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Original Article

Dysregulated lymphocyte subsets and increased inflammatory cytokines in the hip joint of patients with non-traumatic osteonecrosis of the femoral head

Cheng-Zhi Ha[1], Zhen-Feng Yuan[1], Jie Ding[2], Qi Xu[1], Hong-Peng Yang[1], Wei Li[1], Bao-Jie Li[3], Da-Wei Wang[1]

[1]Department of Joint and Sports Medicine, The Affiliated Liaocheng People's Hospital of Shandong First Medical University, Liaocheng 252000, Shandong, China; [2]The Central Laboratory, The Affiliated Liaocheng People's Hospital of Shandong First Medical University, Liaocheng 252000, Shandong, China; [3]Bio-X Center, Shanghai Jiao Tong University, Shanghai 200000, China

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Abstract: Objective: This study aimed to explore the bone immune microenvironment in patients with non-traumatic osteonecrosis of the femoral head (NT-ONFH) by detecting the changes in the lymphocyte subtypes and inflammatory cytokines in the hip joint. Methods: Patients with NT-ONFH and control individuals with fresh femoral neck fractures, admitted to our hospital from January 2018-December 2019, were recruited. Local bone tissue, hip joint fluid, and hip joint synovial tissue samples were collected. Pathology analysis by hematoxylin and eosin (H&E) staining, lymphocyte subtyping through flow cytometry, inflammatory cytokine measurement by ELISA, and assessment of immune complexes (ICs) by immunofluorescence staining were performed. Results: Among the 98 patients with NT-ONF; 21, 34, and 43 presented with hormonal, alcoholic, and idiopathic ONFH, respectively. The number of stage II, III, and IV patients were 15, 30, and 53, respectively. H&E staining revealed collapsed bone trabecula, empty bone lacuna and bone cell necrosis in the NT-ONFH group. Regularly arranged trabecular bones without necrosis or fibrous hyperplasia were observed in the control group. In NT-ONFH bone tissue, the ratios of $CD3^+$ and $CD3^+CD8^+$ in lymphocytes were significantly higher (both $P<0.001$). The ratio of $CD19^+$ cells in lymphocytes and the ratio of $CD3^+CD4^+/CD3^+CD8^+$ was markedly lower (both $P<0.001$). $CD19^+$ lymphocytes percentage in idiopathic NT-ONFH was significantly higher than that in alcoholic and hormonal NT-ONFH ($P<0.001$). Patients with stage II and III NT-ONFH had a significantly higher percentage of $CD3^+CD8^+$ cells than those with stage IV NT-ONFH (P=0.002). Patients with NT-ONFH had significantly higher levels of IL-2, IL-6, IL-10, and TGF-β1 in the hip joint fluid than the control subjects (P=0.001, 0.012, 0.017, and 0.026, respectively). ICs were observed on the synovium of patients with NT-ONFH, but not controls. Conclusion: The dysregulated $CD3^+CD8^+$ and $CD19^+$ lymphocyte subsets in bone tissue of the femoral head and increased inflammatory cytokines such as IL-2, IL-6, IL-10, and TGF-β1 in the hip joint may play an important role in NT-ONFH pathogenesis.

Keywords: Autoimmune, osteonecrosis, femoral head, inflammation, cytokines

Introduction

Osteonecrosis of the femoral head (ONFH), also known as avascular/aseptic necrosis of the femoral head, can be divided into two major categories: traumatic and nontraumatic. The etiological factors for traumatic ONFH includes a femoral head and neck fracture, acetabular fracture, hip dislocation, and severe hip sprain or contusion. Nontraumatic ONFH (NT-ONFH) is reportedly associated with glucocorticoid use, alcohol consumption, lipid metabolism disorders, abnormal coagulation, sickle cell anemia, and autoimmune diseases [1-3]. While the causes of some NT-ONFH cases may be clear, such as alcoholic and hormonal ONFH, a considerable number of patients have idiopathic ONFH for which the etiologies cannot be identified [4]. Idiopathic ONFH accounts for approximately 30% of all diagnosed cases in China [5, 6].

Many theories regarding the pathogenesis of NT-ONFH have been proposed over the past 20 years. Intraosseous hypertension, intravascular fat or gaseous emboli, and extravascular

compression through increased marrow fat stores are accepted theories [7]. Although NT-ONFH is closely related to the disrupted blood supply to the femoral head, it has not been confirmed whether this is the cause or just a result of ONFH. Moreover, there are currently no theories that fully and independently explain all the pathological phenomena of NT-ONFH.

In 2000, Arron and Choi, through summarizing the literature, proposed the concept of osteoimmunology to illustrate the relationship between the skeletal system and immune system, thus suggesting that immune disorders lead to abnormal bone metabolism [8]. This concept is demonstrated in autoimmune diseases, including rheumatoid arthritis [9], systemic lupus erythematosus [10], and ankylosing spondylitis [11]. A recent study suggested that immune cells, such as cluster of differentiation $CD3^+$ and $CD3^+CD8^+$ T cells, occurred in significantly higher numbers in patients with non-traumatic ONFH than in healthy controls [12]. Another study found that patients with sickle cell disease with osteonecrosis had increased circulating $CD4^+$ T cells and that an increased proportion of these cells produce a broad spectrum of pro-inflammatory cytokines to maintain the inflammatory state [13]. Some experts have found that T cells secrete cytokines to regulate bone remodeling in an autocrine or paracrine manner [14]. For example, T cells can increase the receptor activator of nuclear factor kappa-B ligand (RANKL)/osteoprotegerin ratio and play an important role in osteoclast formation, which may facilitate bone resorption [15]. B cells also produce RANKL and secrete OPG, and studies have shown that mice lacking bone marrow B cells often develop osteoporosis because they lack the OPG secreted by B cells [16]. These studies have shown that lymphocyte subsets-mediated immunity may be involved in the pathogenesis of ONFH. Most previous studies on bone immunity focused on the connection between the entire skeletal system and immune system; however, the relationship between the local bone tissue microenvironment and the immune system is poorly understood. We hypothesize that a disturbance in the local immune microenvironment of the femoral head is involved in the pathogenesis of NT-ONFH. To test this hypothesis, we examined changes in immune cells and inflammatory cytokines in the local bone tissue, synovial membrane, and synovial fluid of patients with ONFH, and compared the results with those of the control patients.

Materials and methods

Study subjects

In this retrospective study, patients with NT-ONFH (experimental group) and patients with fresh femoral neck fracture (control group) who were admitted to Liaocheng People's Hospital from January 2018 to December 2019 were enrolled. All NT-ONFH patients who were recruited in this study 1) met the ONFH diagnostic criteria in "Expert Consensus on Diagnosis and Treatment Criteria for Adult Femoral Head Necrosis [17]" without any previous hip surgery history, and 2) had either ARCO stage I and II requiring femoral head decompression or ARCO stage III and IV requiring total hip arthroplasty. All control patients were diagnosed with fresh femoral neck fracture via X-ray, CT, and other imaging methods without any previous hip surgery history, and required total hip arthroplasty or semi-hip arthroplasty. Patients who had serious complications in the heart, brain, lungs, liver or kidneys, or took immunosuppressive or anticoagulant drugs within the last 6 months were excluded. The study was approved by the Medical Ethics Committee of Liaocheng People's Hospital. All study participants provided written informed consent.

Experimental reagents

Immunofluorescence staining kits and ELISA kits for IL-2 (F10100-B), IL-6 (F7042-B), IL-10 (F0065-B), IL-1β (F2040-B), IL-17 (F9825-A), IL-4 (F0051-B), TGF-β (F1767-B), TNF-α (F0121-A) and IFN-γ (F30672-A) were purchased from Wuhan Bode Bioengineering Co. LTD (Wuhan, China). Fluorescence-labeled antibodies, CD3-FITC, CD4-APC, CD8-PE, CD16-PE, CD19-APC, CD45-PerCP and CD56-PE (Catalog: 662965) were obtained from BD Biosciences China (Shanghai, China). Fluorescence-labeled anti-IgA (ZA-0446), anti-IgG (ZF-0306), anti-IgM (ZF-0307), anti-C1q (ZF-0303) and anti-C3 (ZF-0301) antibodies were provided by the Beijing ZSGB Biotechnology Co. LTD. (Beijing, China). Phosphate buffered saline (PBS) was obtained from Sigma-Aldrich China (Beijing, China) and hematoxylin & eosin (H&E) staining

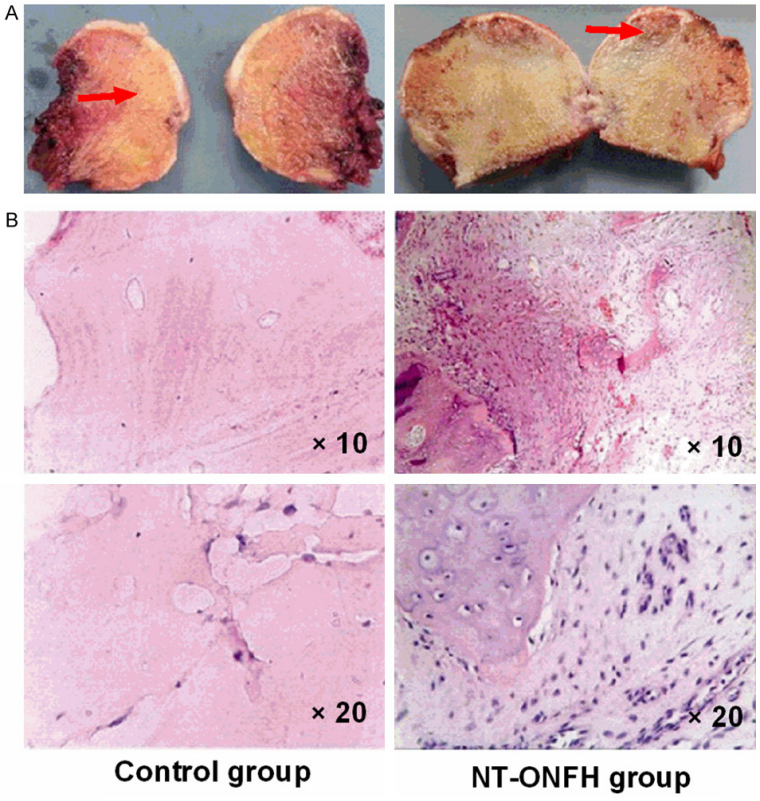

Figure 1. Bone tissue sample and H&E staining from the femoral head. A. Bone tissue sample of control group and NT-ONFH group (represents the sampling area). B. Pathological changes in the bone tissue sample were assessed by H&E staining (×10; ×20).

reagents was from the Phygene Life Sciences Company (Beijing, China).

Parameters assessed

Pathological changes in the lesion were assessed by H&E staining. Lymphocyte subsets were assayed by flow cytometry. Cytokines in hip fluid were measured by ELISA. Deposition of immune complexes (ICs) on the synovial membrane surface was analyzed by immunofluorescence staining.

H&E staining

The femoral head was divided into two parts with a pendulum saw. Bone tissue from the necrotic area in the non-traumatic ONFH group and bone tissue from control patients (**Figure 1A**) were analyzed by H&E staining. The bone tissue was placed in EDTA solution for decalcification for 1-2 weeks until it became completely soft. Subsequently, the bone tissue was embedded in paraffin and sectioned. The slices were drip-stained with hematoxylin solution for 30-60 s and then washed with tap water for several seconds. They were then treated with 1% hydrochloric acid differentiation solution and returned to blue with 0.3% ammonia solution. After completing the staining procedure, the slices were washed with tap water for several seconds. For eosin staining, the slices were incubated in eosin dye for 30 s and then rinsed with tap water. Finally, the dyed slices were dehydrated and sealed.

Determination of lymphocyte subsets by flow cytometry

The experimental group samples from the bone tissues (200 mg) of the necrotic part of femoral head of non-traumatic ONFH. The control group samples from the bone tissues (200 mg) of the normal bone tissue in the femoral head of the fresh femoral neck fracture patients. The bone tissue fragments were chewed into pieces as small as possible with a bone biter. Samples were filtered through 300 μM mesh filters to prepare single cell suspensions that were then washed thoroughly with PBS. Subsequently, cells were incubated with fluorescence-labeled antibodies against CD3, CD4, CD8, CD16, CD19 or CD56 alone or in combination. Stained cells were analyzed using a BD FACSCanto Flow Cytometer (BD Biosciences China). The percentage or the ratio of lymphocyte subsets were calculated.

Cytokine measurement by ELISA

Sample collection: The joint fluid was obtained during hip arthroplasty. After the posterior joint capsule was exposed, a 5 mL syringe was used to take the joint fluid. If the joint fluid could not be extracted, 10 mL of normal saline was injected followed by the movement of the hip joint, and the drainage was collected. The sample was stored at a -80°C freezer until further analysis.

Table 1. Comparison of baseline data between the NT-ONFH and control group

	Sex Ratio (male/female)	Age (years)	BMI (kg/m^2)	Surgical side (left/right/both)
NT-ONFH group	59/39	54.60±5.91	26.46±4.08	47/41/10
Control group	28/21	67.20±4.66	26.44±4.42	27/22/0
(t/χ^2)	0.13	-13.02	0.032	5.40
P	0.726	0.000*	0.975	0.067

*P<0.05, NT-ONFH group VS control group, the difference was statistically significant.

ELISA: All ELISA assays were performed at the Central Laboratory of our hospital. The concentrations of IL-2, IL-6, IL-10, IL-1β, IL-17, IL-4, TGF-β, TNF-α and IFN-γ in the hip fluid were measured by a commercially available ELISA kit (Wuhan Bode Bioengineering Co. LTD, China), following the manufacturer's instructions in the same way.

Immunofluorescence staining analysis of ICs

IC deposition on the synovial surface was evaluated with immunofluorescence staining. Synovial tissues were collected during surgery, embedded with OCT and immediately frozen in liquid nitrogen. Cryosections of 5 μM thickness were prepared in a cryostat. The section was rinsed with PBS for 3×5 min followed by blocking with 10% normal goat serum at 37°C for 30 min. After one wash with PBS, the slide was incubated with fluorescence labeled antibodies (1:40 dilution for anti-IgA, -IgG, -IgM and -C3 antibodies, and 1:150 for anti-C1q antibodies) at 37°C for 1 h in the dark. Finally, the sample was washed with PBS for 3×3 min, and anti-fluorescence quenching mounting medium was added. The fluorescence staining was observed under a fluorescence microscope and analyzed with photographs taken.

Statistical methods

The SPSS 24.0 software (IBM, Armonk, NY, USA) was used for statistical analysis of all data. Parametric continuous variables are expressed as the means ± SD. An independent sample t-test or non-parametric test (Mann-Whitney U test) were used for statistical analysis of continuous variables in the two groups. Multiple groups of continuous variable data were compared using ANOVA or a non-parametric test (Kruskal-Wallis rank sum test). The chi-square test or Spearman correlation analysis were used for the classification of variables, such as the composition ratio. P<0.05 was considered statistically significant.

Results

Comparison of baseline data of patients

There were no significant differences in the sex ratio, BMI, and surgical parameters between the ONFH and control groups (P>0.05). The average age was 52.63±8.51 years in the ONFH group and 62.41±12.22 years in the control group, and the difference was statistically significant (P<0.05). The control group represented a majority in the age group >60 years (**Table 1**).

Among the 98 patients in the ONFH group, there were 21 cases of hormonal ONFH, 34 of alcoholic ONFH, and 43 of idiopathic ONFH. The stage II, III and IV patients were 15, 30 and 53, respectively. The control group was 49 patients with fresh femoral neck fracture. The femoral neck fractures were classified by Garden classification. Of all femoral neck fracture patients, 8.16% (4) were Type I fracture, 26.53% (13) Type II fracture, 48.98% (24) Type III fracture, and 16.33% (8) Type IV fracture.

Pathological changes in local bone tissue in the femoral head

Bone tissue H&E staining revealed reduced density in cartilage layers, collapsed trabecular bone, defective or empty lacunae and necrotic or ruptured osteocytes with nuclear condensation in the experimental group. Hyperemia with significant edema in the surrounding bone tissue and bone marrow necrosis were also observed in ONFH. In contrast, dense cartilage, regularly arranged trabecular bones, and normal bone structure were seen in the control patients (**Figure 1B**).

Table 2. Comparison of lymphocyte subsets in the bone tissue in the two groups

	NT-ONFH (n=98)	Control (n=49)	*P* value
$CD3^+$ (%)	79.26±2.06	70.45±3.70	0.001
$CD3^+CD4^+$ (%)	32.35±3.99	33.27±3.96	0.187
$CD3^+CD8^+$ (%)	42.50±3.73	30.23±3.47	<0.0001
$CD16^+CD56^+$ (%)	9.91±3.24	10.65±3.46	0.209
$CD19^+$ (%)	9.08±1.18	17.97±3.39	<0.0001
$CD3^+CD4^+/CD3^+CD8^+$	0.75±0.13	1.16±0.12	<0.0001

Table 3. Lymphocyte subsets in NT-ONFH patients with different etiologies

	Alcoholic (n=21)	Hormonal (n=34)	Idiopathic (n=43)	*P* value
$CD3^+$ (%)	79.66±2.38	80.73±2.42	79.64±2.33	0.106
$CD3^+CD4^+$ (%)	31.85±3.26	32.12±2.99	31.74±3.12	0.866
$CD3^+CD8^+$ (%)	42.15±2.55	43.26±2.75	42.63±2.02	0.233
$CD16^+CD56^+$ (%)	9.92±1.89	10.65±2.55	9.67±2.85	0.130
$CD19^+$ (%)	8.66±1.70	7.91±1.49	10.21±2.08	<0.0001*
$CD3^+CD4^+/CD3^+CD8^+$	0.76±0.09	0.74±0.14	0.75±0.13	0.743

*Idiopathic ONFH compared with Alcoholic and Hormonal ONFH. The statistical method is one-way ANOVA and the post hoc tests was LSD-t.

Table 4. Lymphocyte subsets in patients with different stages of NT-ONFH

	Stage II (n=15)	Stage III (n=30)	Stage IV (n=53)	*P* value
$CD3^+$ (%)	80.70±3.18	79.29±3.04	78.90±3.54	0.191
$CD3^+CD4^+$ (%)	32.64±3.26	31.63±3.43	32.84±3.37	0.291
$CD3^+CD8^+$ (%)	44.39±3.08	42.88±3.18	41.21±3.49	0.002*
$CD16^+CD56^+$ (%)	9.06±1.41	9.94±1.71	10.15±1.56	0.068
$CD19^+$ (%)	8.87±2.03	9.44±1.30	9.71±1.57	0.189
$CD3^+CD4^+/CD3^+CD8^+$	0.73±0.21	0.74±0.14	0.78±0.21	0.325

*The stage IV patients compared with the stage II and III patients. The statistical method is one-way ANOVA and the post hoc test was LSD-t.

Changes in lymphocyte subsets in bone tissue

The percentages of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD16^+CD56^+$, and $CD19^+$ lymphocytes in the ONFH group were 79.26%, 32.35%, 42.50%, 9.91%, and 9.08%, respectively. The percentages of $CD3^+$ and $CD3^+CD8^+$ cells were significantly higher while the percentage of $CD19^+$ cells was significantly lower in the ONFH group than that in the control group. The percentages of $CD3^+CD4^+$ and $CD16^+CD56^+$ cells showed no significant difference between the two groups. The $CD3^+CD4^+/CD3^+CD8^+$ ratio in the ONFH group was 0.75±0.13, which was significantly lower than that in the control group (*P*<0.05; **Table 2**).

Lymphocyte subsets in ONFH patients with different etiologies

There was no significant difference in the percentages of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD16^+CD56^+$ cells among alcoholic, hormonal and idiopathic ONFH samples. The ratio of $CD3^+CD4^+/CD3^+CD8^+$ in the alcoholic, hormonal and idiopathic ONFH patients was not significantly different. The percentage of $CD19^+$ cells in idiopathic ONFH bone tissue was significantly higher than that in alcoholic and hormonal ONFH (*P*<0.05), while there was no significant difference in the percentage of $CD19^+$ cells between alcoholic and hormonal ONFH patients (*P*=0.543; **Table 3**).

Lymphocyte subsets in patients with different stages of ONFH

There was no substantial difference in the percentage of $CD3^+$, $CD3^+CD4^+$, $CD16^+CD56^+$ and $CD19^+$ lymphocytes among patients with different stages of ONFH. The percentage of $CD3^+CD8^+$ cells in stage IV patients was significantly lower than that in stage II and III patients (*P*<0.05). There was no significant difference in the $CD3^+CD4^+/CD3^+CD8^+$ ratio among patients with different levels of ONFH necrosis (**Table 4**).

Expression of inflammatory cytokines in hip joint synovial fluid

The levels of IL-2, IL-6, IL-10 and TGF-β1 in the articular fluid of patients with NT-ONFH were significantly higher than those in the control group (*P*<0.05). The levels of TNF-α, IFN-γ, IL-1β, IL-17 and IL-4 in the articular fluid were not

Table 5. The expression differences of related cytokines in hip fluid

	NT-ONFH group (pg/mL)	Control group (pg/mL)	*P* value
IL-6	5.74±8.15	0.56±0.15	0.001*
IL-2	1.63±0.51	0.61±0.04	0.012*
IL-10	3.52±0.17	1.28±0.21	0.017*
TGF-β1	1.95±0.15	0.89±0.28	0.026*
TNF-α	1.52±0.18	1.54±0.20	0.624
IFN-γ	1.89±0.12	2.52±0.11	0.897
IL-1β	1.52±0.12	1.51±0.10	0.356
IL-17	1.53±0.18	1.21±0.20	0.181
IL-4	0.62±0.03	0.67±0.02	0.521

*$P<0.05$, NT-ONFH group VS Control group, the difference was statistically significant. The statistical method is an independent sample t-test.

markedly different between the two groups ($P>0.05$) (**Table 5**).

IC deposition on the synovium of the hip joint

ICs (IgA, IgG, IgM, C1q, and C3) on the synovium surface in the hip joint were observed in patients with ONFH but not in controls (**Figure 2**).

Discussion

Inflammation is an evolutionarily conserved physiological response involving immune and non-immune cells to eliminate exogenous or endogenous inflammatory stimuli [18]. When inflammation becomes chronic, the adaptive immune response is activated [19]. Immune mechanisms play further roles in the resolution of inflammation and in the healing process, including tissue repair and regeneration. Thus, innate immunity and acquired immunity must be coordinated to return the injured tissue to homeostasis [20]. Knowledge of how immune mechanisms and inflammatory responses are regulated is critical for understanding the pathogenesis of complex diseases, such as non-traumatic ONFH.

Previous studies have shown that ONFH is an inflammatory disease [21]. The inflammatory cascade is initiated by the adjacent viable tissues, which leads to fibrous vasculature growth in the regions of cell death [22]. Examination of the bone marrow reveals necrosis of hematopoietic cells, endothelial cells and lipocytes. Osteocytes undergo atrophy and eventually die, leading to increased empty lacunae with time. The release of lysosomes acidifies the surrounding tissue, as dying lipocytes release free fatty acids which saponify with extracellular calcium to form insoluble soaps [23]. This is reflected in the results in our H&E staining of idiopathic ONFH bone tissue. The inflammatory process is a complex cascade of events mediated by multiple inflammatory factors [24]. In this study, we discovered the elevation of IL-2, IL-6, IL-10, and TGF-β1 in hip fluid of NT-ONFH patients, indicating that local inflammatory reactions may participate in the pathogenesis of NT-ONFH. Among these upregulated inflammatory factors, IL-6, in particular, has been known to play a pivotal role in synovitis, bone erosion, immune regulation, hematopoiesis and inflammation [25, 26]. IL-6 induces the generation of Th17 cells from naive T cells together with TGF-β, and inhibits TGF-β-induced Treg (iTreg) differentiation [27]. It has been shown that IL-6 is a major regulator for the differentiation of osteoclast progenitor cells into mature osteoclasts. IL-6 also directly stimulates both RANKL and OPG mRNA production in bone, and enhances prostaglandin production [28]. IL-6 inhibits osteoblast differentiation through activation of the JAK/STAT3, SHP/MEK2, and SHP/AKT pathways [29]. Thus, IL-6 is a key bridging molecule between inflammatory responses and immune regulation.

Bone remodeling occurs asynchronously throughout the skeleton at many anatomically distinct sites named basic multicellular units. In these units, tiny packets of bone are removed by osteoclasts and, subsequently, replaced by a new bone matrix produced by osteoblasts, with subsequent mineralization of the matrix to form strong new bone material [30, 31]. Chronic infections and disorders of the immune system involving an inappropriate immune response are commonly associated with local or systemic bone loss. The lymphocytic immunoregulatory and anti-inflammatory actions may contribute to bone remodeling [32]. In this study, it was found that the percentage of $CD3^+$ and $CD3^+CD8^+$ lymphocytes increased in the NT-ONFH group, while the percentage of $CD19^+$ lymphocytes decreased, and the ratio of $CD3^+CD4^+/CD3^+CD8^+$ decreased. There was no significant difference in the percentage of $CD3^+CD4^+$ and $CD16^+CD56^+$ lymphocytes, sug-

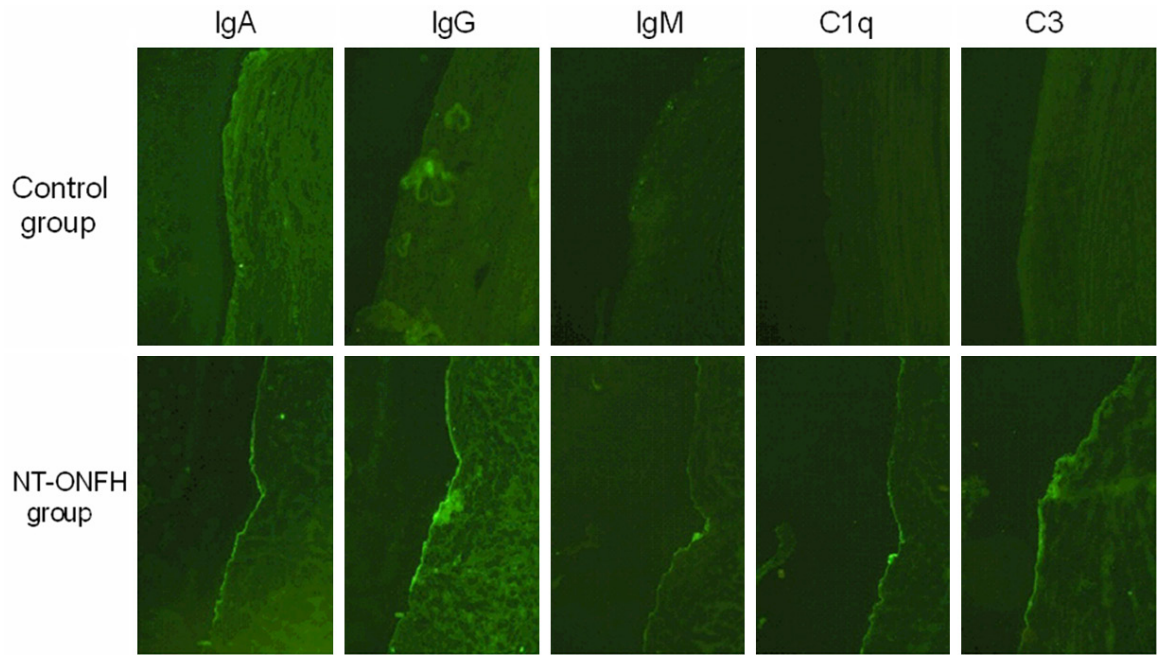

Figure 2. Immunofluorescence staining results of hip joint synovium from the control group and NT-ONFH group.

gesting an immune imbalance in local necrotic bone tissues in NT-ONFH patients. In addition, NT-ONFH with different etiologies showed similar changes in T lymphocyte subtypes, which further suggests that dysregulated local immune environment in the femoral head may be one of the common mechanisms underlying NT-ONFH pathogenesis. Some studies have shown that osteoblast stimulation and osteoclast inhibition by T cells are closely related to T cell subsets [33]. $CD4^+$ T cells promote bone formation, $CD8^+$ T cells promote bone resorption, and $CD4^+$ T cells are more beneficial to bone remodeling than $CD8^+$ cells [34]. This is consistent with our findings that the percentage of $CD8^+$ T cells increased in NT-ONFH bone tissue, while there was no significant change in $CD4^+$ T cells, indicating that bone absorption outperforms bone formation, resulting in bone tissue necrosis. It has been confirmed that $CD4^+$ T cells can promote osteoblast differentiation by secreting IFN-γ [35]. TGF-β activity is negatively correlated with osteoblast differentiation [36]. In this study, TGF-β in NT-ONFH hip fluid was found to increase significantly, while IFN-γ was not, indicating decreased osteoblast activity in necrotic bone tissue. With the increase of NT-ONFH necrosis, the percentage of $CD8^+$ T cells decreased, and we speculated that this phenomenon was related to the decreased activity of osteoclasts. The main residual bone tissue in IV stage was necrotic tissue, and the number of osteoblasts and osteoclasts decreased sharply. However, a recent study shows that $CD8^+$ T cells exert a protective effect on bone tissue by expressing OPG to inhibit osteoclast generation [37], therefore, the exact role of $CD8^+$ T cells in femoral head necrosis remains to be elucidated. Different from previous studies, we found that the percentage of $CD19^+$ cells (B cells) in ONFH necrotic bone tissue decreased, suggesting that $CD19^+$ cells are also involved in bone metabolism. B cells, which are abundant in bone marrow [38], are a source of both RANKL and its decoy ligand, OPG. Their production of OPG in particular is increased by T cell stimulation with CD40L to maintain bone mass [39]. Changes in B and T cells lead to downregulation of OPG in osteoblasts, resulting in osteonecrosis [40]. Taken together, the RANKL-Rank-OPG system serves as a potential link between normal immune responses and bone metabolism.

ICs and autoantibodies may be involved in the pathological process of NT-ONFH. Autoantibodies can combine with circulating antigens to form ICs, and the deposition of soluble ICs in various organs has been demonstrated as an important mechanism for many systemic dis-

eases, such as glomerulonephritis [41], ankylosing spondylitis [42], and rheumatoid arthritis [43]. ICs, through circulation, can deposit on the synovial tissue of the hip joint. Indeed, we observed ICs in NT-ONFH, suggesting a role for ICs in the pathogenesis of NT-ONFH. In addition, we showed local immunoglobulin deposition in the hip joint of NT-ONFH. ICs can activate the complement system to produce inflammatory mediators, resulting in inflammatory vascular response, leukocyte outflow and infiltration, and destruction of target cells, all of which can contribute to necrosis of the femoral head. Further studies are warranted regarding IC roles in the pathogenesis of NT-ONFH.

However, this study had some limitations. First, the control group was not composed of healthy controls in this study. The patients with femoral fracture were selected as the control group, and the representation of the control group was inadequate. The traumatic factors may affect the lymphocyte subsets and inflammatory cytokines in the hip joint. These confounding variables were difficult to address in our study. Second, although the changes in lymphocyte subset numbers and inflammatory cytokines were investigated, it cannot be assumed to reflect functional immune deviation. Finally, our study was conducted in a single center with a limited number of patients, and some of the differences were not statistically significant; thus, a large-scale multicenter study is needed. Therefore, studies comparing the levels of immune cell subpopulations in human tissues are needed. Furthermore, studies are also needed to assess the functional status of the T cells and the role of cytokines in immune cells. Additionally, studies are required to ensure the reproducibility of our findings.

In conclusion, the dysregulated $CD3^+CD8^+$ and $CD19^+$ lymphocyte subsets in bone tissue of the femoral head and increased inflammatory cytokines such as IL-2, IL-6, IL-10, and TGF-β1 in the hip joint may play an important role in NT-ONFH pathogenesis, suggesting that a disturbed bone immune microenvironment is involved in the tissue lesions of the femoral head. These findings may help us better understand the pathogenesis of NT-ONFH and improve its diagnosis, prevention, and treatment.

Disclosure of conflict of interest

None.

Address correspondence to: Da-Wei Wang, Department of Joint and Sports Medicine, The Affiliated Liaocheng People's Hospital of Shandong First Medical University, No. 67 Dongchang West Road, Liaocheng 252000, Shandong, China. E-mail: wdw62@sohu.com

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