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Original Article

T helper 17 and T helper 1 cells are increased but regulatory T cells are decreased in subchondral bone marrow microenvironment of patients with rheumatoid arthritis

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Abstract: Objectives: The present study is to investigate the profiles of Th17, Th1 and Treg cells in bone marrow of patients with rheumatoid arthritis (RA). Methods: Flow cytometry was used to analyze the frequencies of Th17, Th1 and Treg cells in paired peripheral blood and bone marrow of 26 RA patients and 11 osteoarthritis (OA) patients, as well as 10 healthy controls. In addition, the disease activity was analyzed by the 28-joint disease activity score (DAS28). Results: The frequencies of Th17 and Th1 cells were significantly elevated in bone marrow of RA patients. Importantly, Th17 and Th1 cells were significantly elevated in bone marrow compared with the matched peripheral blood from RA patients. However, Treg cells were significantly decreased in bone marrow of RA patients compared with the matched peripheral blood of RA patients and bone marrow of osteoarthritis patients and healthy controls. Moreover, the frequencies of tumor necrosis factor-α-producing T cells were significantly elevated in bone marrow from RA patients. Additionally, Th17 and Th1 cells in bone marrow were positively correlated with DAS28, while Treg cells were negatively correlated with DAS28. Conclusions: The present study demonstrates that Th17 and Th1 cells are markedly increased in bone marrow from RA patients. By contrast, Treg cells are significantly decreased in bone marrow from RA patients. These results suggest that local abnormality of Th17, Th1 and Treg cells in bone marrow of RA patients may contribute to bone destruction in skeletal system.

Keywords: Rheumatoid arthritis, bone marrow, T helper 17 cells, regulatory T cells, T helper 1 cells

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by persistent synovitis and bone destruction in multiple joints [1]. The abnormality of T cells contributes greatly to joint pathology in RA. CD4+ T helper cells are involved in bone destruction of RA. RA is traditionally deemed as a Th1-associated disease [2]. Th17 cells are implicated in the pathogenesis of RA [3, 4], and elevated in both peripheral blood and synovial fluid of patients with RA, suggesting that Th17 cells play a crucial role in RA [5, 6]. Moreover, the level of Th17 cells is higher in synovial fluid in comparison to peripheral blood in RA [6]. Interleukin (IL)-17A is a representative Th17 cytokine that is implicated in the development of RA. It is demonstrated that IL-17A, by synergizing with TNF-α and IL-1, contributes to the inflammatory process of RA.

Treg cells, which are characterized by expressing Foxp3 in the nuclei, have the ability to suppress CD4+ and CD8+ T cells by means of cell contact-dependent mechanisms [7-10]. Moreover, Treg cells also play crucial roles in T-cell tolerance by releasing anti-inflammatory cytokines including IL-10 and TGF-β [11-13]. The mounting evidences strongly suggest that Treg cells are involved in the pathogenesis of RA. The frequency of Treg cells in the peripheral

Table 1. Demographic and clinical characteristics of RA patients

Characteristics	Values
No. of patients	26
Age (year)	62.4 ± 7.3
Sex (male/female)	5/21
Disease duration (year)	12.9 ± 7.4
RF positive	19/26 (73.1%)
Anti-CCP positive	18/26 (69.2%)
ESR (mm/h)	74.2 ± 26.3
CRP (mg/L)	54.3 ± 26.9
No. of swollen joints	11.3 ± 6.2
No. of tender joints	12.3 ± 7.0
DAS28	6.7 ± 1.2

Note: RF, rheumatoid factor; anti-CCP, anti-cyclic citrullinated peptide antibody; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; DAS28, Disease Activity Score in 28 joints.

blood of RA patients is still conflicting [14, 15]. However, the situation of Treg cells in synovial fluid is clear, and the percentage of Treg cells in the synovial fluid of RA patients is higher than that in the peripheral blood [16]. However, the situation of Treg cells in bone marrow environments in RA patients remains unclear.

Inflammation may target bone tissues and result in structural and functional adverse effects in inflammatory bone tissues [17]. At bone level, RA is characterized by focal erosions of marginal and subchondral bones as well as generalized osteoporosis [2]. Bone marrow abnormality is observed in many inflammatory (autoimmune) diseases. For example, bone marrow dysplasia is reported in systemic lupus erythematosus patients, and appears to be associated with disease activity [18]. In addition, bone marrow is observed to function as a reservoir for CD4+ memory T cells, and plays a critical role in inducing colitis in murine colitis models [19]. As T helper cells are considered to exist in bone marrow, their contribution in situ is possible. There are plenty of reports about the situation of T cell subsets (Th1, Th17 and Treg cells) in peripheral blood and synovial fluid environment. However, relatively little is known about the profile of these T cells in bone marrow environment of RA. In order to explore the abnormality of T cells in bone marrow environment of RA, we examine the frequency of Th1, Th17 and Treg cells in bone marrow of patients with RA and investigate their correlation with disease activity in the present study.

Materials and methods

Patients

A total of 26 patients with active RA were included as patient group in this study according to the criteria of the American College of Rheumatology [20]. Each patient with active RA was defined by a DAS28 score ≥ 2.6 [21]. The patient group was consisted of 21 women and 5 men, with a mean disease duration of 12.9 ± 7.6 years. The mean age of the patients was 62.4 ± 7.4 years. Demographic and key clinical information of RA patients were summarized in **Table 1**. In addition, eleven osteoarthritis (OA) patients (8 females and 3 males; mean age, 64.1 ± 3.4 years) were included as disease controls. For healthy controls, 10 trauma patients (7 females and 3 males; mean age, 62.3 ± 5.1 years) who had no systemic inflammatory disease or immune abnormalities were included. Paired samples of bone marrow and peripheral blood were obtained from the same RA patient, OA patient and healthy control. Bone marrow samples were obtained from RA patients and OA patients during total knee arthroplasty. None of OA patients and healthy controls had any systemic inflammatory diseases or immunological background. The study took place between March 2013 and September 2015 in the Department of Orthopedics, Shandong Provincial Qianfoshan Hospital, Shandong University, China. The study was approved by the Institutional Review Boards of the Hospital. Informed consent was obtained from each patient before the patient was included in the study.

Samples

Bone marrow blood (5 ml) was aspirated from tibial proximal epiphysis by needle puncture at the time of operation. Simultaneously, 5 ml sample of venous blood was collected from the matched patient. In the course of bone marrow blood collection, peripheral blood contamination was considered probable. Therefore, we performed a preliminary experiment by examining T cell subsets of different volumes of bone marrow blood in the same patient. When the volume obtained from bone marrow did not exceed 5 ml, the frequencies of T cell subsets

showed no significant difference and the bone marrow blood was not contaminated by peripheral blood (data not shown). Therefore, the first 5 ml of bone marrow blood was believed to properly exhibit the profile of T cell subsets in the bone marrow and could be analyzed in our study.

Flow cytometry

Intracellular cytokines were studied using flow cytometry to reflex cytokine-producing cells. Briefly, heparinized peripheral whole blood (400 μl) and bone marrow blood with equal volume of Roswell Park Memorial Institute (RPMI)-1640 medium were incubated for 4 h at 37°C and 5% CO_2 in the presence of 25 ng/mL of phorbol myristate acetate (PMA), 1 μg/mL of ionomycin, and 1.7 μg/ml Golgiplug (Monensin; all from Alexis Biochemicals, San Diego, CA, USA). PMA and ionomycin are pharmacological T-cell-activating agents that mimic signals generated by T-cell receptor (TCR) complex and have the advantage of stimulating T cells of any antigen specificity. Monensin was used to block intracellular transport mechanisms, thereby leading to the accumulation of cytokines in the cells. After incubation, the cells were stained with PE-Cy5-conjugated anti-CD4 monoclonal antibodies at room temperature in the dark for 20 min. The cells were next stained with FITC-conjugated anti-interferon (IFN)-γ monoclonal antibodies, PE-conjugated anti-IL-17 monoclonal antibodies and APC-conjugated anti-TNF-α monoclonal antibodies (eBioscience, San Diego, CA, USA) after fixation and permeabilization. Isotype controls were given to enable correct compensation and to confirm antibody specificity. Stained cells were analyzed by flow cytometric analysis using a FACScan cytometer equipped with CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

$CD4^+CD25^+Foxp3^+$ T cells as Treg cells were evaluated using Human Regulatory T-cell Staining Kit (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol. Before staining, peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were isolated by centrifugation over Ficoll-Hypaque gradients. Then, single-cell suspension was incubated with a cocktail of anti-CD4-FITC monoclonal antibody and anti-CD25-APC monoclonal antibody for 30 min in the dark at 4°C to stain the surface. After being washed with 2 ml cold staining buffer, the PBMCs were incubated with 1 ml freshly prepared Foxp3 fixation/permeabilization buffer (eBioscience, San Diego, CA, USA) for 60 min at 4°C in the dark. The cells were washed with 2 ml freshly prepared 1× permeabilization buffer twice. The cells were then blocked in normal rat serum for 15 min and stained using anti-Foxp3-PE monoclonal anti-body (PCH101) or PE-conjugated rat IgG2a (used as isotype control) for 45 min in the dark at 4°C. After washing the cells twice, 300 μl cold flow cytometry staining buffer was added to resuspend the cells. Stained cells were analyzed by flow cytometric analysis using a FACScan cytometer equipped with CellQuest software (BD Bioscience, Franklin Lakes, NJ, USA).

Clinical assessment

Disease activity score in 28-joints (DAS28) [21] was calculated in our study. Each patient with active RA was defined by a DAS28 score ≥ 2.6. At the time of clinical assessment for disease activity, blood samples were collected for the measurement of levels of C-reactive protein (CRP).

Statistical analysis

Results were expressed as means ± SD. Statistical significance was determined by ANOVA, and difference between two groups was determined by Newman-Keuls multiple comparison test (*q* test). For comparison of paired samples, Wilcoxon signed rank test was used. Pearson correlation test was used for correlation analysis. All tests were performed by SPSS 17.0 software (IBM, Armonk, NY, USA). *P* values less than 0.05 were considered statistically significant.

Results

Frequencies of Th17 cells in bone marrow of RA patients are higher than those in peripheral blood of RA patients

Flow cytometry was used to analyze the intracellular expression of IL-17, IFN-γ and TNF-α after in vitro activation by PMA/ionomycin in CD4 T cells from peripheral blood and bone marrow. Th17 was defined as $CD4^+IFN\gamma^-IL17^+$ T cells to exclude Th1 cells. The percentage of

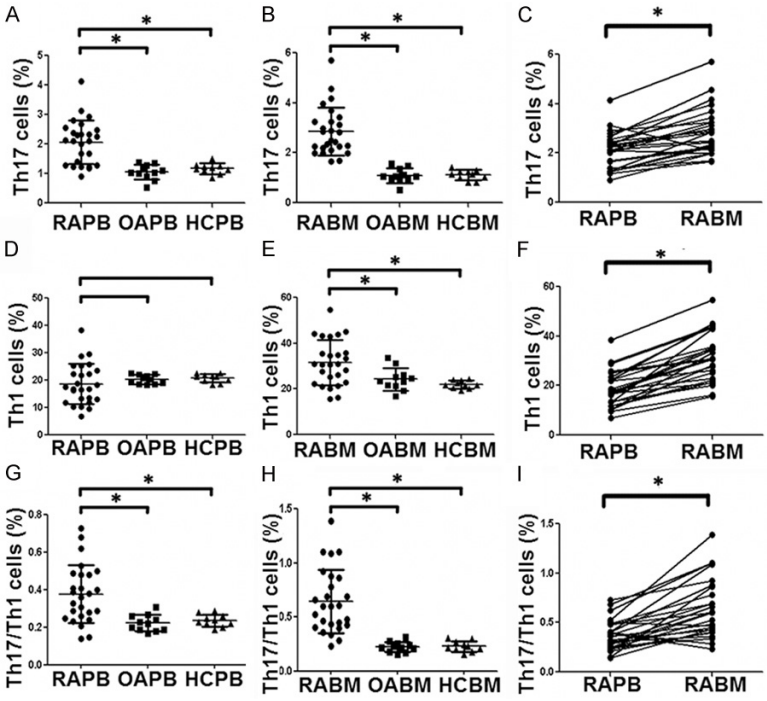


Figure 1. Frequencies of Th17, Th1 and Th17/Th1 cells in both peripheral blood and bone marrow blood of RA patients, OA patients and healthy controls. (A-C) The frequencies of Th17 cells in (A) peripheral blood and (B) bone marrow blood in RA patients, OA patients and healthy controls, as well as (C) the frequencies of Th17 cells in peripheral blood and bone marrow blood of the same RA patient. (D-F) The frequencies of Th1 cells in (D) peripheral blood and (E) bone marrow blood in RA patients, OA patients and healthy controls, as well as (F) the frequencies of Th17 cells in peripheral blood and bone marrow blood of the same RA patient. (G-I) The frequencies of Th17/Th1 cells in (G) peripheral blood and (H) bone marrow blood in RA patients, OA patients and healthy controls, as well as (I) the frequencies of Th17 cells in peripheral blood and bone marrow blood of the same RA patient. RAPB, peripheral blood of RA patients; OAPB, peripheral blood of OA patients; HCPB, peripheral blood of healthy controls; RABM, bone marrow of RA patients; OABM, bone marrow of OA patients; HCBM, bone marrow of healthy controls. *P < 0.05.

Th17 cells in peripheral blood from RA patients was significantly elevated compared to that in peripheral blood from OA patients (P < 0.001) or healthy controls (P < 0.001) (**Figure 1A**), and the percentage of Th17 cells in bone marrow blood from RA patients was significantly higher compared to that in bone marrow blood from OA patients (P < 0.001) or healthy controls (P < 0.001) (**Figure 1B**). However, the percentages of Th17 cells in peripheral blood from OA patients and healthy controls were not significantly different from those in bone marrow from OA patients and healthy controls, respectively (**Figure 1A** and **1B**). By contrast, the percentage of Th17 cells in bone marrow from RA patients was significantly higher than that in the paired peripheral blood from RA patients (P < 0.001) (**Figure 1C**). The result suggests that elevated frequencies of Th17 cells in bone marrow of RA patients may reflect their increased proliferation in situ.

Frequencies of Th1 cells and Th17/Th1 cells in bone marrow of RA patients are higher than those in peripheral blood of RA patients

To determine whether there are differences in profiles of T cell subsets at different levels of bone marrow (from superficial bone marrow to deep bone marrow that appears distant from the cartilage-pannus junction), we examined the T subsets in bone marrow samples from different depths. The frequencies of Th1 cells in the peripheral blood of RA patients were not markedly increased compared with those of OA patients or healthy controls (**Figure 1D**), but the frequencies of Th1 cells in bone marrow of RA patients were significantly higher than those in OA patients (P=0.014) or healthy controls (P =0.002) (**Figure 1E**). Consistent with the result of Th17 cells, the percentage of Th1 cells in bone marrow was significantly higher than that in paired peripheral blood samples from RA patients (P < 0.001) (**Figure 1F**). In addition, the frequencies of CD4 T cells co-expressing IFN-γ and IL-17 (named Th17/Th1 cells) were also analyzed. The percentage of Th17/Th1 cells in peripheral blood of RA patients was significantly elevated compared to that in OA patients (P=0.001) or healthy controls (P=0.003) (**Figure 1G**). Similarly, the percentage of Th17/Th1 cells in bone marrow of RA patients was significantly higher than that in OA patients (P < 0.001) or healthy controls (P < 0.001) (**Figure 1H**). In addition, the percentage of Th17/Th1 cells in bone marrow blood of RA

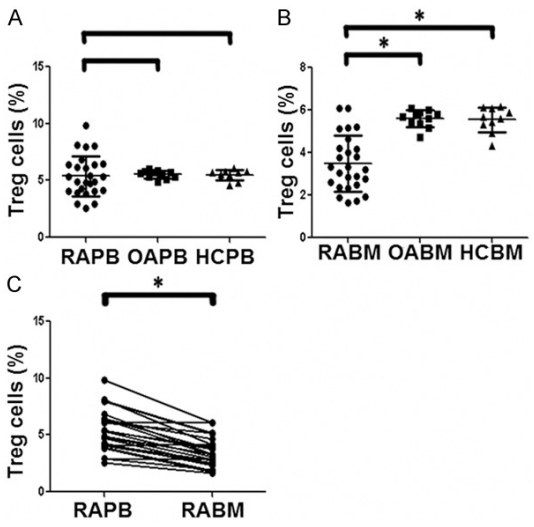


Figure 2. Frequencies of Treg cells in both peripheral blood and bone marrow blood of RA patients, OA patients and healthy controls. (A) The frequencies of Treg cells in (A) peripheral blood and (B) bone marrow blood in RA patients, OA patients and healthy controls. (C) The frequencies of Treg cells in peripheral blood and bone marrow blood of the same RA patient. RAPB, peripheral blood of RA patients; OAPB, peripheral blood of OA patients; HCPB, peripheral blood of healthy controls; RABM, bone marrow of RA patients; OABM, bone marrow of OA patients; HCBM, bone marrow of healthy controls. *P < 0.05.

patients was significantly increased compared to that in peripheral blood of RA patients (P < 0.001) (**Figure 1I**). The results indicate that frequencies of Th1 cells and Th17/Th1 cells in bone marrow of RA patients are higher than those in peripheral blood of RA patients.

Frequencies of Treg cells in peripheral blood of RA patients were higher than those in bone marrow of RA patients

To measure the frequencies of Treg cells, flow cytometry was also used. Treg cells were defined as $CD4^+CD25^+Foxp3^+$ T cells in this study. The data showed that the percentage of Treg cells in peripheral blood from RA patients was not significantly different from that from OA patients or healthy controls (**Figure 2A**). By contrast, the percentage of Treg cells in bone marrow from RA patients was significantly lower than that from OA patients (P < 0.001) or healthy controls (P < 0.001) (**Figure 2B**). In addition, the percentage of Treg cells in bone marrow of RA patients was significantly lower than that in peripheral blood of RA patients (P < 0.001) (**Figure 2C**). The results suggest that frequencies of Treg cells in peripheral blood of RA patients were higher than those in bone marrow of RA patients.

Frequencies of Th17 and Th1 cells co-expressing TNF-α in bone marrow of RA patients are significantly higher than those in peripheral blood of RA patients

To detect TNF-α-producing T helper cells, flow cytometry was performed. Significant elevated expression of TNF-α in CD4+ T cells was found in bone marrow of RA patients. The data showed that the frequencies of TNF-α-producing CD4+ T cells in bone marrow of RA patients was significant elevated compared to those in peripheral blood of RA patients (P < 0.001) (**Figure 3A**). Moreover, the frequencies of TNF-α-producing Th1 cells were in bone marrow of RA patients were markedly higher than those in peripheral blood of RA patients (P < 0.001) (**Figure 3B**). Similarly, the percentage of TNF-α-producing Th17 cells in bone marrow of RA patients was significantly higher than that in peripheral blood of RA patients (P < 0.001) (**Figure 3C**). The percentages of TNF-α-producing CD4+ T cells, TNF-α-producing Th1 cells and TNF-α-producing Th17 helper cells in bone marrow from RA patients were significantly elevated compared to OA patients (P=0.005, P=0.025 and P < 0.001, respectively) or healthy controls (P=0.009, P=0.035 and P < 0.001, respectively) (**Figure 3D-F**). Moreover, the percentages of both TNF-α-producing CD4+ T helper cells and TNF-α-producing Th1 helper cells in peripheral blood of RA patients, OA patients and healthy controls were not significantly different from each other (**Figure 3G** and **3H**). However, the percentage of TNF-α-producing Th17 helper cells in peripheral blood from RA patients was significantly elevated compared to OA patients (P < 0.001) or healthy controls (P < 0.001) (**Figure 3I**). These results indicate that frequencies of Th17 and Th1 cells co-expressing TNF-α in bone marrow of RA patients are significantly higher than those in peripheral blood of RA patients.

Frequencies of each T subpopulation of RA patients have positive correlation between bone marrow and peripheral blood samples

To analyze correlations between peripheral blood and bone marrow blood for the frequencies of each T subpopulation of RA patients,

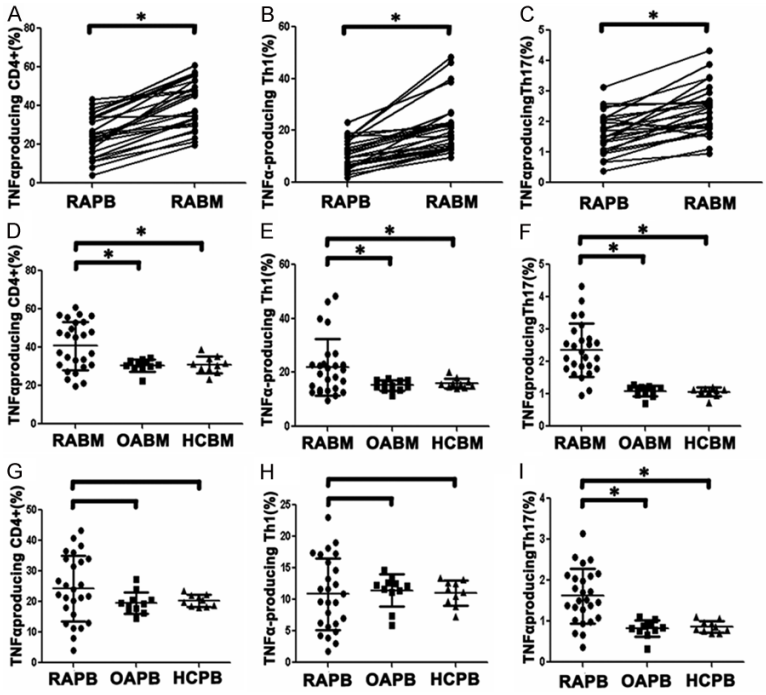


Figure 3. Expression of TNF-α in CD4+ T cells in both peripheral blood and bone marrow blood of RA patients, OA patients and healthy controls. (A-C) The frequencies of (A) TNF-α-producing CD4+ T cells, (B) TNF-α-producing Th1 cells and (C) TNF-α-producing Th17 cells in peripheral blood and bone marrow blood of the same patient. (D-F) The frequencies of (D) TNF-α-producing CD4+ T cells, (E) TNF-α-producing Th1 cells and (F) TNF-α-producing Th17 in bone marrow blood in RA patients, OA patients and healthy controls. (G-I) The frequencies of (G) TNF-α-producing CD4+ T cells, (H) TNF-α-producing Th1 cells and (I) TNF-α-producing Th17 in peripheral blood in RA patients, OA patients and healthy controls. RAPB, peripheral blood of RA patients; OAPB, peripheral blood of OA patients; HCPB, peripheral blood of healthy controls; RABM, bone marrow of RA patients; OABM, bone marrow of OA patients; HCBM, bone marrow of healthy controls. *P < 0.05.

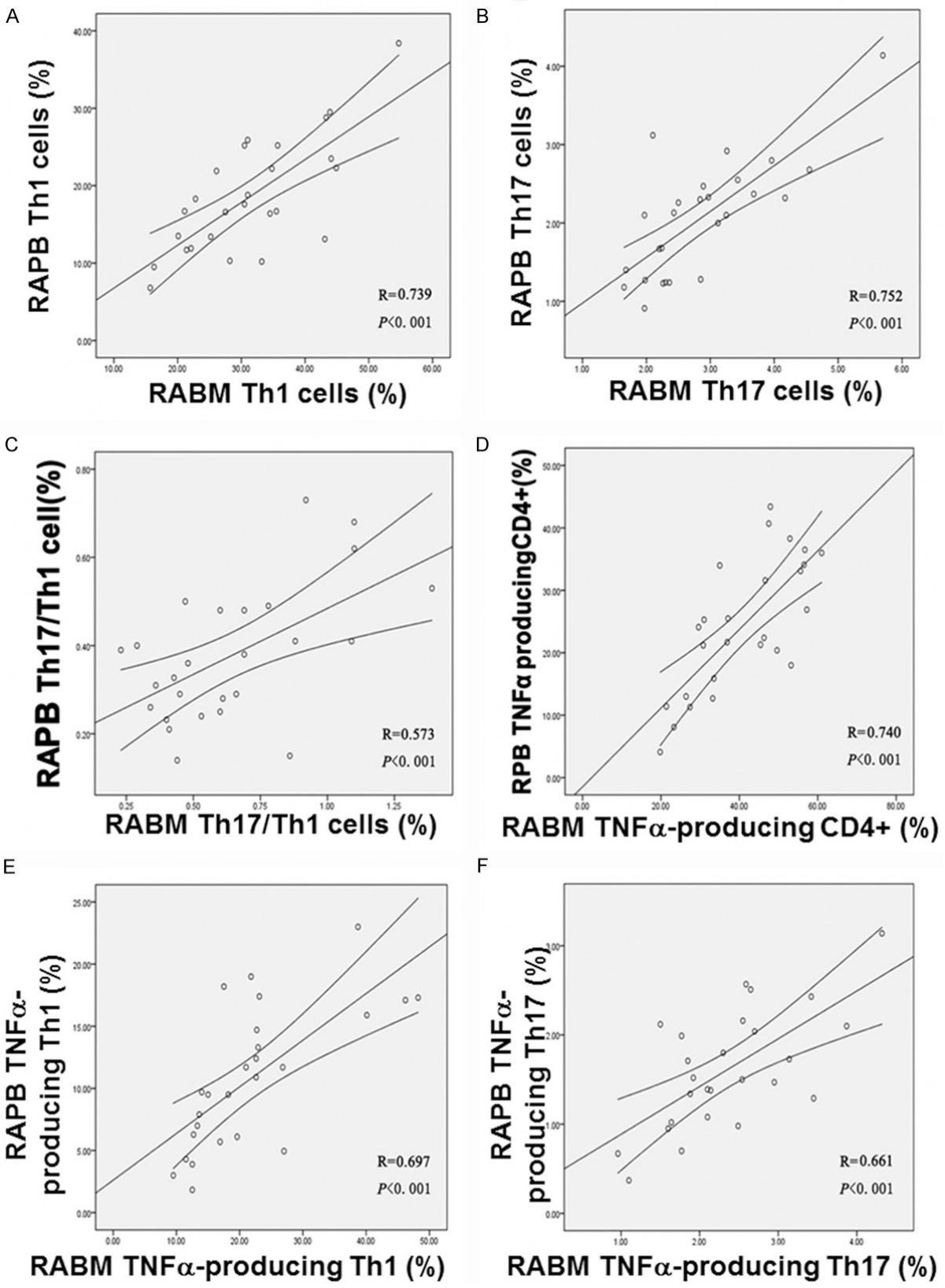
Pearson correlation test was used. In RA patients, frequencies of Th1 (r = 0.739, P < 0.001) (**Figure 4A**), Th17 (r = 0.752, P < 0.001) (**Figure 4B**) and Th17/Th1 cells (r = 0.574, P = 0.002) (**Figure 4C**) in peripheral blood were found to have significant positive correlation with those in bone marrow. The frequencies of TNF-α-producing T helper cells (r = 0.740, P < 0.001) (**Figure 4D**), TNF-α-producing Th1 cells (r = 0.697, P < 0.001) (**Figure 4E**) and TNF-α-producing Th17 cells (r = 0.660, P < 0.001) (**Figure 4F**) in peripheral blood were also positively correlated with their frequencies in bone marrow of RA patients. In addition, frequency of Treg cells in bone marrow was positively correlated with that in peripheral blood of RA patients (r = 0.792, P < 0.001) (**Figure 4G**). These results suggest that frequencies of each T subpopulation of RA patients have positive correlation between bone marrow and peripheral blood samples.

T helper subset is negatively correlated with Treg subset in bone marrow of RA patients

To investigate correlations between T helper subset and Treg subset in bone marrow of RA patients, Pearson correlation test was carried out. The data showed that Th17 (r = 0.611, P = 0.001), Th1 (r = 0.502, P = 0.009) and Th17/Th1 cells (r = 0.424, P = 0.031) were inversely correlated with Treg cells in bone marrow of RA patients (**Figure 5A** and **5B**). Similarly, Treg cells exhibited negative correlation with TNF-α-producing Th1 cells (r = 0.602, P = 0.001) and TNF-α-producing Th17 cells (r = 0.562, P = 0.003) in bone marrow of RA patients (**Figure 5C** and **5D**). The result indicates that T helper subset is negatively correlated with Treg subset in bone marrow of RA patients.

Th17 cells, Th1 cells, TNF-α-producing T helper cells, TNF-α-producing Th1 cells and TNF-α-producing Th17 cells have positive correlation with CRP level or DAS28 in bone marrow, while Treg cells have negative correlation with CRP level or DAS28

To determine the correlations between each T cell subset and disease activity in different samples of RA patients, we performed Pearson correlation test. In patients with RA, Th1 and Th17 cells were positively correlated with either CRP level or DAS28 in bone marrow (r = 0.526, P = 0.006; r = 0.632, P = 0.001 or r = 0.447, P = 0.022; r = 0.594, P = 0.001, respectively) (**Figure 6A**, **6B**, **6G** and **6H**). Consistently, TNF-α-producing T helper cells, TNF-α-producing Th1 cells and TNF-α-producing Th17 cells exhibited positive correlations with either CRP level or DAS28 in bone marrow (r = 0.560, P = 0.003; r = 0.720, P < 0.001; r = 0.529, P = 0.005 or r = 0.622, P = 0.001; r = 0.506, P = 0.008; r = 0.636, P < 0.001, respectively) (**Figure 6C-E**, **6I-K**). On the contrary, Treg cells were inversely correlated with either CRP level


A
RAPB Th1 cells (%)
RABM Th1 cells (%)
R=0.739
P<0. 001
B
RAPB Th17 cells (%)
RABM Th17 cells (%)
R=0.752
P<0. 001
C
RAPB Th17/Th1 cell(%)
RABM Th17/Th1 cells (%)
R=0.573
P<0. 001
D
RPB TNFα producingCD4+(%)
RABM TNFα-producing CD4+ (%)
R=0.740
P<0. 001
E
RAPB TNFα-producing Th1 (%)
RABM TNFα-producing Th1 (%)
R=0.697
P<0. 001
F
RAPB TNFα-producing Th17 (%)
RABM TNFα-producing Th17 (%)
R=0.661
P<0. 001

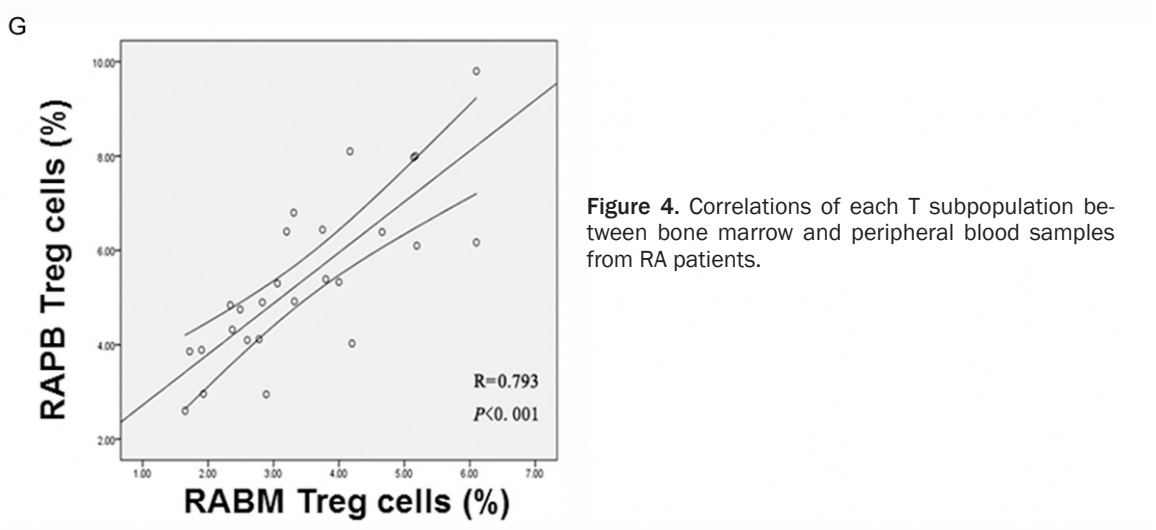


Figure 4. Correlations of each T subpopulation between bone marrow and peripheral blood samples from RA patients.

or DAS28 in bone marrow (r = -0.630, P = 0.001 or r = -0.797, P < 0.001, respectively) (**Figure 6F** and **6L**). These results suggest that Th17 cells, Th1 cells, TNF-α-producing T helper cells, TNF-α-producing Th1 cells and TNF-α-producing Th17 cells have positive correlation with CRP level or DAS28 in bone marrow, while Treg cells have negative correlation with CRP level or DAS28.

Discussion

In the present study, we have examined the co-localization of many important T cells and the profiles of these T cells in bone marrow of subchondral bone of RA patients for the first time. In addition, the role of these T cells in bone erosion and cartilage destruction is also investigated. It has been demonstrated that Th17 cells and Th1 cells contribute to the development of RA [22]. Consistence with previous findings [23, 24], the percentages of Th17 cells are significantly increased in the peripheral blood of patients with RA. More importantly, the percentage of Th17 cells in bone marrow is markedly higher than that in peripheral blood of RA patients. It is reported that Th17 cells also have the expression of chemokine receptor CCR6 [25, 26], which is necessary for T cell migration to the lesion sites. Increased expression of CCL20 [27], the ligand of CCR6, is observed in subchondral bone tissue biopsies of RA patients [28], being consistent with the situation in synovial fluid from RA patients. It is considered that excessive bone resorption by osteoclasts contributes to bone destruction in RA, and CD4+ T cells that migrate to the lesion sites can directly and indirectly regulate the formation of osteoclasts [29, 30]. Th17 cells are the conjunction between T cells and bone destruction in RA [31, 32]. Accordingly, significantly elevated Th17 cells are observed in bone marrow in contrast peripheral blood, suggesting the destructive effect of Th17 cells in situ. Th17 cells can induce receptor activator for nuclear factor-κB ligand (RANKL) expression on osteoblasts. It is well known that RANKL can induce the formation of osteoclasts and activate mature osteoclasts, leading to the destruction of bone [33].

In addition, the association between Th17 cells and disease activity, including DAS28 and CRP, are also analyzed in the present study. Many studies show that the degree of focal bone erosions has an association with the severity of RA [34, 35]. Frequencies of Th17 cells in bone marrow may reflect the extent of focal bone erosion, as well as the severity and disease activity of RA. All of above provide evidence that Th17 cells play an important role in the course of osteoclastogenesis, and elevated Th17 cells in bone marrow contribute to focal bone erosion and osteopenia in RA. Aberrant Th17 cells profiles in subchonral bone marrow have been demonstrated in one single joint in our study. In addition to focal bone loss, RA patients suffer from systemic osteoporosis in the axial and appendicular skeleton, reflecting systemic inflammation of RA.

Consistent with the result of a previous study [36], no significant elevation of peripheral Th1 cells is observed in RA patients. Th1 cells are

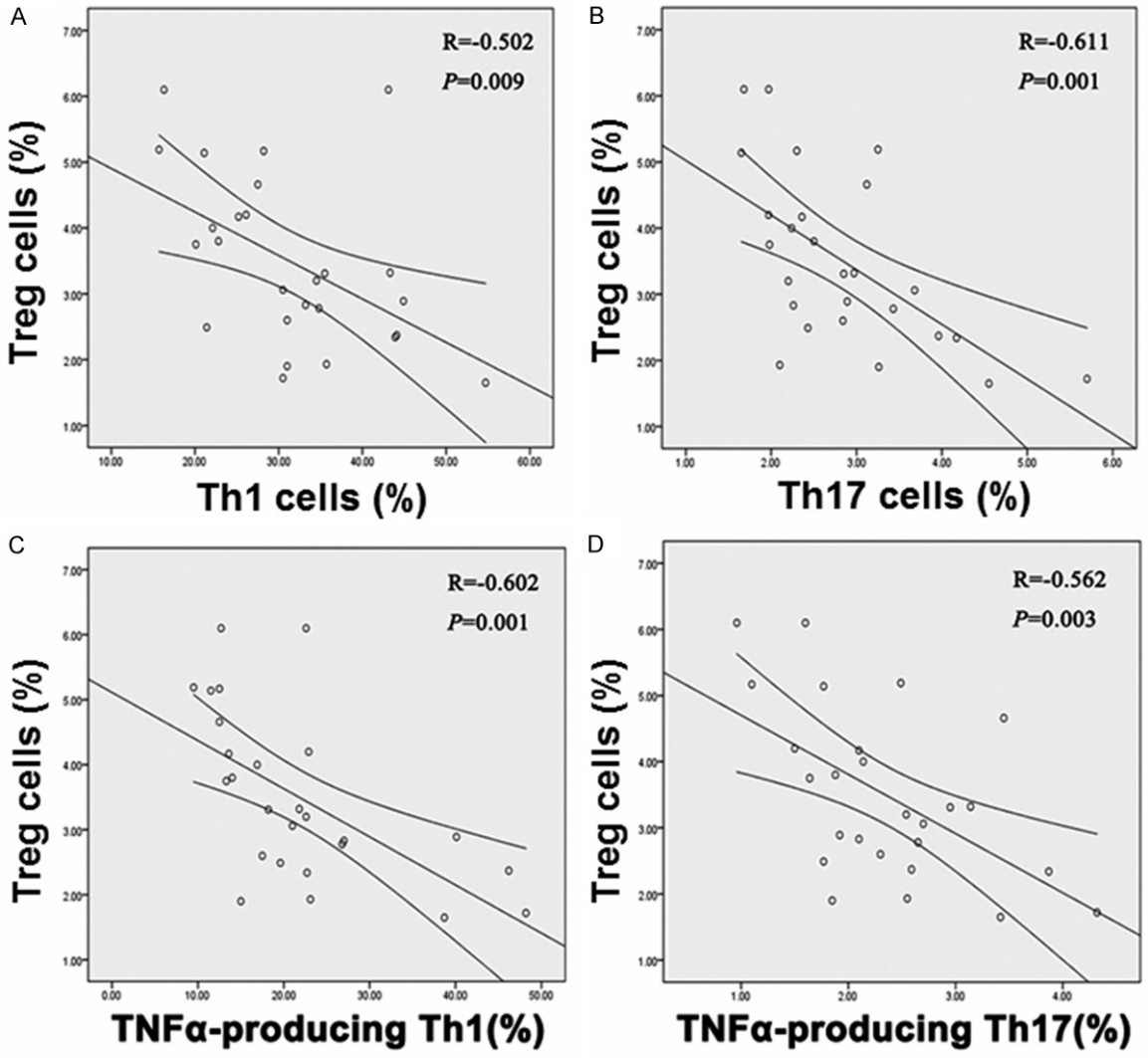


Figure 5. Correlations between the frequencies of each Th cell subset and Treg cells in bone marrow of RA patients.

shown to predominate in the joints of RA patients [37]. In the present study, Th1 cells are significantly elevated in bone marrow of RA patients. Increased Th1 cells in bone marrow of RA patients can express more RANKL that can induce the formation of osteoclasts and activate mature osteoclasts [38]. Furthermore, TNF-α-producing Th1 cells are also elevated in bone marrow from RA patients. Overexpression of TNF-α in Th1 cells contributes to local bone destruction in RA patients. Bone marrow TNF-α-producing Th17 cells induce osteoclast differentiation and contribute to bone destruction in inflammatory bowel disease [39]. Of note, TNF-α is shown to contribute to impaired Treg cell function [40]. Moreover, TNF-α is shown to promote Th17 cell differentiation through IL-6 and IL-1β in RA [41]. TNF-α-induced Treg cell dysfunction is associated with increased Th17 and Th1 cells through FOXP3 dephosphorylation within the synovium from RA [42]. Pathogenic conversion of Treg cells into Th17 cells is observed in autoimmune arthritis [43]. Whether the function of Treg cells is also impaired by TNF-α in bone marrow of RA patients, which is consistent with the mechanism in inflamed synovium, needs further investigation.

With regard to the proportion of Treg cells, controversial results exist in peripheral blood of RA patients compared to healthy individuals [44, 45]. In contrast to the situation in peripheral circulation, the frequencies of Treg cells are elevated in the synovial fluid compared with those in peripheral blood in patients with RA. It has been demonstrated that Treg cells exert their suppressive function of osteoclastogene-

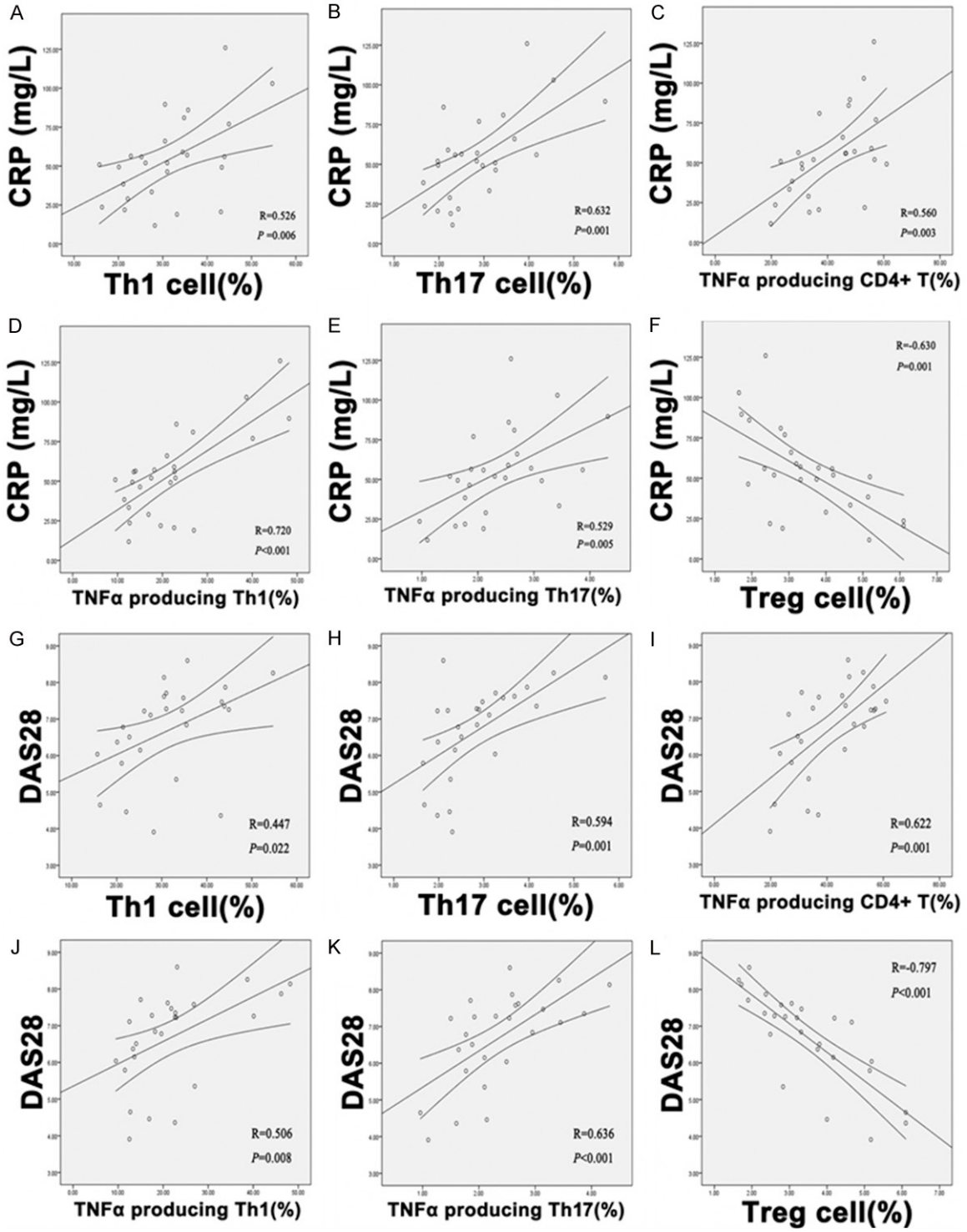


Figure 6. Correlations of each T cell subset with CRP level (A-F) or 28-joints Disease Activity Score (DAS28) (G-L) in bone marrow of RA patients. (A) Correlation of Th1 cell percentage with CRP level. (B) Correlation of Th17 cell percentage with CRP level. (C) Correlation of TNFα producing CD4+ cell percentage with CRP level. (D) Correlation of TNFα producing Th1 cell percentage with CRP level. (E) Correlation of TNFα producing Th17 cell percentage with CRP level. (F) Correlation of Treg cell percentage with CRP level. (G) Correlation of Th1 cell percentage with DAS28. (H) Correlation of Th17 cell percentage with DAS28. (I) Correlation of TNFα producing CD4+ cell percentage with DAS28. (J) Correlation of TNFα producing Th1 cell percentage with DAS28. (K) Correlation of TNFα producing Th17 cell percentage with DAS28. (L) Correlation of Treg cell percentage with DAS28.

sis partially via IL-4 and IL-10 secretion, but mainly in a cell-cell contact manner via cytotoxic T lymphocyte antigen 4 [46]. Nevertheless, other studies show that Treg cells inhibit osteoclastogenesis in a cell-cell contact manner by the secretion of TGF-β and IL-4 [47]. As mentioned above, it is indicated that Treg cells can inhibit osteoclastogenesis both in vitro and in vivo. Moreover, reduced number of Treg cells can lead to weakened anti-osteoclastogenesis functions in bone tissue. However, the exact mechanism by which Treg cells exert suppressive effects on osteoclastogenesis in RA remains to be fully clarified in the future.

Recently, more and more evidence support the point of view that immune system plays a central role in regulating bone homeostasis, generation and destruction [48]. Our results have shown a severe pro-inflammatory environment in bone marrow of RA patients. Therefore, immunocyte infiltration in bone marrow may contribute to cartilage erosion from subchondral bone side. Our results demonstrate that inflammatory cells at the subchondral site resulted in cartilage erosion from bone front. Actually, both synovial and subchondral inflammation may lead to bidirectional erosion of cartilage in knee joints of RA patients. Patients with OA also suffer from joint destruction, but T cell profiles in bone marrow of OA patients are similar to those of healthy controls. Therefore, a completely different mechanism may be involved in joint destruction in RA and OA. In conclusion, the present study provides a new insight into the mechanism of bone destruction of RA patients. In addition, subchondral bone may be proven a promising therapeutic target for RA.

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Disclosure of conflict of interest

None.

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