, and one of the representative figures of the flow cytometry was shown.

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Correlation of CCR4 and CCR6 expression levels on Tregs with RA clinical indexes

| Clinical indexes | CCR4/Tregs | | CCR6/Tregs | |
|------------------|-------------|-------------|-------------|-------------|
| | <i>r</i> | <i>P</i> | <i>r</i> | <i>P</i> |
| CRP | 0.07 | 0.71 | 0.04 | 0.83 |
| ESR | 0.31 | 0.17 | 0.25 | 0.19 |
| RF | 0.09 | 0.64 | 0.13 | 0.50 |
| DAS28 | 0.42 | 0.03 | 0.58 | 0.02 |

Figure 3. Expression levels of CCR4 and CCR6 on Tregs were both positively correlated with the DAS28 score of RA patients.

CD4 (eBioscience, San Diego, CA, USA), PE-cyochrome 5 (PE-Cy5)-labeled mAb against CD25 (eBioscience), and with phycoerythrin-labeled mAb specific for one of the chemokine receptors CCR4, CCR5, CCR6, CCR7 and CCR9 (R&D Systems, Minneapolis, MN, USA). After staining for surface molecules, intracellular staining was performed with fluorescein isothiocyanate (FITC)-labeled mAb against Foxp3, by using a fixation and permeabilization solution (eBioscience) according to the manufacturer's instructions. Then the cells were washed again and analyzed with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using CellQuest software (Becton Dickinson).

Statistical analysis

The Student's *t* test was used for statistical analyses, unless indicated otherwise. SPSS-18.0 software was used in the statistical calculations. $P < 0.05$ was considered significantly.

Results

Frequency of Tregs in RA patients

PBMCs were separated from peripheral blood (PB) of active RA patients and healthy controls

(HC), then the surface marker of CD4⁺CD25^{high}FoxP3⁺ T cells (Treg) cells were labeled and detected using flow cytometry. Frequency of Tregs was shown as percentages of Tregs among PBMCs. Although there were controversial reports on frequency of Tregs in RA patients [15, 16], our study showed that the frequency of Tregs in PB of active RA patients was significantly decreased compared to healthy controls [(1.21±0.43)% vs (3.50±0.98)%, $P < 0.05$, **Figure 1**].

Expression of CCR4 and CCR6 is increased on Tregs in PB of RA patients and correlated positively with disease activity

As expected, the expression of inflammation trafficking chemokine receptor CCR4 and CCR6 on Treg cells were both higher in PB of active RA patients than those of healthy controls [CCR4: (91.13±2.98)% vs (79.45±4.72)%, $P < 0.05$; CCR6: (67.33±7.53)% vs (42.73±5.60)%, $P < 0.05$, **Figure 2**]. The expression levels of CCR4 and CCR6 on Treg cells in active RA patients had significantly positive correlation with the disease activity, when assessed by DAS28 score (CCR4: $r = 0.42$, $P < 0.03$; CCR6: $r = 0.58$, $P < 0.02$, **Figure 3**).

CCR4 and CCR6 on Tregs in rheumatoid arthritis

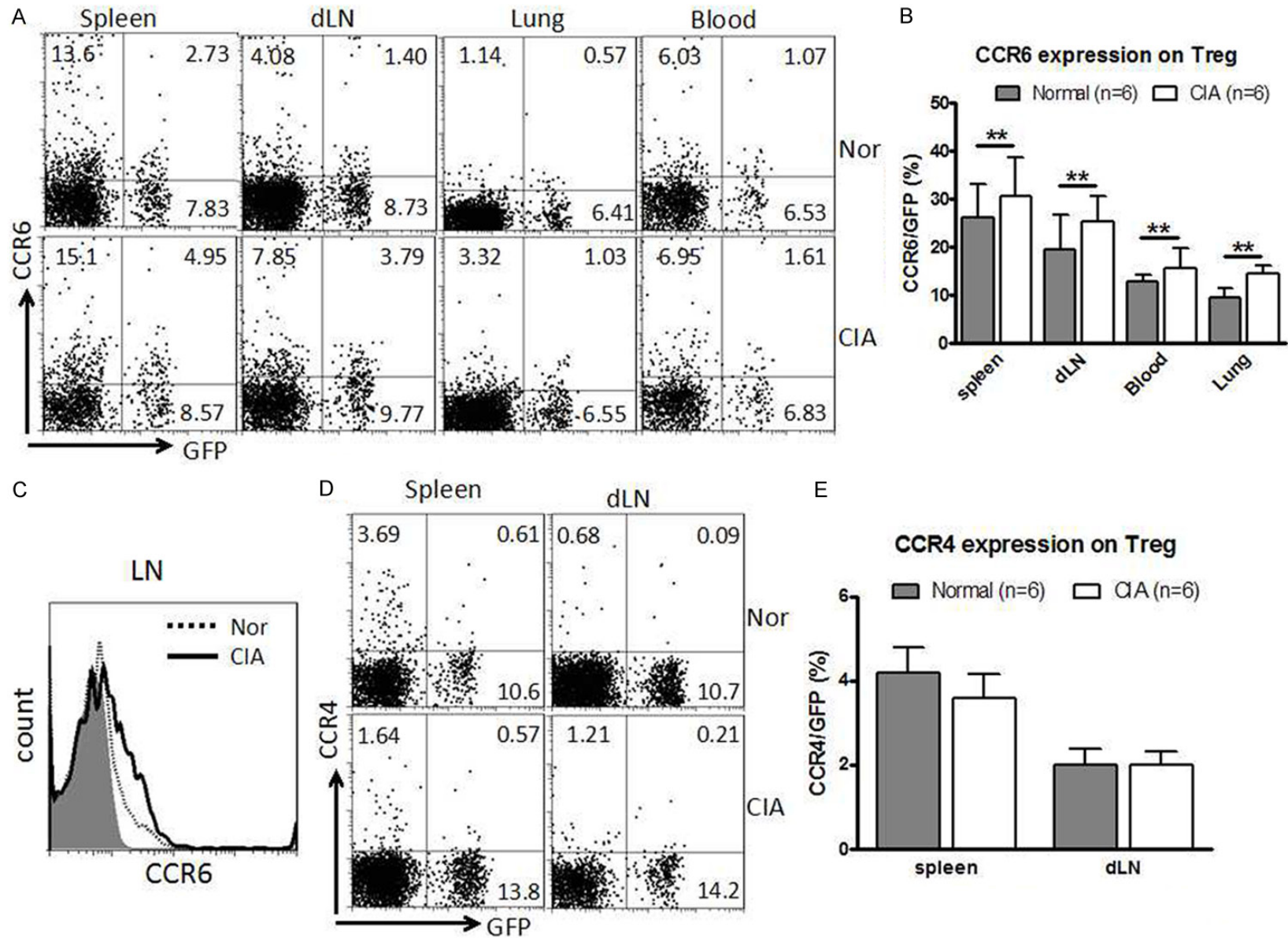


Figure 4. A: The flow data representative of CCR6 and GFP expression gated on CD4 positive cells from different tissue as indicated. B: Frequency of CCR6⁺ expression on GFP⁺ cells in the spleen, dLNs, lung and blood. C: Representative histogram of CCR6 expression on dLNs of CIA model or control mice. D: Representative flow data of CCR4⁺ cell frequency in the spleen and dLNs. E: Frequency of CCR4 expression on GFP⁺ cells in the spleen, dLNs, lung and blood.

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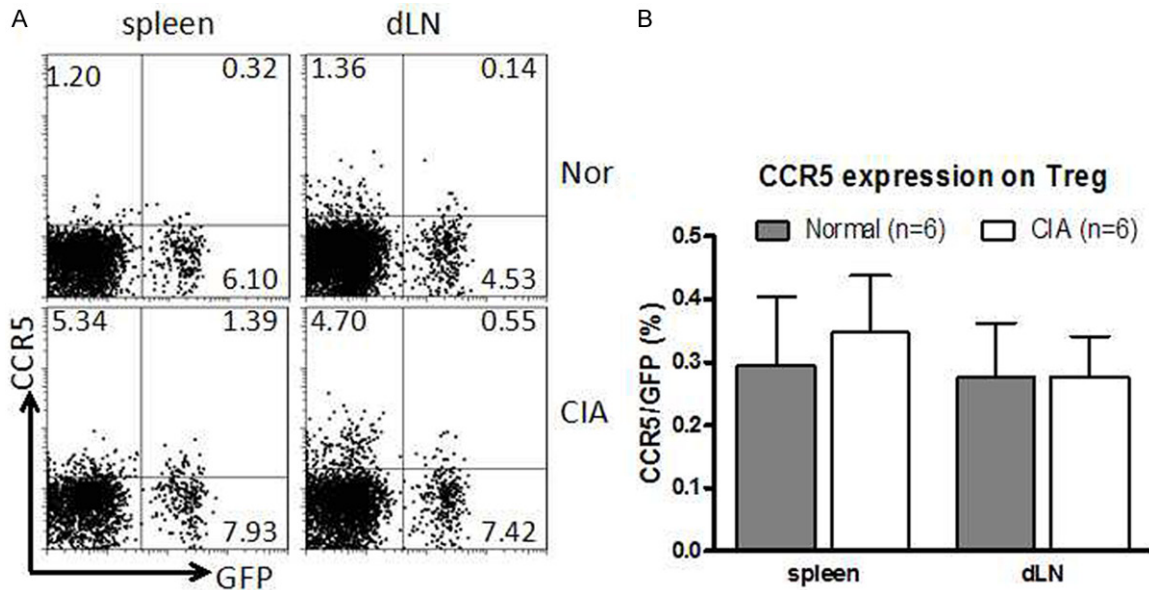


Figure 5. A: Representative flow data of CCR5⁺ cell frequency in the spleen and dLNs. B: Frequency of CCR5 expression on GFP⁺ cells in the spleen, dLNs, Lung and blood.

Heterogeneous expression of chemokine receptors on Tregs in PB, dLNs, spleen and lung of CIA models

Collagen-induced arthritis (CIA) model was induced in DBA/1 Foxp3^{efp} reporter mice as previously reported [17-19]. Frequency of CCR6 expression on GFP(Foxp3)⁺ cells in the spleens, dLNs, lungs and blood of CIA were all increased compared to GFP⁺ cells in normal mice ($P < 0.01$, **Figure 4A-C**). Frequency of CCR4 expression on GFP⁺ cells was lower in the spleen, dLNs and blood of CIA than those of normal mice. Conversely, the level of CCR4 expressed on Tregs in lungs from CIA was higher than that from normal mice although this difference is not significant ($P > 0.05$, **Figure 4D, 4E**). Frequency of CCR5 expression on GFP⁺ cells in the spleens and dLNs of CIA were also increased than those of normal mice, but there was no significant difference ($P > 0.05$, **Figure 5A, 5B**). No clear CCR7 or CCR9 expression on Treg cells from spleen/LN of either normal or CIA mice was observed (data not shown).

Discussion

One of the fundamental features of the immune system is its ability to preserve a delicate balance between effector responses and mechanisms of immunoregulation. The identification

of regulatory cells has opened up a vast field of research into the potential of these cells for controlling autoimmunity [20]. Treg cells play a critical role in inhibiting deleterious responses against self-antigens. Certain "regulatory" chemokines may control the termination of inflammatory responses by recruiting a specific subset of Treg cells that can limit tissue damage and prevent autoimmunity. Manipulating the recruitment of Treg cells may be useful in a variety of pathological conditions to achieve tolerance in autoimmunity [8].

In this study, we found that the frequency of Tregs in PB of active RA patients was significantly decreased compared to healthy controls, which was in accordance with some reports [6, 21]. There are two possible reasons for the reduction of Tregs in PB of RA patients. One possibility is that the conversion and apoptosis of Tregs increased, another one is that Tregs migrated to the inflammatory joints or other lymph organs from peripheral blood, leading a relative reduction in the peripheral blood. We have recently revealed that Treg cells can convert to Th1 and Th17 cells [19]. Additionally, apoptosis possibly contributes to this reduction as well (Li N et al, in press).

Chemokines play essential roles in migration and homing of lymphocyte sub-populations,

which express specific sets of chemokine receptors in accordance with their lineage and functional maturation, imparting unique migration and tissue homing properties. Chemokine receptor expression is exquisitely regulated depending on the stage of activation and differentiation of T cells and coordinates tissue localization and encounters with APCs [22]. Given the central role of chemokines in the regulation of immunity, we postulated that some of these molecules could participate in turning off adaptive immune responses by recruiting cells with immunoregulatory functions.

Tissue-specific migration is achieved by specific expression patterns of chemokine receptors. Skin, gut and lymphoid tissue homing potential was increased on Tregs by expressing CCR4, CCR6, CCR7 and CCR9 [23]. Treg cells play essential roles in the suppression of alloimmunity depending on the chemokine receptors CCR2, CCR4, and CCR5. In the allograft, Treg cells were activated and subsequently migrated to the dLNs in a CCR2, CCR5 and CCR7 dependent fashion [24]. In this study, we found that the expression of CCR4 and CCR6 on CD4⁺CD25^{high}Foxp3⁺Treg cells were both significantly greater in PBMCs from RA patients when compared to that from controls. Besides, the expression levels of CCR4 and CCR6 on Treg cells in active RA patients had significantly positive correlation with the disease activity, when assessed by DAS28 score, as shown in **Figure 3**.

Notably, Jiao et al [21] found that CD4⁺CD25⁺Foxp3⁺T cells in synovial fluid (SF) expressed high levels of CCR4, CCR5 and CXCR4, especially CCR4. However, CCR4⁺ regulatory T cells were decreased in the RA PB, which is contrary to our result. Hirahara et al reported that almost all CD4⁺CD25^{high}Foxp3⁺ regulatory T cells in normal human PB expressed high levels of CCR4 [25]. One possible explanation for the low expression of CCR4 in PB of RA patients is that such cells may migrate to SF, according to a report showing that human blood Treg cells express CCR4 and migrate in response to their ligands *in vitro* [8]. Lee et al also demonstrated that the recruitment of Foxp3-expressing Treg cells to an allograft tissue is dependent on the chemokine receptor CCR4 [26].

Specific expression of CCR4 on Treg cells may allow their migration toward APCs and activat-

ed T cells leading to inhibition of APC function or suppression of responding T cells. CCR4 is crucial for regulating immune balance and known to be expressed selectively on Th2 cells and Treg cells [8, 27]. There is evidence that blood borne human CD4⁺CD25⁺Treg cells exhibit a distinctive chemotactic response profile and chemokine receptor expression. Treg cells exhibit chemotactic responsiveness to several inflammatory and lymphoid chemokines, but they are specifically hyperresponsive to chemokines that engage the chemokine receptor CCR4 [8]. With special regard to the relationship between CD4⁺CD25⁺Tregs and CCR4, Yagi et al reported that human FOXP3-transduced CD4⁺CD25⁻ naive T cells upregulate the expression of CCR4 and acquire the functions of CD4⁺CD25⁺Treg cells [28]. FOXP3⁺ naturally occurring CD4⁺CD25⁺Treg cells are mainly present in the CD4⁺CD25^{high}CCR4⁺ population, but not in the CD4⁺CD25^{high}CCR4⁻ population. Human peripheral CCR4⁺ T cells can be divided into two separate functional subpopulations (Th2 and Treg cells) according to their level of expression of CD25; that is, the CCR4⁺CD25^{low} population is greatly enriched in Th2 cells, whereas the CCR4⁺CD25^{high} population is greatly enriched in Treg cells [29].

Accordingly, we considered that the decreased level of Tregs may be one of the reasons for the uncontrollable autoimmune reaction in RA, and increased expression of CCR4 and CCR6 on peripheral Tregs in our present study imply the cells obtained the ability to migrate to inflammatory joints, reflecting the body feedback regulation when local autoimmune reaction was out of control. Furthermore, there were only 11 cases in the study of Jiao et al, maybe much more patients should be enrolled. Indeed, there was also a limitation in our study because the CCR4 and CCR6 expression on synovial fluid mononuclear cells (SFMCs) were not included.

Trafficking and migration to tissues and secondary lymphoid organs are required for Treg cell function *in vivo* [30, 31]. So, we further observed some CCR expression in the CIA model *in vivo*, by using the DBA/1 Foxp3^{gfp} reporter mice. Notably, the levels of CCR6 on GFP-high (i.e., Foxp3-high) cells in many secondary lymphoid organs such as spleens, dLNs, lungs and blood in CIA were all significantly

greater than those in normal control mice ($P < 0.01$, **Figure 4A-C**). However, other chemokines, including CCR4 and CCR5 expression was not significantly different between CIA and control and CCR7 or CCR9 expression on Treg cells is undetectable in both groups of mice, suggesting that the mouse cells do not completely reflect the results generated from human cells.

These results suggest that Foxp3⁺ regulatory T cells in peripheral tissues and lymph nodes exhibit a distinct chemokine receptor pattern that may contribute to its migration and accumulation in the inflammation joints.

In an autoimmune diabetes model, Treg cells control T cell priming within the LN [32]. Treg cells used CCR2, CCR4 and CCR5 to migrate first into the inflamed allograft. Then, Treg cells used CCR2, CCR5 and CCR7 to migrate from the islet to the dLN. This pattern permitted Treg cells to differentiate and display optimal suppressive function to inhibit DCs migration from the islet to the dLNs and then inhibit effector T cell responses in both the islets and the dLNs [24]. Several studies identified Treg cells within inflamed tissues and transplanted grafts, suggesting these cells control effector T cells in peripheral tissues at sites of ongoing immune responses [25, 33, 34].

Such disparate results may be due to differences in the numbers, activation status, and types of Treg cells. Manipulation of Treg cell differentiation and dynamic trafficking may be therapeutically beneficial for immunotherapy designed to engage their suppressive function. Although the mechanisms regulating expression of these chemokine receptors and trafficking of Foxp3-expressing regulatory T cells within the rheumatoid joints require further study, our results may indicate the new areas for mechanistic studies of immunological modulations of regulatory T cells in RA.

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Disclosure of conflict of interest

None.

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