Original Article
Increased frequency of peripheral blood IL-21+ follicular helper T cells and increased serum IL-21 levels in patients with non-traumatic osteonecrosis of the femoral head

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Abstract: Background: There was no study about follicular helper T (TFH) cells in non-traumatic osteonecrosis of the femoral head (NONFH). Objectives: This study examined the roles of TFH cells and serum IL-21 in the pathogenesis of NONFH. Methods: The study included 28 consecutive patients with NONFH and 15 healthy control subjects. Flow cytometry was used to measure the frequencies of peripheral blood inducible costimulator (ICOS)+, programmed death 1 (PD-1)+, and IL-21+ TFH cells in NONFH patients with and without long-term steroid use or alcohol abuse. The disease progression, the extent of femoral head collapse, the serum IL-21, C-reactive protein, and fibrinogen levels, and the erythrocyte sedimentation rates were quantified. Results: The percentages of IL-21+ TFH cells and serum IL-21 levels in patients of each group were significantly higher than those in controls. Higher serum IL-21 levels were positively associated with the index of femoral head collapse in the steroid use and alcohol abuse groups. Conclusions: IL-21+ TFH cells may participate in the pathogenesis of NONFH. Increased serum IL-21 may be associated with and act as a potential indicator for the extent of femoral head collapse, particularly in patients with late-stage osteonecrosis of the femoral head (FICAT: III-IV) who use steroids or abuse alcohol.

Keywords: TFH cell, IL-21, NONFH

Introduction
Non-traumatic osteonecrosis of the femoral head (NONFH) is a severe chronic disease characterized by progressive collapse of the femoral head. It irreversibly affects adults in the third or fourth decade of life, leading to a high degree of disability during the late stages of disease [1]. There are 5 to 7.5 million patients with osteonecrosis of the femoral head (ONFH) in China, and most of these patients have NONFH [2]. Although hip replacement provides effective pain relief and restores function in NONFH, it places a heavy economic burden on society and is associated with long-term complications.

The mechanism of NONFH pathogenesis is unknown. Among the numerous risk factors that have been associated with an increased risk of ONFH, steroid use and alcohol intake have been identified as major causative factors [3]. These risk factors may exert their effects through intravascular coagulation, which leads to ischemia and subsequent death of the bone and bone marrow cells [4].

Immunologic factors also play important roles in ONFH pathogenesis [5-9]. Increased levels of serum cytokines, such as interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, have been detected with histopathologic changes of the femoral head in animal models of NONFH [7]. Vasculitis arising from autoimmune diseases, such as systemic lupus erythematosus
(SLE) and rheumatoid arthritis (RA), has been identified as a cause of ONFH [6-9].

Anti-phospholipid antibodies may contribute to ONFH pathogenesis by promoting thrombotic vasculopathy at terminal bone arteries [8]. Korompilias examined 40 patients with NONFH and found anti-cardiolipin antibodies in 37.5% of patients [5]. Okazaki indicated that osteonecrosis in animal models of NONFH may be related to B cell proliferation and antibody secretion [7]. A higher frequency of B cells may be associated with the development of human NONFH [10].

As a subset of CD4+ T cells, follicular helper T (T\textsubscript{FH}) cells are crucial regulators of B cell differentiation, antibody production, and humoral immunity [11]. T\textsubscript{FH} cells express chemokine (C-X-C motif) receptor 5 (CXCR5), inducible costimulator (ICOS), and programmed death (PD)-1 [12]. Although the identification of T\textsubscript{FH} cells remains controversial, a previous study found that CXCR5+CD4+ T cells shared the functional properties of T\textsubscript{FH} cells. Therefore, CXCR5+CD4+ T cells were considered to be T\textsubscript{FH} cells [13].

The CXCR5, ICOS and PD-1 Receptor-ligand binding mediates the T\textsubscript{FH} cell-mediated activation of B cells within lymphoid germinal centers (GCs), where B cells progress through the developmental processes that lead to the generation of long-lasting memory B cells and plasma cells [14]. The binding of CXCR5 to the cytokine CXCL13 is crucial for B cell follicular homing. ICOS positively regulates B cell differentiation, whereas PD-1 negatively regulates T\textsubscript{FH} cell function. IL-21, a cytokine secreted by T\textsubscript{FH} cells, modulates B cell differentiation and proliferation. Dysfunctions of T\textsubscript{FH} cells and IL-21 have been reported in patients with certain autoimmune diseases [15-18], suggesting that T\textsubscript{FH} cells contribute to SLE and RA pathogenesis. Moreover, T\textsubscript{FH} cells and IL-21 play important roles in the joint inflammation and osteonecrosis of RA [17, 18].

We hypothesized that T\textsubscript{FH} cells are involved in ONFH pathogenesis because: 1) T\textsubscript{FH} cell-related autoimmune diseases (e.g., SLE and RA) are risk factors for ONFH, 2) B cells are associated with NONFH development, and 3) T\textsubscript{FH} cells are crucial regulators of B cell differentiation. In this study, we examined the involvement of T\textsubscript{FH} cells in NONFH pathogenesis by measuring the percentages of peripheral blood T\textsubscript{FH} cells, including ICOS+ T\textsubscript{FH} cells, PD-1+ T\textsubscript{FH} cells, and IL-21+ T\textsubscript{FH} cells, as well as the serum IL-21 concentration, in 28 ONFH patients and 15 control subjects.

Material and methods

Patients and controls

Twenty-eight consecutive patients with NONFH were recruited at the inpatient service of a university hospital. Patients were divided into three groups based on their reported risk factors. The steroid group included 10 patients with long-term steroid use, who had undergone informal treatment with an average of 5-year use history of steroid-containing medications for strain-related physical pain. The alcohol abuse group included 10 patients with long-term alcohol abuse, who reported consuming more than 0.25 kg/d of white spirits every day for the past 10+ years. The NONFH without known risk factors group included 8 patients who had no known risk factors for NONFH (i.e., no trauma, steroid use, alcohol abuse, vascular disease, desmosis, or metabolic disease). Fifteen age- and ethnicity-matched subjects were recruited to the control group.

NONFH patients were diagnosed according to criteria proposed by Mont [19]. For each individual, the stage of disease progression (0-IV) was determined by FICAT guidelines [20]. As known, the femoral head is approximately circular. Pelvic radiographs of patients and controls were obtained. Then, the extent of femoral head collapse was calculated as the ratio of the radius of the femoral head (B) to the depth of the collapse of the femoral head (A) [21]. Subjects with traumatic femoral head necrosis, bone tumor, ankylosing spondylitis, developmental dysplasia or osteoarthritis of the hip, chronic inflammatory or autoimmune disease, allergy, or recent infection were excluded. No patient had received steroids, immunosuppressants, or other medication for the month prior to blood sample collection. No patient had received any treatment modality that could influence the extent of femoral head collapse.

Laboratory methods

Fasting venous blood samples were obtained from participants before treatment. Sera were
**Table 1.** Demographic and Clinical Characteristics of NONFH Patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control group</th>
<th>Steroid group</th>
<th>Alcohol abuse group</th>
<th>No risk factor group</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>15</td>
<td>10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48 (36-70)</td>
<td>50 (37-60)</td>
<td>49 (40-65)</td>
<td>52 (36-64)</td>
</tr>
<tr>
<td>No. of males</td>
<td>NA</td>
<td>5.5 (2.5-16)</td>
<td>7.5 (2.5-19)</td>
<td>6 (3-21)</td>
</tr>
<tr>
<td>Duration of disease (years)</td>
<td>NA</td>
<td>2.58 (1.03-4.64)</td>
<td>4.5 (0.82-38.66)*</td>
<td>5.2 (0.8-43.1)*</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>4.34 (1.5-22)</td>
<td>20 (2.1-14)*</td>
<td>45.5 (10-100)*</td>
<td>29.4 (8-90)*</td>
</tr>
<tr>
<td>WBC (10^9/L)</td>
<td>5.3 (4.35-9.68)</td>
<td>5.85 (4.22-7.22)</td>
<td>6.68 (4.53-8.64)</td>
<td>5.8 (4-9.05)</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>1.68 (1.39-3.25)</td>
<td>3.23 (2.18-5.17)*</td>
<td>4.28 (1.75-4.96)*</td>
<td>3.51 (2.03-6.06)*</td>
</tr>
<tr>
<td>FIB (g/l)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Index of femoral head collapse</td>
<td>NA</td>
<td>0.315 (0.18-0.55)</td>
<td>0.425 (0.12-0.62)</td>
<td>0.4 (0.18-0.58)</td>
</tr>
</tbody>
</table>

Data are shown as median (range) or as raw numbers. CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; WBC: White blood cell counts; FIB: Fibrinogen. *p < 0.05 vs. the HC; #p < 0.05 vs. the alcohol buse group; NA: not applicable. Normal ranges of individual measures are CRP: 0-3 (mg/l), ESR: 0-15 (mm/hour), FIB: 1.8-4 (g/l), and WBC: 4-10 (10^9/L).

Prepared by centrifuging samples with Ficoll-Paque Plus (Amersham Biosciences, Little Chalfont, UK). The number of white blood cells (WBCs), the erythrocyte sedimentation rate (ESR), and the serum C-reactive protein (CRP) concentration for each sample were measured with an analyzer (Siemens Healthcare Diagnostics Products, GmbH, Germany). The fibrinogen (FIB) level was measured by the von Clauss method (Sysmex CA1500, Japan).

**PBMC stimulation**

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation with Ficoll-Paque Plus (Amersham Biosciences). PBMCs (4 x 10^6 cells/ml) were cultured in RPMI-1640 media with 10% fetal calf serum (FCS) (Hyclone, Waltham, MA, USA) in 24-well U-bottom tissue culture plates (Costar, Corning Corp., NY, USA). Cells were stimulated with or without 50 ng/mL of phorbol myristate acetate (PMA) and 2 g/mL of ionomycin (Sigma, St. Louis, MO, USA) for 1 h. Cells were cultured with Brefeldin A (10 g/ml, GolgiStop, BD Sciences, San Jose, CA, USA) for 5 h and subjected to intraplasmic staining and flow cytometric analyses.

**Flow cytometry**

Human PBMCs (5 x 10^6 cells/tube) were stained with Alexa Fluor 647 anti-CXCR5, PE/CY7 anti-CD4, PE anti-CD278, and FITC anti-CD279 (BD Pharmingen, San Diego, CA, USA) at room temperature (RT) in the dark for 30 min. Control staining was done with FITC anti-IgG1, PE anti-IgG1, PE/CY7 anti-IgG1, and Alexa Fluor 647 anti-IgG1 (BD PharMingen). Cell gating was set to isolate CD4+ cells. The number of CXCR5+CD4+ cells per sample was analyzed with FlowJo software (7.6.2) [22].

Stimulated PBMCs were harvested and stained simultaneously with Alexa Fluor 647 anti-CXCR5 and PE/CY7 anti-CD4 at RT in the dark for 30 min. Subsequently, cells were fixed, permeabilized, and stained with PE anti-IL-21 (BD Pharmingen). Frequencies of IL-21+ T FH cells were determined by flow cytometric analysis. Identical lots of antibodies and protocols were used for all samples, and the samples were analyzed blindly.

**Measurement of serum IL-21 by ELISA**

Serum IL-21 concentrations were measured with a human IL-21 ELISA kit following the manufacturer's instructions (Roche Diagnostics, Lewes, UK). Briefly, sera were diluted 1:4, ELISAs were performed, and concentrations were calculated with the standard curve generated with the provided recombinant IL-21. The detection limit of the IL-21 ELISA kit was 0.5 ng/L.

**Statistical analysis**

Data were expressed as ranges, median values, or individual values. The difference between two groups was analyzed by the Mann-Whitney U nonparametric test. Potential correlations between variables were evaluated by using Spearman's rank correlation test in SPSS 19.0 for Windows (SPSS, Chicago, IL, USA). A two-tailed P value of < 0.05 was considered statistically significant.
Results

Patient characteristics

A total of 28 NONFH patients and 15 control subjects were recruited for this study. The ratio of males to females was significantly higher in the alcohol abuse group than in the other groups, presumably as a result of social conventions. When compared with control subjects, all of the groups of NONFH patients had
TFH cell and IL-21 levels in patients with NONFH

Increased frequencies of different T_{FH} cell subsets and serum IL-21 levels in NONFH patients

Flow cytometry did not reveal any significant differences in the frequencies of peripheral blood CD4^+ T cells between NONFH patients and control subjects (data not shown). However, the percentage of CXCR5^+CD4^+ T_{FH} cells in NONFH patients was higher than that in control subjects (P<0.001, Figure 1). Increased frequencies of ICOS^+ , PD-1^+ , and IL-21^+ T_{FH} cells in the peripheral blood and higher serum IL-21 levels were found in NONFH patients compared to those in the control subjects (P=0.023, < 0.001 and < 0.001, respectively; Figure 1). Thus, higher frequencies of T_{FH} cells, multiple subsets of T_{FH} cells, and increased serum IL-21 levels were associated with the development of NONFH in this population.

Increased frequencies of different T_{FH} cell subsets and serum IL-21 levels in NONFH patients with steroid use and alcohol abuse as risk factors

When we compared the frequencies of peripheral blood CD4^+ T cells between the steroid use group and the control group, we found no significant difference (data not shown). However, the percentage of CXCR5^+CD4^+ T_{FH} cells in the steroid use group was higher than that in the control group (P=0.003, Figure 2A and 2B). The percentages of PD-1^+ , ICOS^+ , and IL-21^+ T_{FH} cells in the peripheral blood and serum IL-21 levels were higher in the steroid use group than those in the control group (P=0.005, 0.02, < 0.001 and < 0.001, respectively; Figure 3A-C). Thus, higher frequencies of T_{FH} cells, multiple subsets of T_{FH} cells, and increased serum IL-21 levels were associated with the development of steroid-induced NONFH.

Similarly, there was no significant difference between the frequencies of peripheral blood CD4^+ T cells between the alcohol abuse and control groups (data not shown). The percentages of T_{FH} cells, PD-1^+ T_{FH} cells, and IL-21^+ T_{FH} cells, and the level of serum IL-21 were higher in the alcohol abuse group than in the control group (P<0.001, 0.006, < 0.001 and < 0.001, respectively; Figures 2A, 2B, 3A-C). However, the ICOS^+ T_{FH} cell frequencies were lower in the alcohol abuse group than in the steroid use group (P=0.023; Figure 3A and 3B). Thus, the increased T_{FH} cell frequencies and serum IL-21 concentrations may have contributed to the alcohol-induced NONFH pathogenesis.

Only the frequencies of IL-21^+ T_{FH} cells and serum IL-21 levels were higher in NONFH patients with no risk factors

In contrast to the steroid use and alcohol abuse groups, only the IL-21^+ T_{FH} cells and serum IL-21
Figure 3. Frequencies of subsets of $T_{FH}$ cells in all three groups of NONFH patients analyzed by flow cytometry. PBMCs isolated from participants were stained with anti-CD4, anti-CXCR5, anti-ICOS, anti-PD-1, and isotype controls. Flow cytometry gating was set for CXCR5+CD4+ $T_{FH}$ cells, and frequencies of ICOS+ and PD-1+ $T_{FH}$ cells were measured. Some PBMCs were stimulated with PMA and ionomycin in the presence of BFA, stained with anti-CD4 and anti-CXCR5, fixed, permeabilized, and further intraplasminally stained with anti-IL-21 or an isotype control. The frequency of IL-21+ $T_{FH}$ cells was characterized by flow cytometry analysis. In addition, serum IL-21 concentrations were measured by ELISA. A. Flow cytometry analysis. B. Quantitative analysis. C. Serum IL-21 levels. Data are presented in chart form, and the horizontal lines indicate the median values. Group 1: Steroid-related NONFH (n=10); 2: alcohol abuse NONFH (n=10); 3: The NONFH without known risk factors (n=8); HC: healthy control group (N=15).
levels were higher in the NONFH without known risk factors group compared to the control group (P=0.002 and < 0.001, respectively; Figure 3A-C). We found no significant difference between the risk factors group and the steroid use group or the alcohol abuse group (data not shown). Thus, the elevated frequency of peripheral blood IL-21+ T\textsubscript{FH} cells and elevated serum IL-21 level may contribute to NONFH pathogenesis when there are no apparent risk factors.

**Correlations between subsets of T\textsubscript{FH} cells, serum IL-21 levels, and clinical indices**

To further test the relevance of peripheral blood T\textsubscript{FH} cells in NONFH patients, we analyzed the associations between the subsets of T\textsubscript{FH} cells and serum IL-21 levels with clinical measurements for each group of patients using Spearman’s correlation test. Serum IL-21 levels were positively correlated with femoral head collapse in the steroid use and alcohol abuse groups (R=0.673, P=0.039 and R=0.760, P=0.015, respectively, Figure 4A and 4B), but not in the NONFH without known risk factors group (data not shown). Serum IL-21 levels were positively correlated with ESR in the alcohol abuse group (R=0.762, P=0.0013; Figure 4C). When correlations with T\textsubscript{FH} cell subsets were performed for all groups, the P-values revealed no correlations (data not shown).

**Discussion**

We found that the frequency of peripheral blood CD4\textsuperscript{+}CXCR5\textsuperscript{+} T\textsubscript{FH} cells in NONFH patients was significantly higher than in control subjects. T\textsubscript{FH} cells are critical regulators of B cell development, GC formation, and antibody production [23, 24]. Previous studies have shown that an imbalanced immune system is responsible for NONFH pathogenesis [5-9]. Higher levels of anti-cardiolipin antibodies, B cell proliferation, and antibody secretion have been related to NONFH in humans and animals. The higher frequency of T\textsubscript{FH} cells suggests that these cells may contribute to disease pathogenesis [5].

To understand the role(s) of T\textsubscript{FH} cell in NONFH development and progression, we characterized the frequencies of ICOS\textsuperscript{+}, PD-1\textsuperscript{+}, and IL-21\textsuperscript{+} T\textsubscript{FH} cells and serum IL-21 levels among groups with differing risk factors. IL-21 is a crucial regulator of immunity [25]. In an epidemiologic survey of ONFH patients, 51% of patients reported systemic steroid use [26]. Long-term steroid use is an important risk factor for NONFH development. In the steroid use group, the percentages of peripheral blood T\textsubscript{FH} cells and of PD-1\textsuperscript{+}, ICOS\textsuperscript{+}, and IL-21\textsuperscript{+} T\textsubscript{FH} cells and the level of serum IL-21 were all significantly higher than those in the control group. The finding of increased frequencies of both PD-1\textsuperscript{+} and ICOS\textsuperscript{+} T\textsubscript{FH} cells seems to be paradoxical because these molecules have opposite regulatory effects on T\textsubscript{FH} cells. Due to the participation of T\textsubscript{FH} cells in NONFH, the increased frequency of PD-1\textsuperscript{+} T\textsubscript{FH} cells might stem from compensative spontaneous regulation of the immune system. In this context, the increased frequency of PD-1\textsuperscript{+} T\textsubscript{FH} cells would negatively regulate inflammatory responses in NONFH patients. In addition, Hu found increased serum TNF-α levels in rabbits with glucocorticoid-induced femoral head necrosis [27]. Rasmussen argued that IL-21
play important roles in joint inflammation and osteonecrosis [17]. Similarly, in the present study, we found that the percentage of IL-21+ T_{FH} cells in the peripheral blood and the serum IL-21 levels were elevated in NONFH patients. Thus, our data provide further evidence that pro-inflammatory cytokines, such as IL-21, contribute to steroid-induced ONFH pathogenesis.

T_{FH} cells and serum IL-21 levels mediate the development and progression of RA [16, 17]. IL-21 promoted bone destruction via osteoclastogenesis in RA patients and in mouse models of arthritis. Thus, IL-21 may be a novel therapeutic target for treating bone destruction in RA patients [28]. It follows that steroid-mediated inhibition of bone regeneration contributes to NONFH pathogenesis. Steroids also enhance bone resorption by regulating osteoclast activity. As osteonecrosis progresses, femoral heads will ultimately collapse [29, 30]. Interestingly, we found that serum IL-21 levels positively correlated with the index of femoral head collapse, but the correlation was weak and borderline significant. This result could be because femoral head collapse is influenced by many factors, including weight and the degree of physical activity.

After steroid use, alcohol abuse is the second most important risk factor for NONFH development. The percentages of peripheral blood T_{FH} cells and PD-1+ and IL-21+ T_{FH} cells, and the level of serum IL-21 were significantly higher in the alcohol abuse group than in the control group, whereas the frequency of ICOS+ T_{FH} cells was significantly lower in the alcohol abuse group than in the steroid group. PD-1+ T_{FH} cells may regulate autoimmunity similarly in the alcohol abuse and steroid groups. Indeed, an increased frequency of PD-1+ T_{FH} cells has been detected in RA patients [31].

The reduced frequency of ICOS+ T_{FH} cells in the alcohol abuse group compared to the steroid group suggests that steroids activate T_{FH} cells to a greater extent than alcohol, or that alcohol indirectly activates T_{FH} cells. Alcohol has been shown to induce the adrenal cortex to secrete large amounts of glucocorticoid through the hypothalamic-pituitary-adrenal axis (HPA) [32, 33], which is known to regulate the immune system. In a study on alcohol-induced osteonecrosis, Okazaki suggested that alcohol abuse activates a pro-inflammatory LPS-mediated signaling pathway initiating mechanisms that destroy the femoral head [7]. It is well-known that LPS activates B cells through TLR4 [34]. Here, we found that the alcohol abuse group showed positive correlations between the serum IL-21 levels and the ESR or the index of femoral head collapse. Thus, IL-21+ T_{FH} cells and IL-21 may be involved in alcohol-induced NONFH pathogenesis.

The pathologies of steroid-induced and alcohol-induced osteonecrosis of the femoral head are similar [35]. In the present study, we found similar changes in the frequencies of the subsets of T_{FH} cells in the steroid and alcohol groups. As with steroid-induced ONFH, alcohol may also contribute to ONFH pathogenesis by activating IL-21+ T_{FH} cells to induce destructive bone resorption.

For the NONFH without known risk factors group, only the frequency of IL-21+ T_{FH} cells and the concentration of serum IL-21 were significantly elevated when compared to the control group. However, these changes were not correlated with femoral head collapse. In unknown risk factors group, ONFH may be the result of a complex etiology of multiple unknown factors, unlike the steroid use and alcohol abuse groups that had relatively few risk factors each.

In conclusion, our findings suggest that the increased percentages of peripheral blood IL-21+ T_{FH} cells and serum IL-21 levels may play important roles in the development and progression of NONFH. In addition, an increased concentration of serum IL-21 may be a potential indicator for femoral head collapse, particularly among patients with late-stage ONFH (FICAT: III-IV) who use steroids or abuse alcohol. On the other hand, corticosteroids were recently shown to inhibit T_{FH} cell expression in SLE patients [36]. These seemingly opposing results may be due to the different diseases or the different dosages or durations of steroid treatment. Importantly, the patients in our research did not receive steroids in the month before their blood samples were collected.

This study is the first to demonstrate that IL-21+ T_{FH} cells and serum IL-21 levels are associated with the development and progression of NONFH. These findings may lay a foundation for further studies on the mechanisms in ONFH.
The limitations of this study included a small sample size and no analysis of $T_{FH}$ cell function. Further studies of $T_{FH}$ cell and other immunocompetent cells in the pathogenic process with a larger population are warranted.

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Disclosure of conflict of interest

None.

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