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Lead Guest Editor: Ziqing Li
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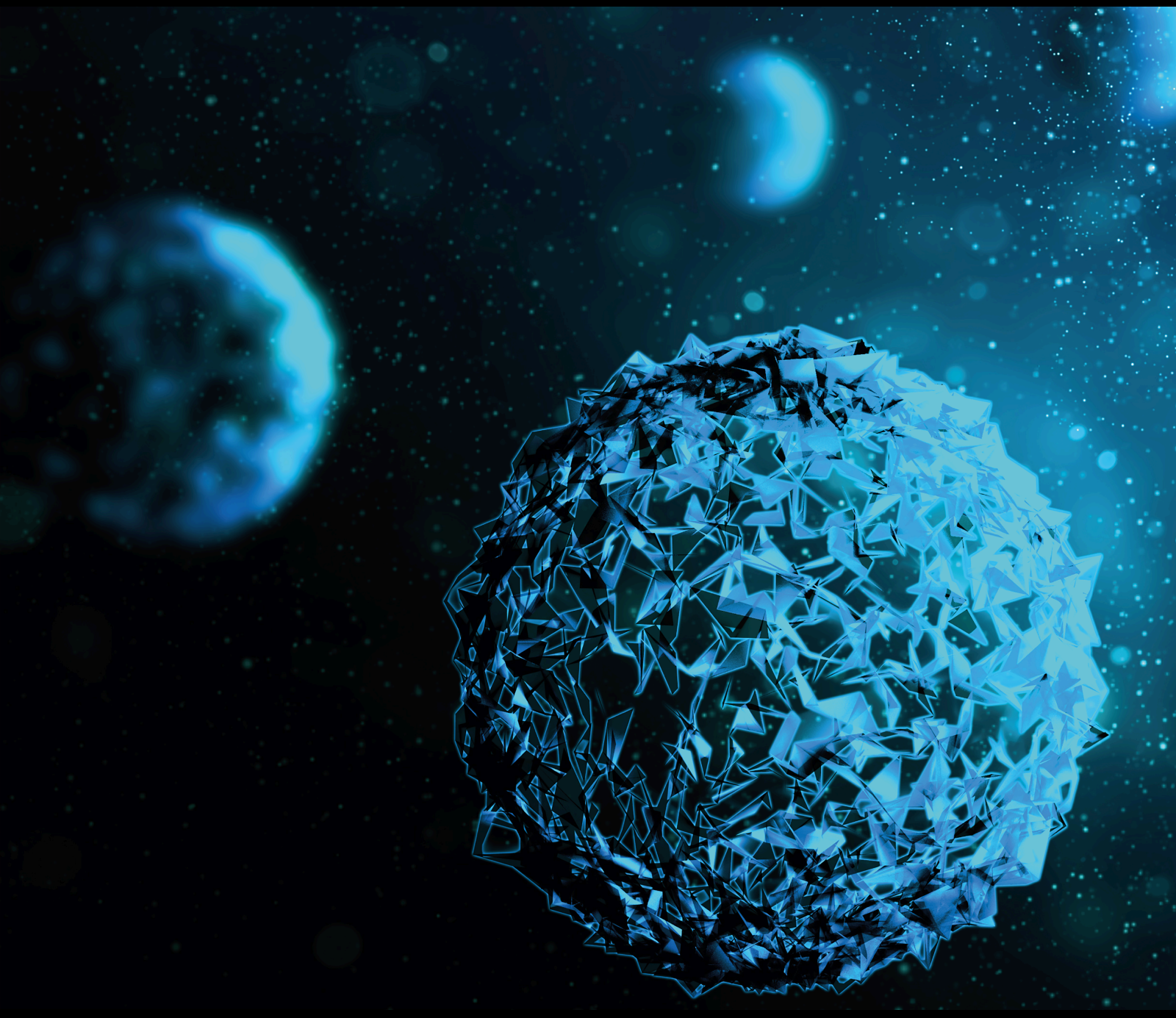

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Editorial Board

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Editorial

Osteoimmunology in Bone Regeneration

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Bone has the unique capacity to heal bony injuries without fibrous scar formation via its regeneration process and therefore maintains physiological and mechanical characteristics. This continuous and well-orchestrated regeneration process is also critical for the upkeep of calcium homeostasis throughout one's lifetime [1, 2]. Stimulating bone regeneration is the central aim in orthopedic surgery and in oral and maxillofacial surgery, in order to overcome large bone defects and to cure metabolic/inflammatory bone diseases. Although tissue-engineered materials or artificial bone can help to retain bone stability, biocompatibility and bioresorbability are still significant obstacles for biomaterials to achieve so as to sync with the natural regeneration process. During recent decades, advances in this field have begun to reveal various regulatory molecules shared by the immune system and skeletal system, which depict interesting crosstalk of signaling transducers between immune cells and bone cells [3, 4]. Therefore, osteoimmunology has developed as an essential interdisciplinary field underlying major discoveries concerning bone regeneration and developing targeted therapies for bone diseases. This special issue is aimed at presenting recent research efforts in the crosstalk between the immune system and the skeletal system and their potential application for bone regeneration.

The study by X. Zhang et al. reports the osteoimmunomodulatory properties of microscale magnesium (Mg ions) in stimulating osteogenesis via the immunomodulation between bone marrow stem cells (BMSCs) and macrophages. The authors found that microscale Mg ions could induce M2 phenotype changes of macrophages and inhibited the TLR-NF-κB signaling pathway by the release of anti-inflammatory cytokines. Meanwhile, microscale Mg ions stimulated the expression of osteoinductive molecules in macrophages and promoted osteogenesis of BMSCs through the BMP/SMAD signaling pathway. The results indicated that manipulating Mg ion concentration to a proper microscale can endow the Mg biomaterial with favorable osteoimmunomodulatory properties, thereby providing crucial evidence for improving and modifying the effect of Mg-based bone biomaterials.

The study by C. Fu et al. identifies the novel function of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) as a critical factor in periodontitis and bone remodeling. The authors showed that PTEN decreased in a ligature-induced mouse periodontitis model and was associated with inflammatory factors interleukin 1 (IL-1) and tumor necrosis factor (TNF-α) in macrophages. Lack of PTEN activated IL-1 and TNF-α, which increased the number of osteoclasts and led to alveolar bone erosion and loss, whereas nanoparticle therapy of PTEN could directly inhibit the inflammatory process and bone erosion in vivo. The study provides a novel insight into understanding the protective effects of PTEN on inflammation and bone remodeling in periodontitis and proposes that PTEN can be used as adjuvant therapy for inflammatory diseases.

The study by J. Yuan et al. investigated the therapeutic effect of genistein on rat temporomandibular joint osteoarthrosis (TMJOA) which was characterized by chronic inflammation and joint cartilage loss. The authors demonstrated that genistein treatment had positive effects on the condylar cartilage renovation, wherein high-dose genistein treatment had better effects on the reversal of OA changes and reduction of the expression of p65 (NF-κB signaling) and inflammatory cytokines (IL-1β and TNF-α) in male TMJOA rat models. Collectively, the study indicated a better therapeutic effect of high-dose genistein on condyle cartilage damage in TMJOA rats via the suppression of NF-κB expression and inflammatory cytokine activation.

The study by J. Xiong et al. reveals the relationship between dyslipidemia and the risk of osteoarthritis (OA) based on the foundation that autoimmune response affects the homeostasis of the internal environment in the human body and causes self-immune regulation. Through a meta-analysis study comprising 22,501 patients with OA (19,733 with hand OA, 2,679 with knee OA, and 89 with hip OA), the authors stated that OA was higher in those with dyslipidemia compared to those who did not have. Therefore, dyslipidemia might be associated with an increased risk of OA.

The review by H. Wang et al. discusses the current understanding of the varied roles of osteoclasts (OCs) in maintaining skeletal health. The authors summarize the coupling factors that affect the interaction and crosstalk of OCs with osteocytes, mesenchymal stem cells (MSCs), and osteoblasts (OBs), in order to provide a different perspective on recognizing OCs when strategies are created to develop ideal therapeutic agents that target bone remodeling disorders characterized by excessive OC activity.

The study by Z. Chen et al. defines the immune cell landscape of different structures of the knee in OA by using cell-type identification by estimating relative subsets of known RNA transcripts (CIBERSORT) for deconvolution of the global gene expression data. The authors suggested that the immune cell composition in knee OA differed substantially in different anatomical structures of the knee. Meanwhile, activated mast cells were mainly associated with high immune cell infiltration in OA. Moreover, M2 macrophages in the synovium and mast cells in subchondral bone may play essential roles in the pathogenesis of OA.

In conclusion, a comprehensive understanding of ongoing efforts would enable researchers to identify the most efficient approaches in the field and eventually lead to the successful discovery of therapeutics. The guest editorial team wishes that this special issue will help in evidencing researches from multiple disciplines in this area and encourage future collaborations from multidisciplinary aspects.

Conflicts of Interest

The editors declare that there are no conflicts of interest regarding the publication of this special issue.

Acknowledgments

We would like to express our great gratitude to all authors and reviewers who contributed to this special issue. Special thanks are due to Dr. Guangpu Yang (The Chinese University of Hong Kong) who served as an academic specialist to our special issue.

Ziqing Li
Puyi Sheng
Chaohong Li
Gongsheng Yuan
Yilun Deng

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Research Article

The Immune Cell Landscape in Different Anatomical Structures of Knee in Osteoarthritis: A Gene Expression-Based Study

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Guest Editor: Yilun Deng

Background. Immunological mechanisms play a vital role in the pathogenesis of knee osteoarthritis (KOA). Moreover, the immune phenotype is a relevant prognostic factor in various immune-related diseases. In this study, we used CIBERSORT for deconvolution of global gene expression data to define the immune cell landscape of different structures of knee in osteoarthritis. *Methods and Findings*. By applying CIBERSORT, we assessed the relative proportions of immune cells in 76 samples of knee cartilage, 146 samples of knee synovial tissue, 40 samples of meniscus, and 50 samples of knee subchondral bone. Enumeration and activation status of 22 immune cell subtypes were provided by the obtained immune cell profiles. In synovial tissues, the differences in proportions of plasma cells, M1 macrophages, M2 macrophages, activated dendritic cells, resting mast cells, and eosinophils between normal tissues and osteoarthritic tissues were statistically significant ($P < 0.05$). The area under the curve was relatively large in resting mast cells, dendritic cells, and M2 macrophages in receiver operating characteristic analyses. In subchondral bones, the differences in proportions of resting master cells and neutrophils between normal tissues and osteoarthritic tissues were statistically significant ($P < 0.05$). In subchondral bones, the proportions of immune cells, from the principle component analyses, displayed distinct group-bias clustering. Resting mast cells and T cell CD8 were the major component of first component. Moreover, we revealed the potential interaction between immune cells. There was almost no infiltration of immune cells in the meniscus and cartilage of the knee joint. *Conclusions*. The immune cell composition in KOA differed substantially from that of healthy joint tissue, while it also differed in different anatomical structures of the knee. Meanwhile, activated mast cells were mainly associated with high immune cell infiltration in OA. Furthermore, we speculate M2 macrophages in synovium and mast cells in subchondral bone may play an important role in the pathogenesis of OA.

1. Introduction

Knee osteoarthritis (KOA) is one of the most frequently common diseases in orthopedic department, which affects 30%-50% of people over 65 years old [1]. Although a series of treatment, such as anti-inflammatory medicine, play a certain role in relieving symptoms, it is difficult to prevent the process of bone degeneration, and total knee arthroplasty is still the mainly curative therapy for KOA [2].

KOA is a chronic degenerative disease characterized by articular cartilage injury and degeneration, together with sclerosis, proliferation and cystic degeneration of subchondral bone, and subsequent narrowing of articular space [3]. In osteoarthritis (OA), various anatomical structures of the knee joint are damaged. OA was used to be considered as "mechanical wear and tear" [4]. However, in recent years, more and more studies have shown that immunological mechanisms play the vital role in the pathogenesis of OA

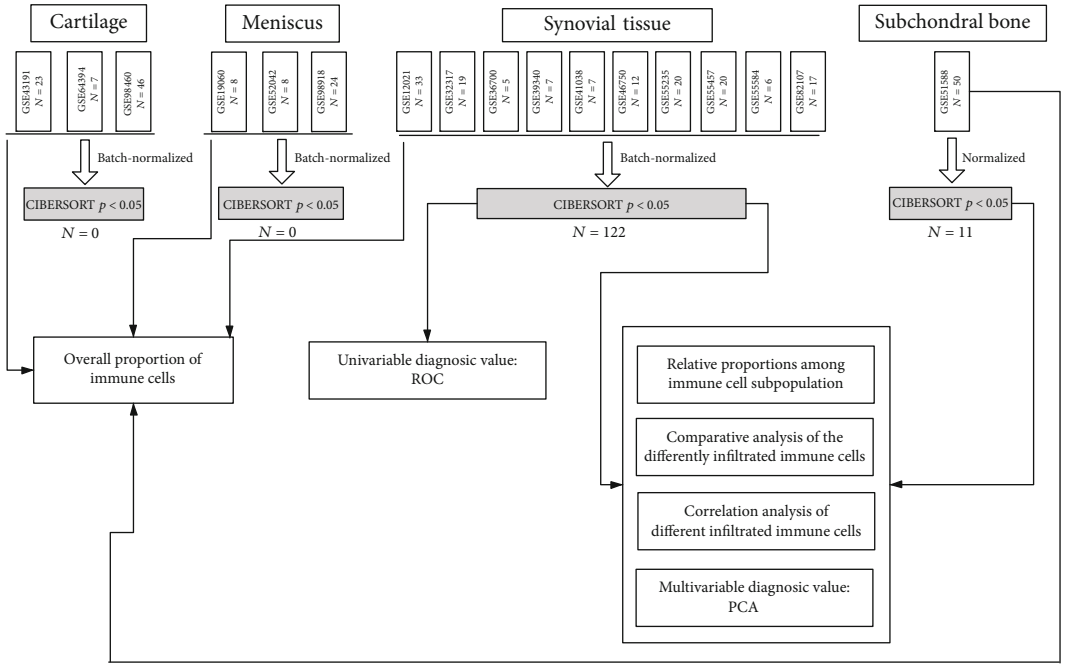


Figure 1: Flowchart detailing the study design. GEO: Gene Expression Omnibus; CIBERSORT: Cell-type Identification By Estimating Relative Subsets Of known RNA Transcripts; PCA: Principle component analyses; ROC: receiver operating characteristic.

[5, 6], and OA is gradually considered as a chronic inflammatory response [7]. In the pathological process of OA, the destruction of bone and cartilage caused by synovitis and inflammation is the hot spots for series studies [8]. Until now, however, the role of various immune cells in osteoarthritis-related microenvironment still has not been clarified.

The function and proportions of infiltrating immune cells vary subtly according to the host's immune status, which is reported to be effective drug targeting and related to clinical outcomes [9–11]. Moreover, the immune phenotype is a relevant prognostic factor in various immune-related diseases [12–14]. Therefore, clarification of local infiltration of immune cells in knee joint contributes to better understand the local immune situation and to develop new treatment methods.

Immune cell composition of solid tissues is usually analyzed by flow cytometry and immunohistochemistry, which have the limitations in small number of detected cells and great need of number of fluorescence channels. The system biology tool Cell-type Identification By Estimating Relative Subsets Of known RNA Transcripts (CIBERSORT) can employ deconvolution of bulk gene expression data from solid tissues, enumerate 22 immune cell types at once, and apply signatures from ~500 marker genes to quantify the relative fraction of each cell type, which means there is a high resolving power for CIBERSORT [12, 15].

Therefore, in the present study, we used CIBERSORT for deconvolution of global gene expression data to define the immune cell landscape of different structures of knee in osteoarthritis.

2. Methods

2.1. Data Acquisition. In the present study, datasets were searched from the Gene Expression Omnibus (GEO) database [16] with the keywords "osteoarthritis" [MeSH Terms] OR "osteoarthritis" [All Fields] AND "Homo sapiens" [porgn] AND "gse" [Filter], uploaded up to 15 September 2019. The study type was described as "expression profiling by array." All selected datasets were genome-wide expression data in different structures of knee of normal or OA patients. Datasets with samples of normal area in OA patients were excluded, considering it is hard to define it as normal or OA tissues. All of the selected studies were approved by their respective institutional review boards. Preprocessing, aggregation, and normalization of raw data were performed according to the robust multiarray average algorithm. Details of the study design are illustrated in Figure 1 as a flowchart.

2.2. Evaluation of Infiltrating Immune Cells in Different Structures of Knee. Normalized gene expression data were used to infer the relative proportions of 22 types of infiltrating immune cells using the CIBERSORT algorithm. Briefly, gene expression datasets were prepared using standard annotation files and data uploaded to the CIBERSORT web portal, with the algorithm run using the default signature matrix at 1000 permutations. CIBERSORT is an analytical tool which accurately quantifies the relative levels of distinct immune cell types within a complex gene expression mixture [15]. CIBERSORT derives a *P* value for the deconvolution for each sample using the Monte Carlo sampling, providing a measure of confidence in the results. A set of barcode gene

expression values (a "signature matrix" of 547 genes) was used by CIBERSORT for characterizing immune cell composition. Here, the original CIBERSORT gene signature file LM22 was applied. The 22 cell types inferred by CIBERSORT include B cells, T cells, natural killer cells, macrophages, dendritic cells, eosinophils, and neutrophils, amongst others.

2.3. Principle Component Analyses (PCA) and Receiver Operating Characteristic (ROC) Analyses. Principle components analysis (PCA) was used to identify major sources of variance in the proportion of different types of infiltrating immune cells between normal patients and OA patients. The major sources of variance could potentially be the diagnostic clues for OA. When the most major variation in PCA result was still low (<30%), receiver operating characteristic (ROC) was performed to assess the diagnostic value of different types of infiltrating immune cells separately. The area under the curve (AUC) under 95% confidence interval was calculated, and the ROC curve was generated.

2.4. Overall Proportion of Immune Cells in Different Tissues. As explained by the creators of CIBERSORT, the CIBERSORT *P* value is empirical and produced for the deconvolution [15]. It is calculated for the actual observed data instead of theoretical data. Moreover, it tests the null hypothesis that none of the cells that comprise the signature matrix in a given sample are present. Thus, it was considered as a parameter that could reflect the proportion of a sample that comprised immune cells versus nonimmune cells, where a greater proportion of nonimmune cells would produce a correspondingly larger *P* value. Also, several researches have confirmed this hypothesis [12, 17, 18]. In the present study, high infiltration of immune cells was defined as CIBERSORT P value ≤ 0.01, medium infiltration of immune cells was defined as $0.01 <$ CIBERSORTP value ≤ 0.05, and low infiltration of immune cells was defined as CIBERSORT P value > 0.05.

2.5. Comparison of the Results Calculated by xCell and CIBERSORT Algorithms. In order to validate the results obtained by CIBERSORT, another algorithm xCell [19] was performed for the quantification of overall immune cell infiltration and xCell abundance scores of those immune cell types which were significantly different between OA tissues and normal tissues in CIBERSORT results. Expression data from different anatomical structures of knee in osteoarthritis was concatenated in different files. xCell ran with the "rnaseq = FALSE" option, and the immune scores and xCell scores were computed. For comparison purposes, only cell types which could be detected by both xCell and CIBERSORT were selected in this validation process.

2.6. Statistical Analyses. Datasets from different structures of knee were analyzed separately. Using a limma R package [20] and a sva R package [21], batch normalization was performed for data from different datasets, including datasets with samples of cartilage, meniscus, and synovial tissue. Dataset with samples of subchondral bone was normalized using a limma R package (Figure 1). Details of batch normalization and normalization are available at https://github.com/Au-CZM. Cases with a CIBERSORT P value of <0.05 were included in further analysis. Immune cell profile was obtained for each sample, and mean values for each group (normal and OA) were calculated. Unpaired T-test was used to evaluate the difference of continuous variables between normal groups and OA groups. Correlations between continuous and categorical variables were evaluated using the Kruskal-Wallis test. Associations between immune cell subsets were tested by the Pearson correlation coefficient. For univariable analyses of the 22 immune cell subsets, adjustment for multiple testing was performed by calculating q-values using the Benjamini-Hochberg method. To analyze if distinct classes of immune cell infiltration are present in different groups, we used hierarchical clustering of immune cell proportions by Ward's method. A combination of the elbow method and the Gap statistic was conducted to explore the likely number of distinct clusters in the data.

All analyses were conducted using R version 3.6, excepting that ROC analyses were performed using SPSS 23.0 statistical software (SPSS Inc., Chicago, IL, USA). All statistical tests performed were two-sided, and the P values < 0.05 were considered as statistical significance.

3. Results

Datasets with samples of 4 anatomical structures of knee joint, including cartilage, synovial tissues, meniscal tissues, and subchondral bone, can be found in GEO. 17 studies were selected in this study, including 3 studies in knee cartilage, 10 studies in knee synovial tissue, 3 studies in knee meniscal tissue, and 1 study in knee subchondral bone (Table 1) [22–31]. By integrated analysis, 12945 genes for cartilage, 3238 genes for synovial tissue, 6598 genes for meniscal tissue, and 9570 genes for subchondral bone were obtained. Using CIBERSORT algorithm, we first investigated the difference of immune infiltration between normal and OA synovial tissue in 22 subpopulations of immune cells.

3.1. Synovial Tissue. In synovial tissue of knee joint, Figure 2(a) summarized the results obtained from 122 samples with a CIBERSORT P value of <0.05. Of these, 30 samples were normal and 92 samples were osteoarthritic.

Overall, in osteoarthritic synovial samples, the most abundant immune cells were M2 macrophages with 30.10%, resting T cell CD4 memory with 23.88%, and activated NK cells with 16.20%, while in normal synovial samples, the most abundant immune cells were M2 macrophages with 26.76%, resting T cell CD4 memory with 24.06%, and activated NK cells with 15.02%.

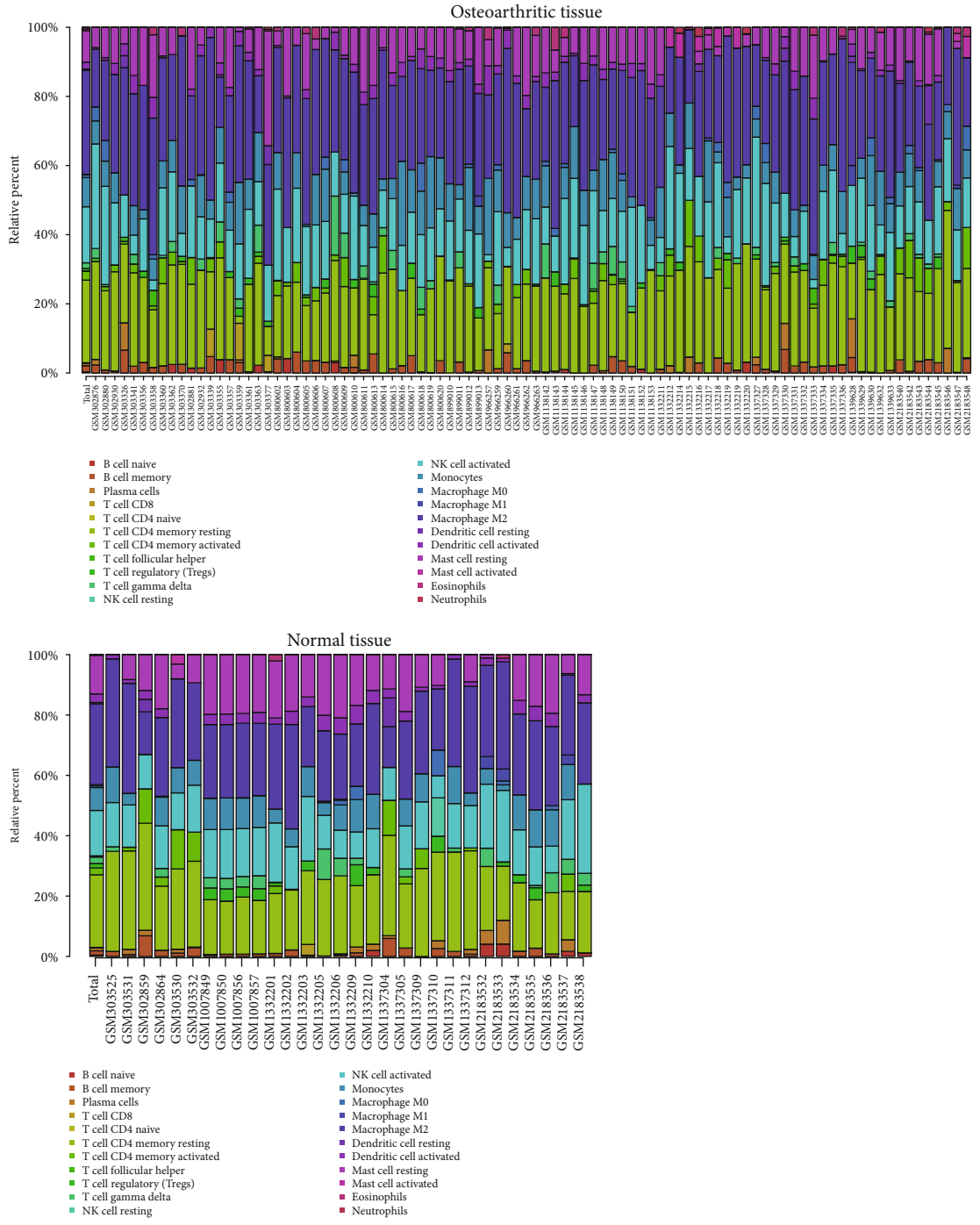
The differences in proportions of plasma cells, M1 macrophages, M2 macrophages, activated dendritic cells, resting mast cells, and eosinophils between normal tissues and osteoarthritic tissues were statistically significant (Figure 2(b), $P < 0.05$). Higher proportion for above significantly changed cells existed in osteoarthritic tissues, compared with in normal tissues.

The proportions of different infiltrating immune cell subpopulations were correlated weakly to moderately (Figure 2(c), absolute value of correlation coefficient < 0.80).

Table 1: Profiling datasets of Gene Expression Omnibus (GEO).

Tissue source	GEO ID	Normal	Case	Platform	Year*	Country	Author**
Knee cartilage	GSE43191	0	23	GPL11532 [HuGene-1_1-st] Affymetrix Human Gene 1.1 ST Array	2016	Spain	Fernández-Tajes J
	GSE64394	5	2	GPL6244 [HuGene-1_0-st] Affymetrix Human Gene 1.0 ST Array	2018	USA	Bhutani N
	GSE98460	0	46	GPL16686 [HuGene-2_0-st] Affymetrix Human Gene 2.0 ST Array	2019	USA	Muhammad Farooq Rai
Knee synovial tissue	GSE12021	13	20	GPL96 [HG-U133A] Affymetrix Human Genome U133A Array GPL97 [HG-U133B] Affymetrix Human Genome U133B Array	2018	Germany	Huber R
	GSE32317	0	19	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	2019	USA	Scanzello CR
	GSE36700	0	5	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	2019	Belgium	Lauwerys BR
	GSE39340	0	7	GPL10558 Illumina HumanHT-12 V4.0 Expression BeadChip	2018	China	Xiaotian C
	GSE41038	4	3	GPL6883 Illumina HumanRef-8 v3.0 Expression BeadChip	2019	Australia	Thomas GP
	GSE46750	0	12	GPL10558 Illumina HumanHT-12 V4.0 Expression BeadChip	2018	Belgium	Lambert C
	GSE55235	10	10	GPL96 [HG-U133A] Affymetrix Human Genome U133A Array	2018	Germany	Thomas H
	GSE55457	10	10	GPL96 [HG-U133A] Affymetrix Human Genome U133A Array	2018	Germany	Kinne RW
	GSE55584	0	6	GPL96 [HG-U133A] Affymetrix Human Genome U133A Array	2018	Germany	Peter S
	GSE82107	7	10	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	2019	Netherlands	de Vries M
Knee meniscus	GSE19060	3	5	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	2019	USA	Sun Y
	GSE52042	0	8	GPL17882 Microarrays Inc. Human MI Ready Array–49K Genomic Array	2014	Germany	Von der Heyde S
	GSE98918	12	12	GPL20844 Agilent-072363 SurePrint G3 Human GE v3 8x60K Microarray	2018	USA	Zhang B
Knee subchondral bone	GSE51588	10	40	GPL13497 Agilent-026652 Whole Human Genome Microarray	2018	USA	Chou CH

*Year: last update date. **Author: contact name.



(a)

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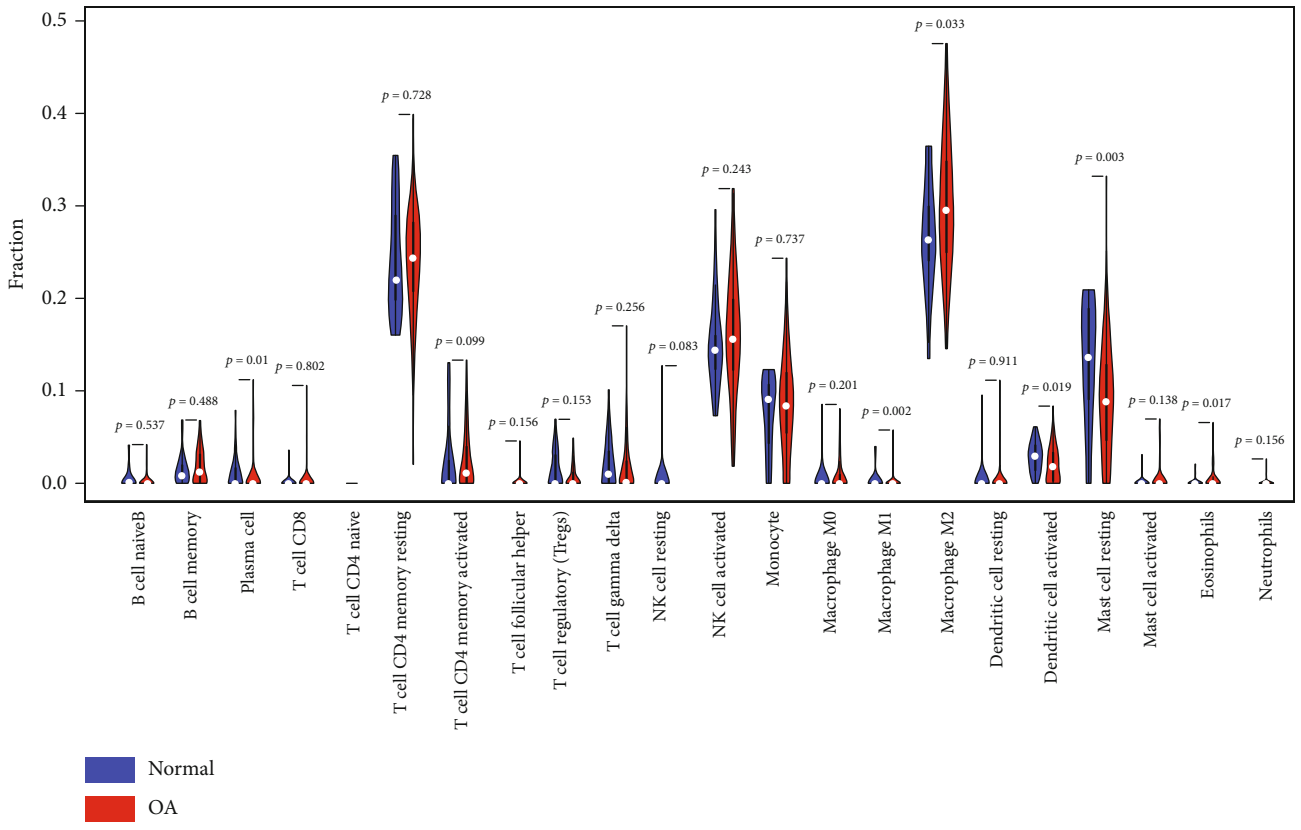

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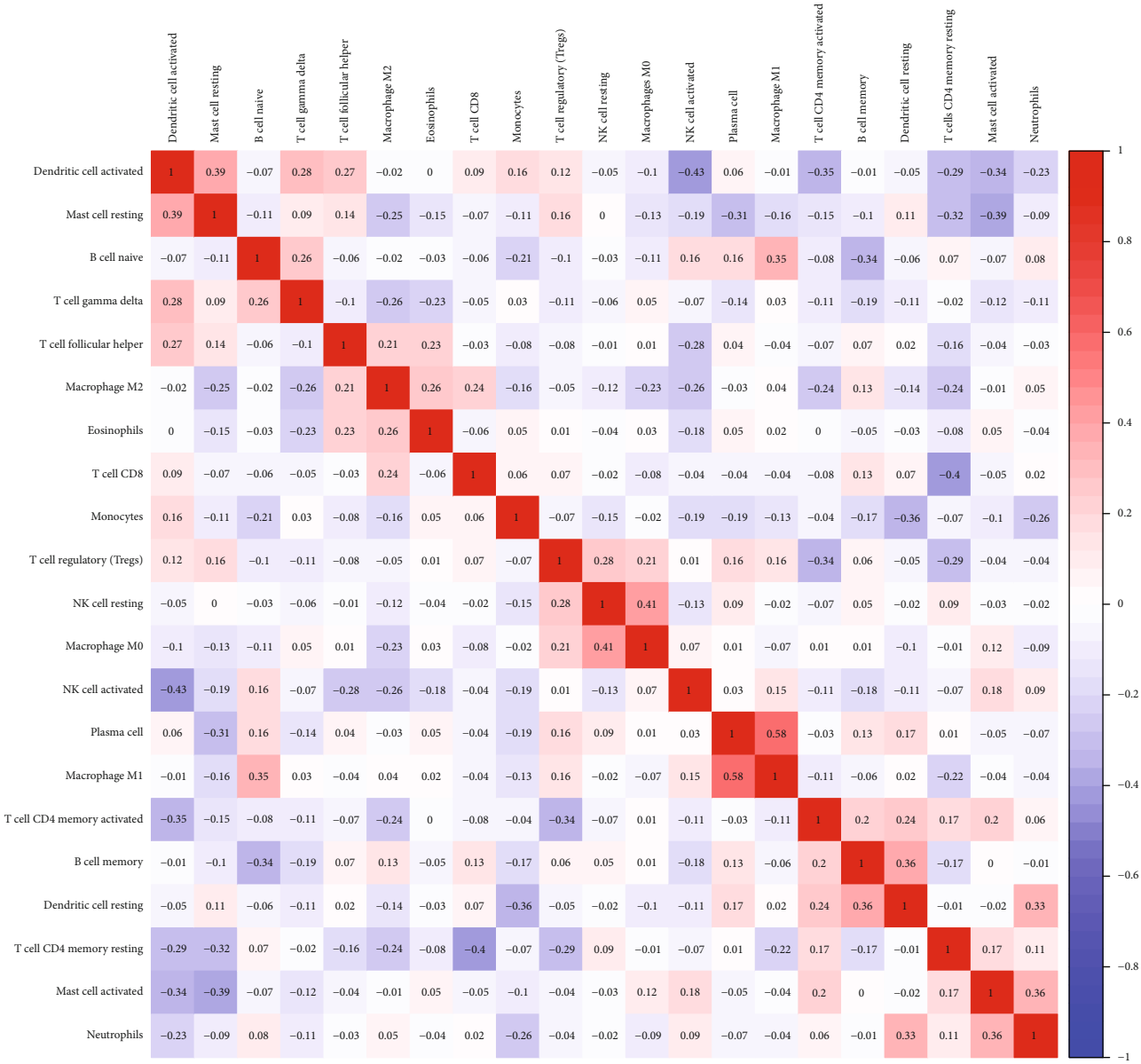

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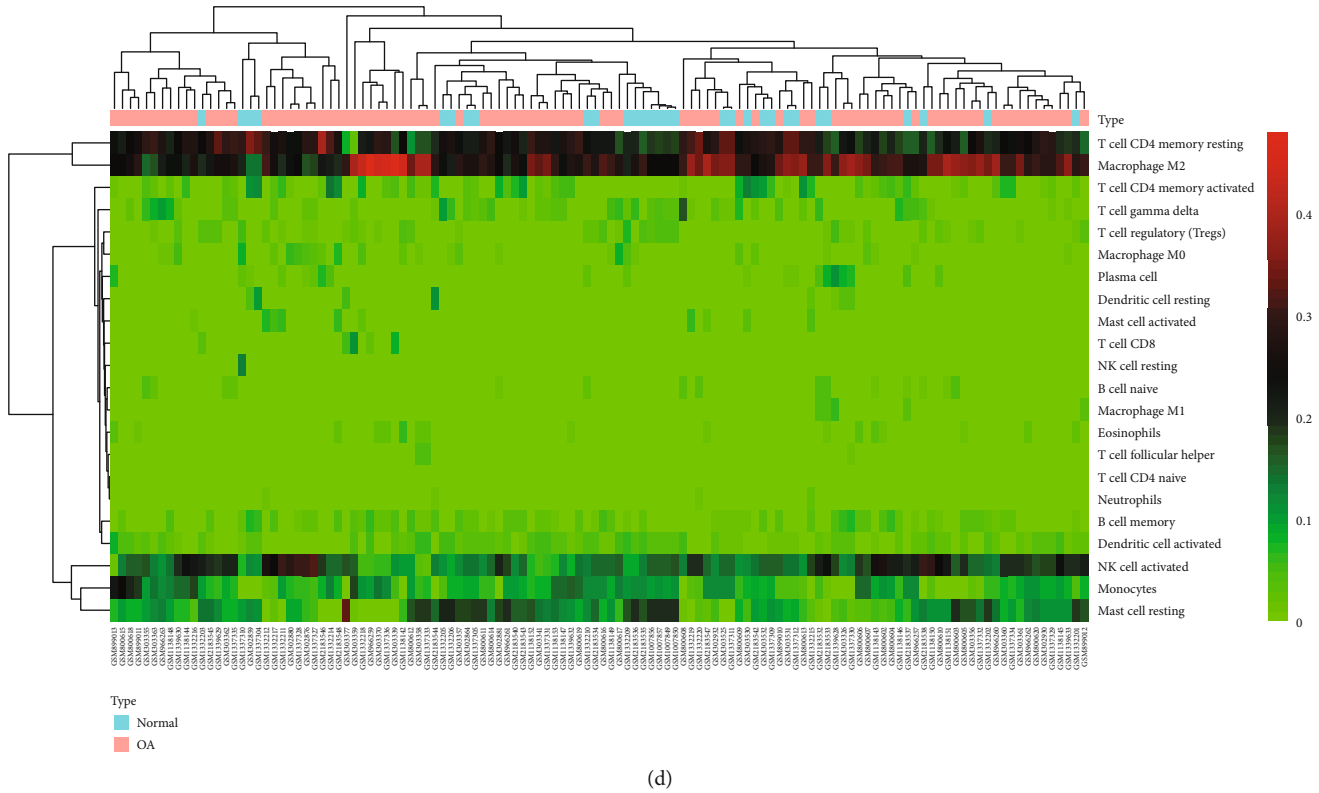


(d)

Figure 2: The landscape of immune infiltration in osteoarthritis in synovial tissue. (a) The composition of immune cells for each sample. Total: the average composition of immune cells. (b) The difference of immune infiltration between osteoarthritic tissue and normal tissue. (c) Correlation matrix of all 22 immune cell proportions. (d) Heat map of the 22 immune cell proportions. OA: osteoarthritis.

Correlations of M2 macrophages with other immune cell populations by calculating the Pearson correlation coefficients in Figure 2(c) were all weak (absolute value of correlation coefficient < 0.30).

As shown in Figure 2(d), using unsupervised hierarchical clustering based on above-identified cell subpopulation, the samples of pathological and normal could not be clearly separated. PCA was used to assess if the proportions of infiltrating immune cell could be used to differentiate the diagnosis of OA. Figure 3(a) showed that the diagnosis of OA could not be apparently attributed to the proportions of different infiltrating immune cell subpopulations. The first principle components (PC) accounted for 25.90% variance. M2 macrophages, resting mast cells, and activated NK cells were the major components of PC1, especially M2 macrophages with more than 0.75 component loading (Figure 3(b)). Figure 3(c) showed that AUC was relatively large in resting mast cells (0.682, (0.560, 0.804)), dendritic cells (0.642, (0.534, 0.750)), and M2 macrophages (0.630, (0.522, 0.738)), in ROC curve analyses. The above-mentioned cells might be related to the pathological mechanism of OA.

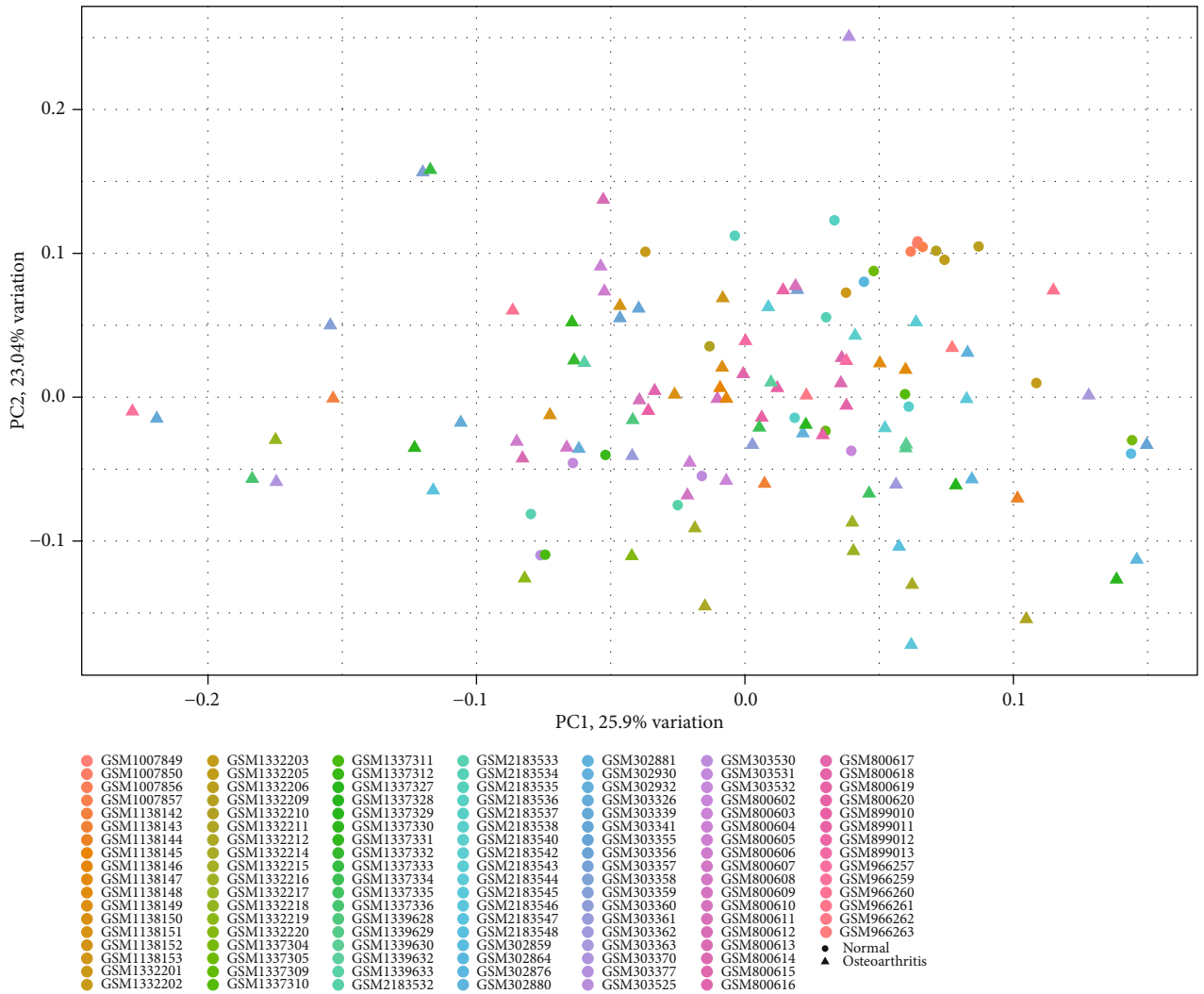
Together, these results indicated that aberrant immune infiltration and its heterogeneous in osteoarthritic synovial tissues as a tightly regulated process might have important clinical meanings. It is worth noting that M2 macrophages had a high proportion in the synovial tissue of knee joint, and its proportion in OA patients and normal people had statistical significance. It might have clinical diagnostic significance for OA. In order to develop a diagnostic method using M2 macrophages, the cutoff was determined using the maximum of the Youden index (0.276) based on ROC analysis. The sensitivity and specificity were 0.609 and 0.667, respectively. The diagnostic value of the method only using M2 as diagnostic criteria is limited but really existed.

Therefore, we speculated that M2 macrophages might play an important role in the pathogenesis of OA. However, the composition of immune cells in synovial tissue could not discriminate clearly between normal and OA groups, and other pathogenic factors still needed further investigation.

3.2. Subchondral Bone. In subchondral bones of knee joint, Figure 4(a) summarized the results obtained from 11 samples with a CIBERSORT P value of <0.05. Of these, 2 samples (GSM1248762, GSM1248767) were normal and other 9 samples were osteoarthritic.

Overall, in osteoarthritic subchondral bones, the most abundant infiltrating immune cells were T cell CD8 with 18.84%, activated mast cells with 17.37%, and activated T cell CD4 memory with 9.12%, while in normal synovial samples, the most abundant infiltrating immune cells were resting mast cells with 76.97%, monocytes with 4.63%, and neutrophils with 4.61%.

The differences in proportions of resting master cells and neutrophils between normal tissues and osteoarthritic tissues



(a)

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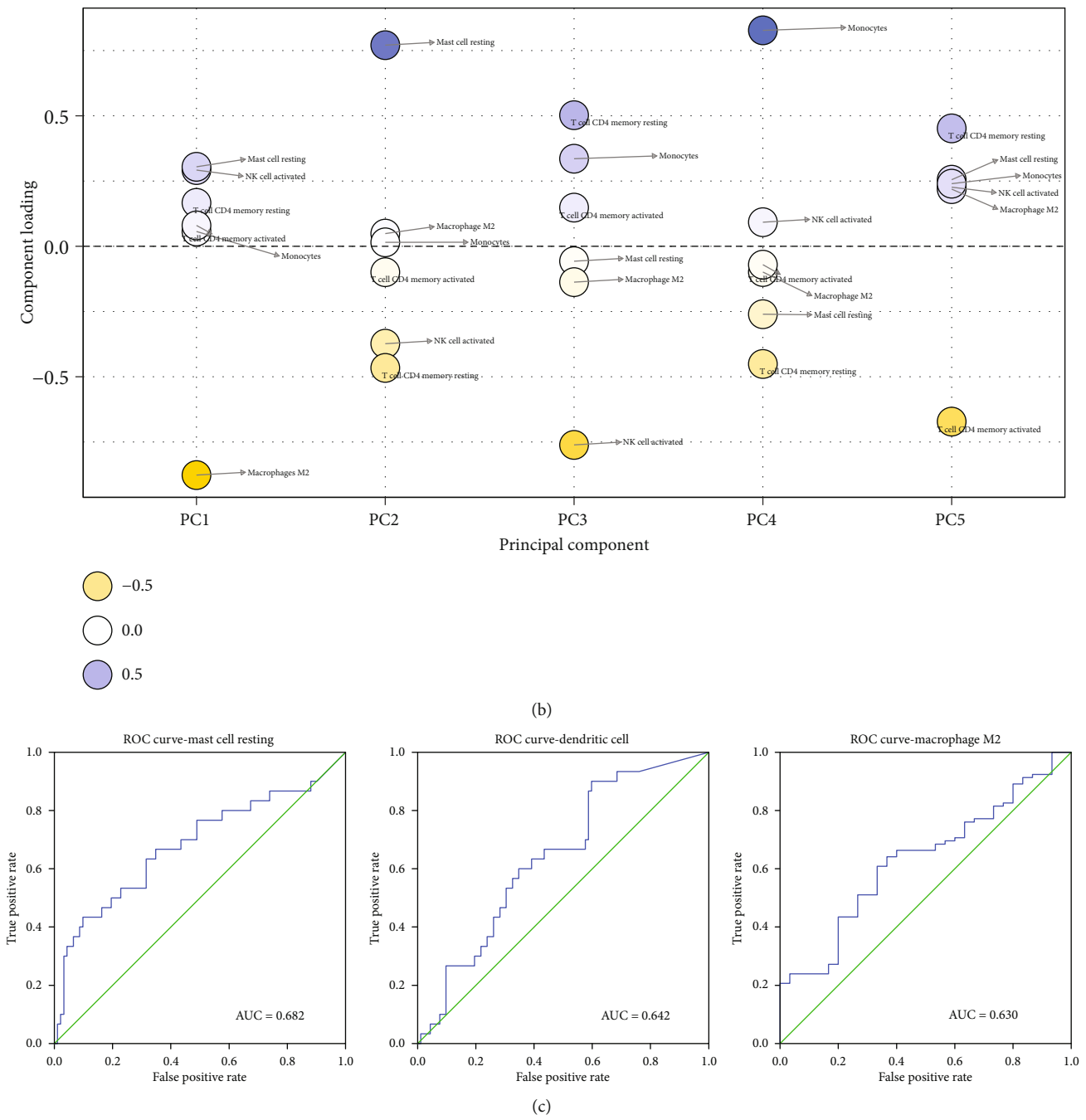
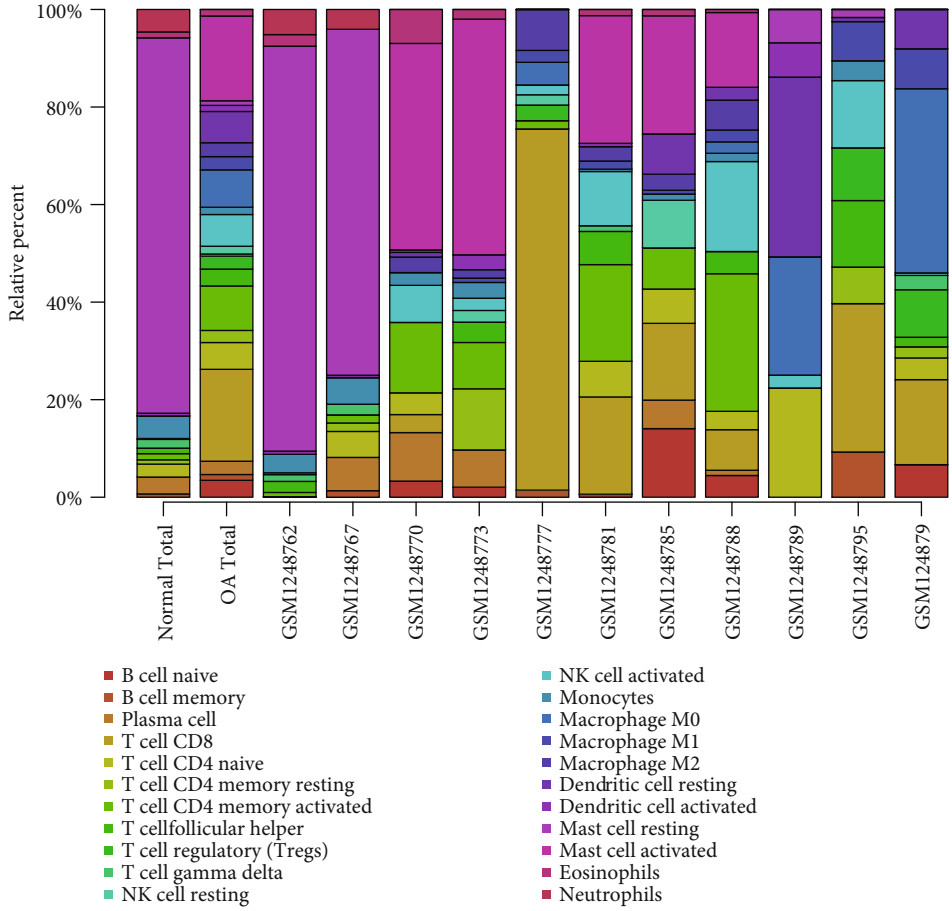


Figure 3: The diagnostic value of composition of infiltrating immune cells for osteoarthritis in synovial tissue. (a) Principle component analysis (PCA). (b) Component loading in PCA results. (c) Receiver operating characteristic analysis.

were statistically significant (Figure 4(b), $P < 0.05$). Lower proportion for above significantly changed cells existed in osteoarthritic tissues, compared with normal tissues. Considering that 2 normal cases were low number samples, we used all 10 normal cases including low overall infiltration of immune cells and found that the differences in proportions of resting master cells and neutrophils between normal tissues and osteoarthritic tissues were still statistically significant with $P = 0.005$ and $P < 0.001$, respectively.

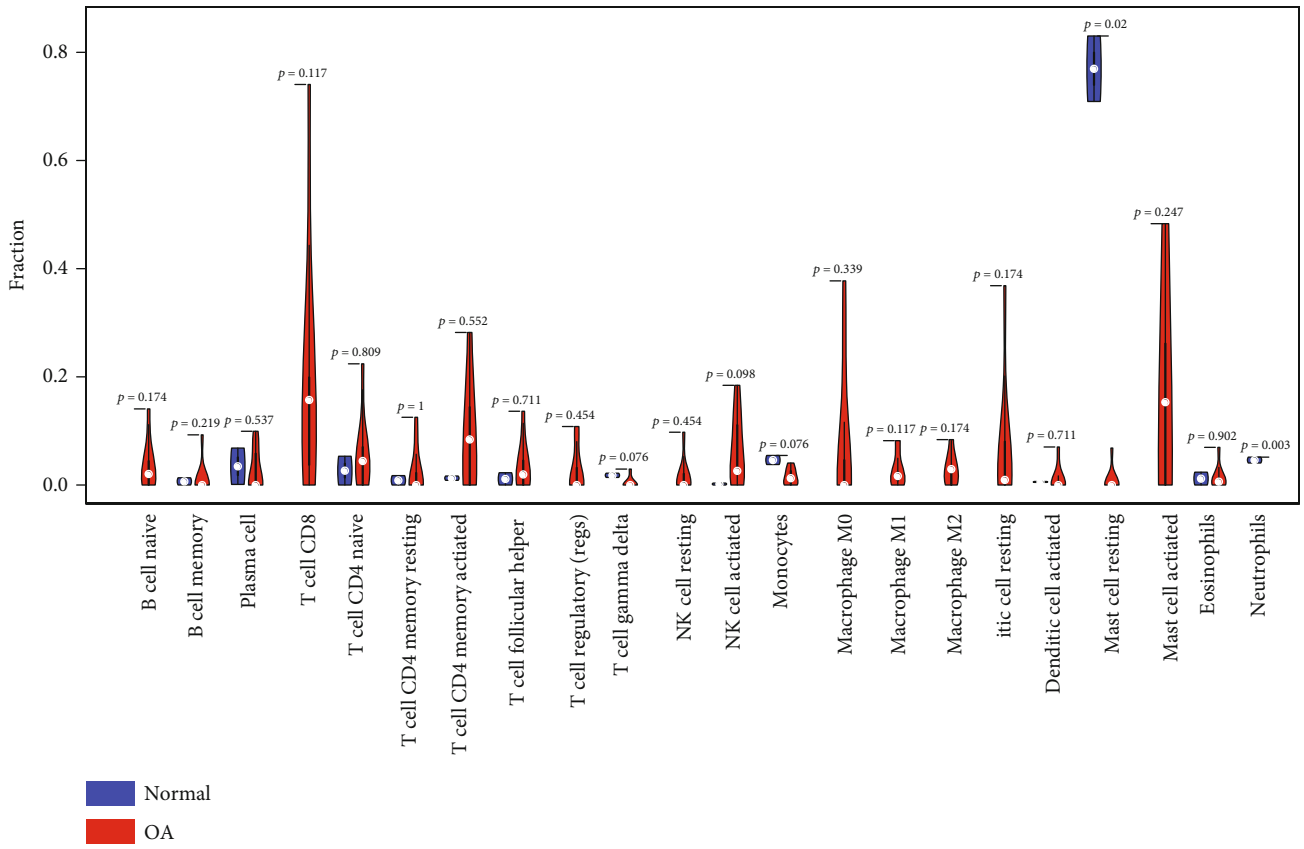
Correlations between proportions of resting dendritic cells and activated dendritic cells, resting dendritic cells and T cell CD4 naive, M1 macrophages and T cell regulatory, and M2 macrophages and neutrophils were strong (absolute value of correlation coefficient > 0.80, Figure 4(c)). There might be a potential interaction between them.

To further elucidate the role of mast cells in the OA immune cell network, correlations of resting and activated mast cells with other immune cell populations by calculating



(a)

Figure 4: Continued.



(b)

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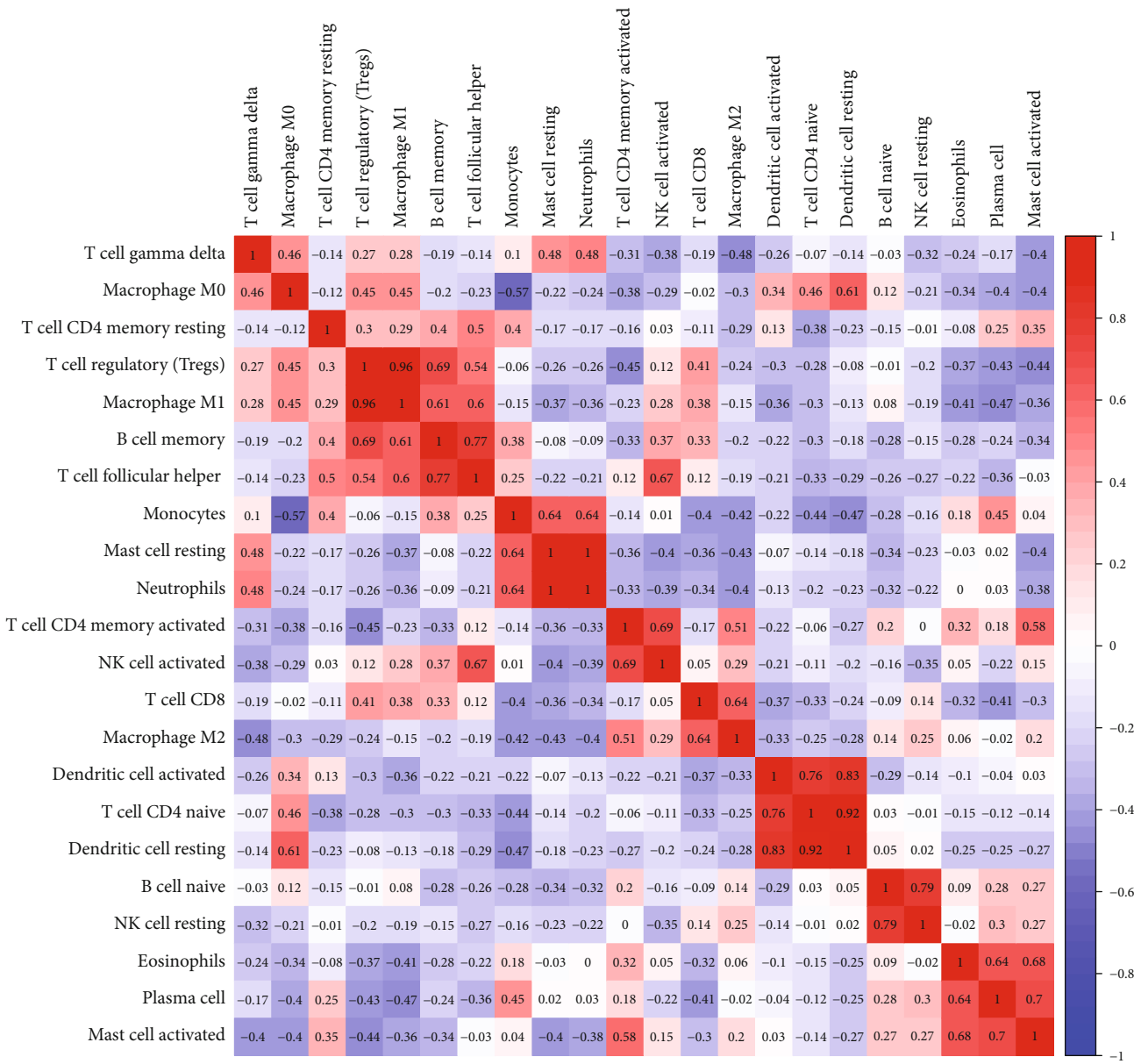

(c)

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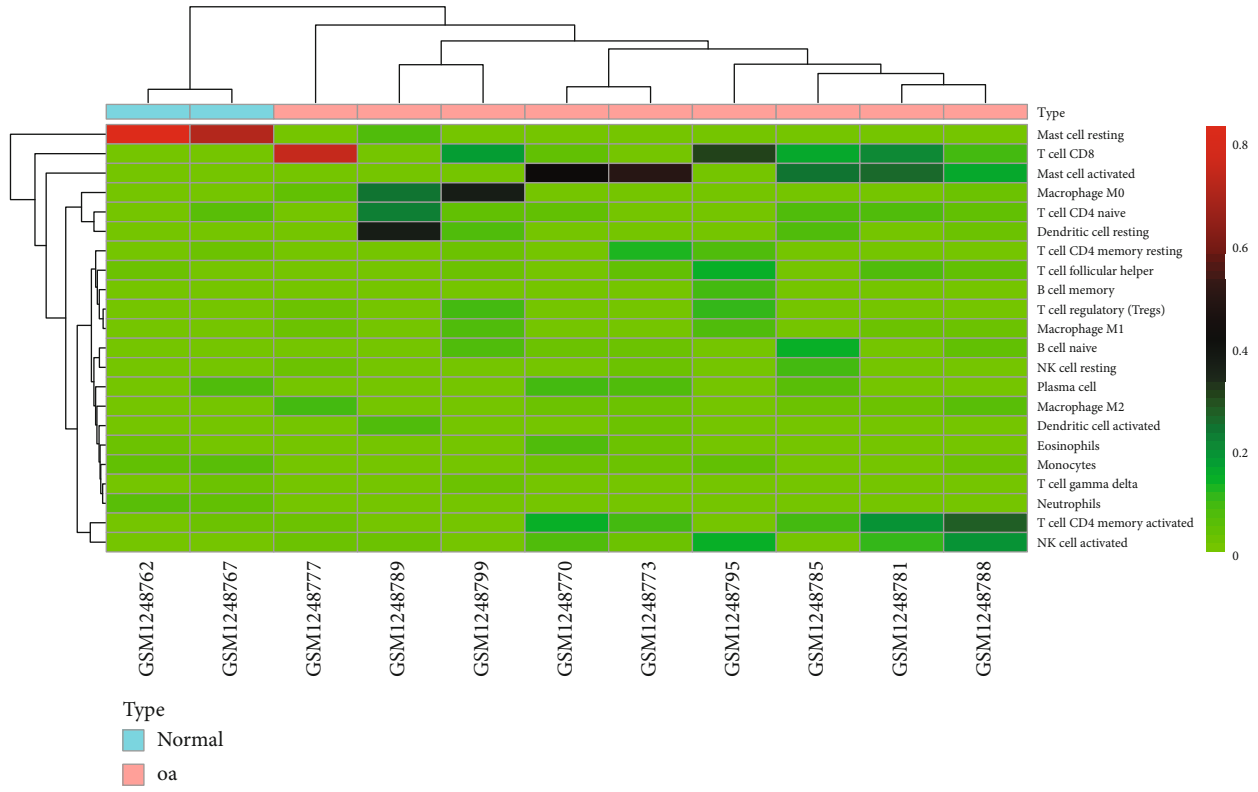


(d)

Figure 4: The landscape of immune infiltration in osteoarthritis in subchondral bone. (a) The composition of immune cells for each sample. Normal total: the average composition of immune cells in normal tissue; OA total: the average composition of immune cells in osteoarthritic tissue. (b) The difference of immune infiltration between osteoarthritic tissue and normal tissue. (c) Correlation matrix of all 22 immune cell proportions. (d) Heat map of the 22 immune cell proportions. OA: osteoarthritis.

the Pearson correlation coefficients in Figure 4(c) attracted our attention. Activated mast cells correlated positively with resting T cell CD4 memory, activated T cell CD4 memory, plasma cells, and eosinophils. However, they correlated negatively with T cell gamma delta, M0 and M1 macrophages, T cell regulatory, B cell memory, resting mast cells, neutrophils, and T cell CD8. Resting mast cells correlated positively with T cell gamma delta, monocytes, and neutrophils, while they correlated negatively with M1 macrophages, activated T cell CD4 memory, activated NK cells, T cell CD8, M2 macrophages, B cell naive, and activated master cells. Among them, statistical tests of correlation analysis were significant between activated mast cells and plasma cells, activated mast cells and eosinophils, resting mast cells and monocytes, and resting mast cells and neutrophils.

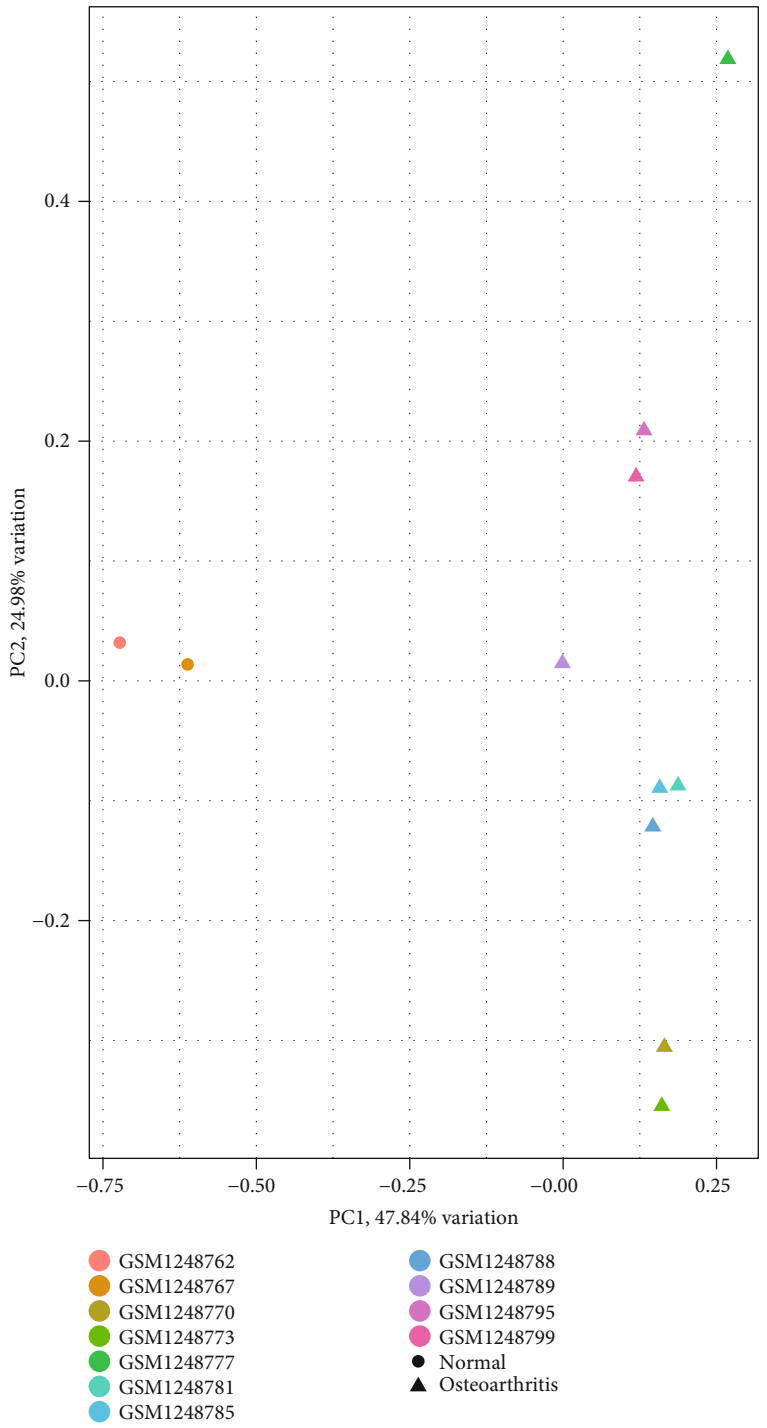
As shown in Figure 4(d), using unsupervised hierarchical clustering based on the above-identified cell subpopulation in subchondral bone, the samples of pathological and normal could be clearly separated into two discrete groups.

In subchondral bones, the proportions of immune cells, from the PCA, displayed distinct group-bias clustering (Figure 5(a)). PC1 appeared to discriminate further between normal and OA groups, with 47.84% variation. Resting mast cells and T cell CD8 were the major component of PC1 (Figure 5(b)). The above-mentioned cells might be related to the pathological mechanism of OA.

Collectively, in subchondral bones, these results indicated that aberrant immune infiltration and its heterogeneous in OA also might have important clinical meanings. It is worth noting that the proportion of master cells was high in knee subchondral bones. And the difference of their proportion in OA patients and normal people had statistical significance. It also had certain clinical diagnostic significance for OA. Therefore, we speculated that master cells played an important role in the pathogenesis of OA. Compared with synovial tissue, the composition of immune cells appeared to discriminate further between normal and OA groups. Thus, the bone immune response in subchondral bones was relatively important pathogenesis.

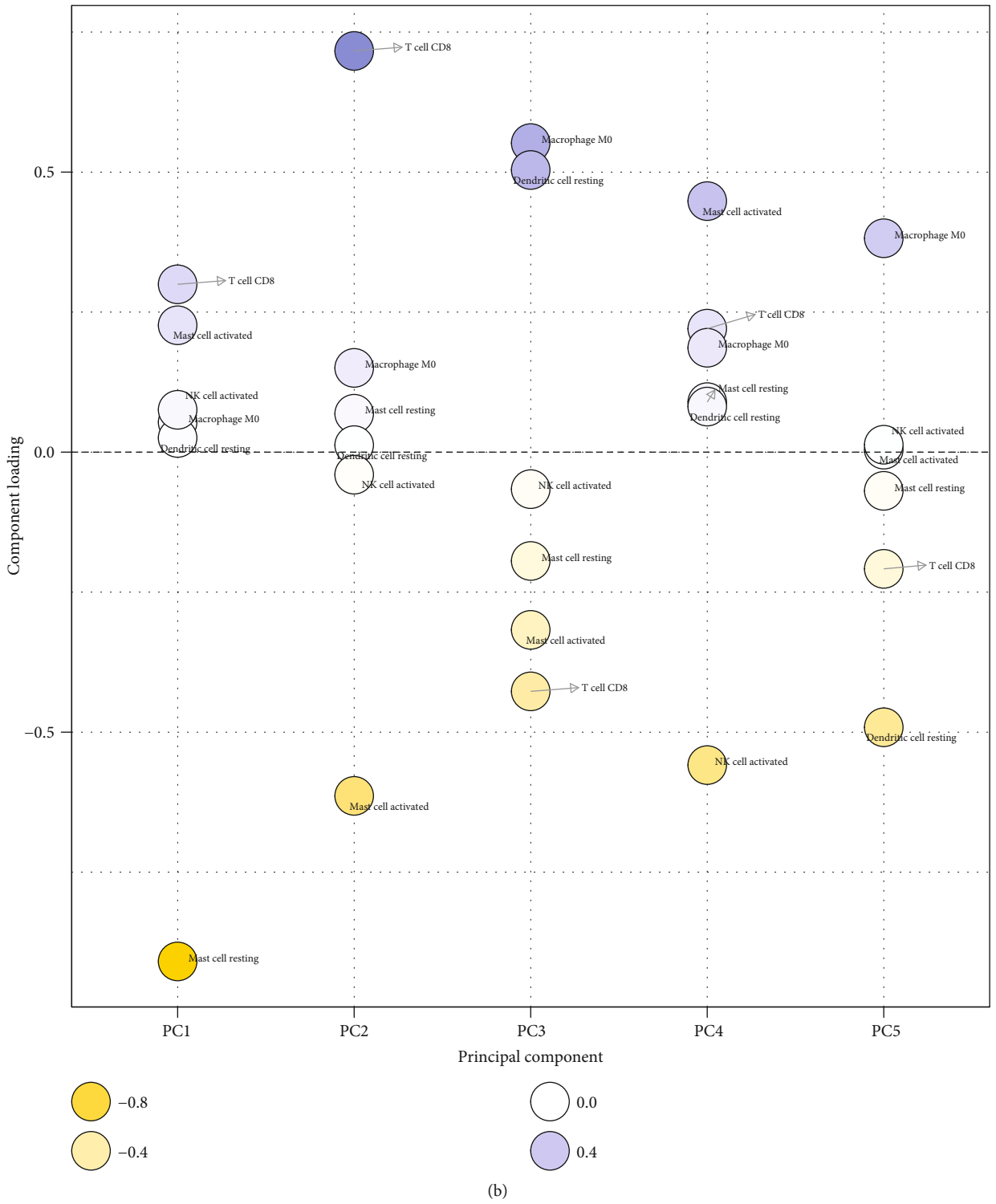
3.3. Meniscus and Cartilage. Using the CIBERSORT algorithm, we found that the CIBERSORT *P* values of 22 subpopulations of infiltrating immune cells in 71 cartilage OA samples and 25 OA meniscus samples from 6 studies were higher than 0.05 (Figure 6(a)).

As mentioned before, the *P* value derived by CIBERSORT could reflect the proportion of a sample that comprises immune cells versus nonimmune cells. This indicated that



(a)

Figure 5: Continued.



(b)

Figure 5: The diagnostic value of composition of infiltrating immune cells for osteoarthritis in subchondral bone. (a) Principle components analysis (PCA). (b) Component loading in PCA results.

there was almost no infiltration of immune cells in the meniscus and cartilage of the knee joint. Therefore, we speculated that in the pathogenesis of OA, cartilage and meniscus lesions mainly came from mechanical injury, humoral immunity, and other factors. Figure 6(a) also showed a high degree of immune cell infiltration in synovial OA tissues (76.47%, CIBERSORT $P \leq 0.01$).

The degree of immune cell infiltration into the tissue is a crucial prognostic factor. To characterize the correlations between immune cell composition and the degree of immune

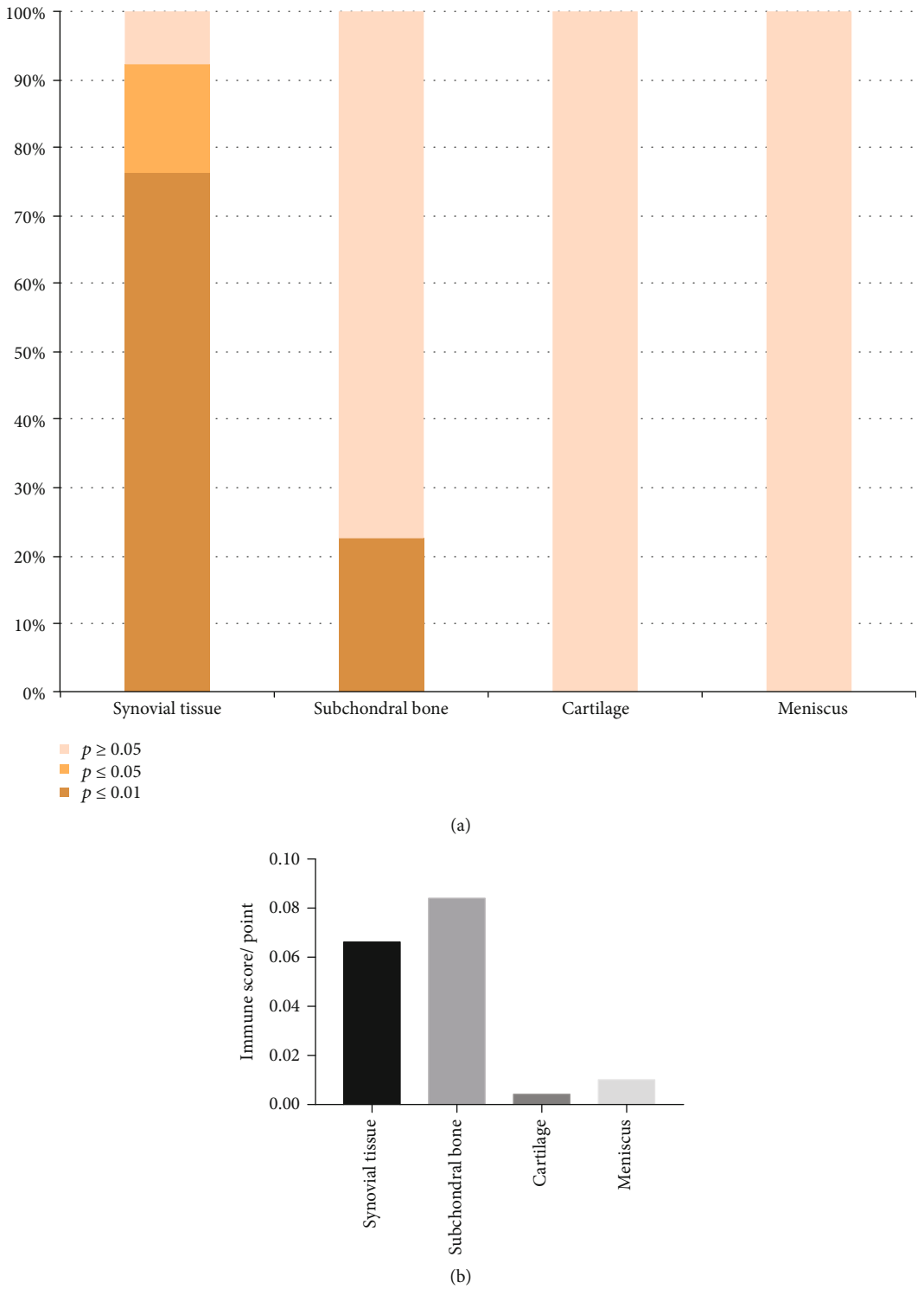


FIGURE 6: Overall infiltration of immune cells in different osteoarthritis tissues. (a) Overall proportion of immune cells in different osteoarthritis tissues. $P \leq 0.01$: high infiltration of immune cells; $0.01 < P \leq 0.05$: medium infiltration of immune cells; $P > 0.05$: low infiltration of immune cells. (b) Immune scores in different osteoarthritis tissues calculated by xCell.

cell infiltration in OA, Pearson correlations of 22 immune cell types with CIBERSORT P values were calculated. Finally, we found that activated mast cells were mainly associated with high immune cell infiltration in OA, no matter whether in synovium (correlation coefficient = 0.713) or subchondral bone (correlation coefficient = 0.359). P values < 0.002 were considered as statistical significance (Bonferroni correction).

3.4. Similar Results Calculated by xCell and CIBERSORT Algorithms. Immune scores obtained by xCell showed

Table 2: Comparison of immune cell abundance in osteoarthritis subchondral bones and osteoarthritis synovial tissues calculated by xCell and CIBERSORT algorithms.

	Cell type	xCell score	Correlation between xCell and CIBERSORT		Qualitative consistency xCell vs. CIBERSORT
			Correlation coefficient	*P* value	
Subchondral bone	Neutrophils	0.0039 ± 0.00175	0.602	<0.001	Yes
Synovial tissue	Plasma cells	0.0066 ± 0.00163	0.555	<0.001	Yes
	Macrophage M1	0.0067 ± 0.00101	-0.087	0.410	No
	Macrophage M2	0.0071 ± 0.00120	0.233	0.026	Yes
	Eosinophils	0.0001 ± 0.00006	0.075	0.479	Yes*

*Qualitative consistency without statistical significance.

immune cell infiltration in the cartilage, and the meniscus was very low (Figure 6(b)), which was similar with the result obtained by CIBERSORT. Moreover, xCell scores of immune cells were mostly consistent with proportions of immune cells calculated by CIBERSORT (Table 2).

4. Discussion

In the present study, we applied CIBERSORT to assess differential immune cell infiltration in osteoarthritic tissues and normal tissues in different structures of knee in osteoarthritis.

We found that M2 macrophages infiltrated in the synovium accounted for a high proportion, so the synovium might be as the immunogenic location for M2 macrophages playing an important role in OA. Previous studies have found that there was a certain correlation between macrophages and OA [32, 33]. Takano et al. found that interleukin- (IL-) 1β induced by macrophages in the synovium could upregulate calcitonin receptor in a mouse OA model, and calcitonin gene-related peptide was involved in the occurrence of arthritis-related pain [34]. Daghestani et al. have assessed the inflammatory phenotypes predicted by macrophage biomarkers in synovial fluid and blood of patients with KOA. And they found that CD14, CD163 in synovial fluid, and CD163 in serum were associated with a large number of active macrophages, while CD163 and CD14 were associated with formation of osteophyte in knee joint. CD14 was also associated with the severity of knee joint space narrowing, while CD14 in synovial fluid and serum was associated with knee pain [35]. There are different types of macrophages, and Mills et al. proposed the M1–M2 terminology in 2000 [36]. Macrophages activated through a pathway opposite to the classical pathway are referred to as M2 or the alternative pathway. It has been demonstrated that stimuli such as CSF-1, IL-4, IL-10, TGF-β, IL-13, fungi, and helminth infections favor M2 subpopulation polarization, delivering IL-10 in high concentrations, and IL-12 in low amounts. A series of studies have found that immune-suppressive, proangiogenic M2 macrophages play a central role in responses to parasites, tissue remodeling, angiogenesis, and allergic diseases [37, 38]. In addition, CD163 is one of the markers of macrophage M2 [39]. Combined with our result that M2 macrophages were correlated to OA, we speculated that infiltrating M2 macrophages in synovium might play a vital role in the pathogenesis of OA.

Subchondral bone is another important component in the progress of OA. Osteosclerosis in subchondral bone caused by abnormal changes of subchondral bone could occur in the early stage of OA. Moreover, some studies have found that subchondral bone might be the initial cause of osteoarthritis [40, 41]. Therefore, current research on the pathogenesis of osteoarthritis and the research about new treatment of KOA have focused on the role of subchondral bone in the pathogenesis and progression of osteoarthritis [42]. Most of previous studies related to subchondral bone and the pathogenesis of osteoarthritis focused on bone metabolism and biomechanical mechanisms [43–45]. Mast cells are the most important effector cells in the innate immune system. They are transformed from hematopoietic cells produced by the precursors of pluripotent bone marrow stem cells. Mast cells have attracted much attention in the field of rheumatoid arthritis [46–48]. Ruschpler et al. have found that mast cells played an important role in the pathogenesis of rheumatoid arthritis [49]. However, compared with studies in rheumatoid arthritis, the number of studies about mast cells in osteoarthritis is far less. In the present study, less resting mast cells and more activated mast cells were found in the subchondral bone of OA patients. Therefore, the immunological study of subchondral bone in osteoarthritis and the influence of mast cells on it should be paid more attention.

Moreover, we found activated mast cells were mainly associated with high immune cell infiltration in OA, in both of synovium and subchondral bone. Mast cells could be activated by different stimuli [50]. However, CIBERSORT enumerates specifically IgE-activated mast cells because the gene expression signature used for deconvolution was obtained from mast cells stimulated by IgE [15]. Mast cells are key regulators of immune effector cells [51]. Therefore, their activation could be a desired aim of immunotherapy. Moreover, we revealed the potential interaction between these immune cells and other immune cells in our study.

The present study only focused on immune cell infiltration. However, other forms of immune response remain non-negligible. In the degeneration of articular cartilage, extracellular matrix, which protects special surface antigens on chondrocyte from the immune system, disappeared and

immune barrier was destroyed. Huber-Lang et al. found that on the surface of posttraumatic debris, a variety of products activated by complements were found on the surface of chondrocyte [52]. Jong et al. have proved that cartilage proteoglycan peptide located in G1 domain could induce T cell reaction and promote cartilage degradation in patients with OA. Moreover, in proteoglycan and Yersinia outer proteins, there is a same amino acid region, 263-283 sites, which could induce immune response in patients with OA [53]. Frisenda et al. used heterogeneous type II collagen to immunize mice, which could induce arthritis, suggesting that cartilage collagen was also a potential target of autoimmune reaction [54]. Combined with the results of our study, it could be speculated that in the cartilage of patients with OA, the immune response might occur more in the form of humoral immunity or other ways that did not require the infiltration of immune cells.

Similarly, type I collagen is one of the components in meniscus. When the meniscus was damaged, the exposed type I collagen could also stimulate autoimmune response [55]. In addition, inflammatory mediators have been shown to be able to cooperate with nitric oxide, inhibit the synthesis of collagen II and proteoglycan, and accelerate their degradation [56]. Combining with the relative lack of blood supply and lymph nodes in the meniscus and cartilage, the results of the present study excluded the influence of immune cell infiltration in the meniscus and cartilage on OA to a certain extent.

xCell is a novel gene signature-based method for inferring 64 cell types including stromal cells and stem cells. Therefore, xCell needs more genes for analysis and calculation than CIBERSORT. In the present study, GSE12021 for synovial tissue was not selected for xCell analysis due to the insufficient number of genes.

There are still some limitations to be acknowledged. First, healthy knee samples were rare, and normal samples in our study were mostly collected after amputation or osteotomy which potentially influences immune infiltration. Second, in order to enlarge our sample size, several studies from different platform were combined. Although we conducted statistical methods to eliminate the bias, heterogeneity in these data still impeded the repeatability to some extent. Third, data in the present study only could provide the correlation analysis between OA and immune cells, instead of the exploration of the cause and effect relationship. However, as mentioned above, previous studies have demonstrated that immunological mechanisms play the vital role in the pathogenesis of OA. Thus, we speculated that different infiltrating immune cell subpopulations were the potential reason of OA. Finally, the present study was based on publicly accessible array datasets. Some basic characteristics of patients including age were missing. Meanwhile, the immunologic function could be influenced by many factors, including age and gender [6]. However, pathological changing is always considered as the main factor of the local infiltration of immune cells. Therefore, sometimes other factors were neglected when analyzed [18, 57, 58]. Our study mainly proposed some new ideas in immunology for researches related to knee osteoarthritis. Further researches are still necessary to validate our speculation.

5. Conclusion

The immune cell composition in OA differed substantially from that of healthy joint tissue, while it also differed in different anatomical structures of the knee. Meanwhile, activated mast cells were mainly associated with high immune cell infiltration in OA. Furthermore, we speculate M2 macrophages in synovium and mast cells in subchondral bone may play an important role in the pathogenesis of OA.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors certify that they have no affiliations with or involvement in any organisation or entity with any financial interest or nonfinancial interest in the subject matter or materials discussed in this manuscript.

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Review Article

Friend or Foe? Essential Roles of Osteoclast in Maintaining Skeletal Health

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Heightened activity of osteoclast is considered to be the culprit in breaking the balance during bone remodeling in pathological conditions, such as osteoporosis. As a "foe" of skeletal health, many antiosteoporosis therapies aim to inhibit osteoclastogenesis. However, bone remodeling is a dynamic process that requires the subtle coordination of osteoclasts and osteoblasts. Severe suppression of osteoclast differentiation will impair bone formation because of the coupling effect. Thus, understanding the complex roles of osteoclast in maintaining proper bone remodeling is highly warranted to develop better management of osteoporosis. This review aimed to determine the varied roles of osteoclasts in maintaining skeletal health and to highlight the positive roles of osteoclasts in maintaining normal bone remodeling. Generally, osteoclasts interact with osteocytes to initiate targeted bone remodeling and have crosstalk with mesenchymal stem cells and osteoblasts via secreted factors or cell-cell contact to promote bone formation. We believe that a better outcome of bone remodeling disorders will be achieved when proper strategies are made to coordinate osteoclasts and osteoblasts in managing such disorders.

1. Introduction

Bone is a dynamic organ that continuously remodels in a well-orchestrated manner to support body-required mechanical characteristics and maintain calcium homeostasis throughout one's lifetime [1, 2]. This constant remodeling process requires delicate coordination from multiple cell types, in which hematopoietic stem cell- (HSC-) derived osteoclast (OC) lineage and bone marrow mesenchymal stem cell- (BMSC-) derived osteoblast (OB) lineage receive the most attention [3–5]. Balance between bone resorption by OCs and bone formation by OBs is usually maintained during the physiological process but dies away under pathological conditions, such as inflammation, diabetes, aging, and cancer, resulting in bone remodeling-related disorders and diseases, such as osteoporosis, periodontitis, inflammatory arthritis, Paget's disease, or tumor-induced osteolytic bone metastasis [6–10]. OCs, the giant cells that are responsible for bone removal in the skeletal family, have always been considered to be the main culprit in these disorders and diseases because of its overactive functionalities under pathological conditions [7, 8]. Therefore,

antiresorptive drugs, such as bisphosphonates, receptor activator of nuclear factor-κB (RANK) ligand (RANKL) inhibitor, estrogen, or selective estrogen receptor modulators, are prevalent therapeutics that target osteolysis and rescue bone loss [11–13].

Recently, with the in-depth study in bone physiology, OCs, the giant (but not a fool), are manifesting more complex identities beyond their resorptive function. In particular, the reciprocal interactions between bone cells are attracting much attentions [14–16], because of the advanced understanding of the bone coupling between osteoclastic bone resorption and osteoblastic bone formation [3, 17, 18]. Through cell-cell contact, cell-bone matrix interaction, and paracrine factors, OCs have crosstalk with other bone cells, stem cells, and immune cells in the bone microenvironment, which affects recruitment, differentiation, and function of not only themselves but also the other cells [19–21]. This effect of OCs on other cells is more apparent during skeletal aging due to deteriorations on mesenchymal stem cell/mesenchymal stromal cell- (MSC-) derived osteogenesis and chondrogenesis, while HSC-derived osteoclastogenesis advances with increasing age, thereby gaining the initiative in the bone remodeling process and functioning predominantly over other factors [22–25]. It should be noted that OC-derived activities have both positive and negative effects, and those "pure" antiresorptive drugs (bisphosphonates or denosumab) for age-related bone disorder usually inhibit bone resorption with a concomitant reduction in bone formation owing to bone coupling, indicating the importance of OCs in maintaining normal bone remodeling after adulthood [11, 26, 27].

This review aimed to determine the essential roles of OC not just as a bone eater during bone remodeling but also as a positive contributor to the bone microenvironment and skeletal health. Specifically, we discuss how OCs contribute to the recruitment and differentiation of MSCs, as well as the following bone formation during remodeling. We hope this review can provide a different perspective on recognizing OCs when strategies are created to develop ideal therapeutic agents that target bone remodeling disorders characterized by excessive OC activity.

2. Osteoclasts and Bone Remodeling

Unlike bone modeling, which does not require coupled activities of OCs and OBs during skeletal growth and development, bone remodeling demands anatomically or spatially coupled activities of OCs and OBs to replace the old and damaged bone and to maintain calcium homeostasis in the body throughout one's life [28]. Each year, approximately 3 to 4 million basic multicellular units (BMUs) responsible for bone remodeling are initiated, and about 1 million of them are highly active as a standby for participating in bone turnover in the adult skeleton [28–30]. The remodeling process inside the BMUs does not occur randomly along the bone surface, but rather at specific sites, and it follows a well-orchestrated sequence of events that are typically divided into five stages: the activation of OC recruitment, initiation of osteoclastic bone resorption, transition from catabolism to anabolism due to OC apoptosis and OB recruitment, formation of the new organic matrix by OBs, and subsequent mineralization over time [28, 31]. In healthy adults, under physiological conditions, bone mass can be stable for one or two decades after reaching the peak volume due to a balance of the bone resorption and bone formation, that is, until age-related imbalance starts (heightened OC activity and reduced OB performance) [6, 22, 28].

OCs, the unique bone-resorbing cells, arise from HSCs and belong to the monocytic family [21, 32]. In the activation phase of bone remodeling, mononuclear OC precursors in the bone marrow or from blood circulation are attracted to prospective resorption sites, where they attach to the matrix surface and further differentiate into mature OCs (giant multinucleated cells) via cell fusion, termed as "multinucleation" [5, 32–34]. Mature OCs start to generate sealing zones on the targeted matrix surface during the resorption phase via the rearrangement of the cytoskeleton and the formation of a dense belt-like structure called the "actin ring" [35, 36]. The actin ring encloses the plasma membrane and makes it into a highly convoluted ruffled border which then serves as an exit site for protons and lysosomal proteases, such as cathepsin K (CTSK) to be secreted into the resorption lacunae, facilitating hydrolyzation and solubilization of the inorganic and organic components of bone [5, 20, 37]. By sensing the concentration of extracellular calcium $[Ca^{2+}]_o$ around the cell and responding to the change of intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, OCs switch between the resorbing state featured by possessing actin rings and the nonresorbing/migrating state featured by scattered podosomes [38–40]. The resorbing activity of OCs gradually declines when basal $[Ca^{2+}]_i$ increases, whereas lower $[Ca^{2+}]_i$ reduces cell motility but enhances the anchoring capacity of the cell onto the bone matrix surface [38, 41]. Once resorption at one site is completed, OCs can move and start a new resorption cycle somewhere else or undergo apoptosis based on their lifespan [32, 36]. Among key molecules and signaling pathways involved in the process of osteoclastogenesis and resorption activity, RANK signaling is dominant through the entire life cycle of OCs and can be further amplified by costimulatory signals from immunoreceptor tyrosine-based activation motif- (ITAM-) associated immunoglobulin-like receptor (IgLR) signaling [38, 42–44]. Details of the RANK signaling network, along with other critical pathways that cooperate with it, such as calcium signaling pathway (Ca^{2+}/calmodulin/calcineurin/NFATc1) and oxidative stress response pathway (ROS/Nrf2/Keap1), have been well summarized in several excellent papers and will not be discussed further in this review [32, 38, 45–47].

Recent advances widely explored the origins of OCs and associated them with aging and other pathological scenarios. It was not until the last decade that researchers started to decipher how aging affects the skeletal system tremendously. While osteogenic and chondrogenic differentiation from MSCs deteriorates, aging upgrades OC progenitors in both quality and quantity, including increased intrinsic expression of c-Fms and RANK, and enlarged OC progenitor pool

[22–25]. As the origin of the OC progenitor, HSCs contribute to the reinforcement of the progenitors' pool by giving a bias toward myeloid development over lymphoid differentiation with increasing age [23, 48]. Madel et al. recently summarized different origins of OCs in an age-dependent manner (Figure 1): the embryonic erythro-myeloid progenitor (EMP) lineage during the embryonic and postnatal period, bone marrow myeloid/monocyte/macrophage (BMMs) lineage during adulthood, and conventional/mature monocytes (MNs), as well as dendritic cells (DCs) under inflammatory conditions which are usually seen in old age [21, 49]. In addition to the promotion of OC progenitors during aging, OC supporting cells, such as OBs, B cells, and T cells, also contribute to osteoclastogenesis by increasing RANKL expression and reducing osteoprotegerin (OPG) level in the bone microenvironment, although the population of these cells decreases with increasing age [24, 50–52]. Therefore, OC is vulnerable to be treated as a "foe" of skeletal health because of hyperactivity, especially in aged individuals. However, it does not negate its substantial role as a "friend" in removing the old and damaged bone, as well as a positive contributor during bone formation after adulthood, which has become more understandable in the last few years.

Several *in vitro* studies indicated that OC-derived factors directly affect MSC recruitment and OB differentiation [53–56]. Karsdal et al. reported that conditioned media (CM) from human OCs increased bone nodule formation in a dose-dependent manner, which was further confirmed by Kreja et al. [53, 54, 56]. Interestingly, they also found that the effect of OCs on MSC migration and OB differentiation can be independent of their resorption activity. Likewise, Henriksen's study indicated that mature OCs were sources of anabolic stimuli for OBs, and their interaction with the matrix can strongly affect the anabolic signals from OCs to OBs [55]. Conversely, a reduced number of OBs and bone formation were found in OC-poor osteopetrosis, indicating a critical role of OCs in regulating bone anabolic function [57]. All these findings suggest complex identities of the giant beyond the resorption function.

3. Osteoclasts and the Initiation of Bone Remodeling

The initiation phase of bone remodeling includes the recruitment of OC precursors, differentiation and functioning of OCs, and maintenance of bone resorption [28, 31]. The initiation of osteoclastogenesis largely depends on the crosstalk between OC precursors and the OB lineage cells. Emerging data supports the central regulatory role of osteocytes in the initial stage of bone remodeling [58–62]. As the most abundant cells in bone that are derived from OBs and embedded in the bone matrix, osteocytes play a role in determining which bone surface OCs are about to resorb [58, 59]. Through a network of osteocyte canaliculi, osteocytes can detect microfractures and microcracks in bone and contact other cells, such as OBs, on the bone surface. Bone fatigue induces apoptosis of osteocytes, which are localized to regions that contain microcracks, and this apoptosis was observed to precede OC invasion in the damaged area, which triggers subsequent bone remodeling in the targeted region [63].

Osteocytes have also crosstalk with OCs via secreted proteins. Osteocytes can control OC function by secreting RANKL and transforming growth factor beta (TGF-β) [64, 65]. RANKL, one of the essential osteoclastogenic factors, is mainly secreted by osteocytes [65–67]. Nakashima et al. [65] demonstrated that osteocytes express a much higher amount of RANKL and have a better capacity to support osteoclastogenesis than OBs and bone marrow stromal cells, which is a strong evidence for the crosstalk between osteocytes and OCs in bone remodeling. The MLO-Y4 osteocyte-like cell line represents a good model for studying the soluble interactions between osteocytes and OCs [64]. When mechanical scratching was applied to MLO-Y4 cells, enhanced secretion of osteoclastogenic factors, RANKL, and the monocyte colony-stimulating factor (M-CSF) was observed. The mechanical scratching of osteocytes induced the formation of tartrate-resistant acid phosphatase- (TRACP-) positive cells on top of the gel along the damaged region. No TRACP-positive cells were formed in the peripheral regions [59]. These findings indicate that soluble factors secreted from damaged osteocytes could locally induce and activate the initial phase of OCs formation.

The initiation of bone remodeling at the targeted bone site is essential for the renewal of an old or damaged bone matrix to prevent the skeleton from aging. Failure to trigger bone remodeling can result in accumulated microdamage and hypermineralization, which leads to reduced bone quality and increased fracture risk. Thus, retaining the crosstalk between OCs and osteocytes is beneficial for skeletal health when managing high turnover bone disorders, such as osteoporosis.

4. Effect of Osteoclasts on Mesenchymal Stem Cell Recruitment and Osteoblast Differentiation

After the old or damaged bone is resorbed by OCs, bone remodeling enters the second phase: the transition of OC to OB activity. In this reversal phase of bone remodeling, the microenvironment created by OC activity provides signals that aid in the cessation of bone resorption and the initiation of bone formation via the recruitment and differentiation of MSCs [17, 68]. The bone resorptive microenvironment is built by multiple factors that are released from the bone matrix during bone resorption or directly secreted by OCs locally, which also contribute to the establishment of the osteogenic microenvironment that promotes the recruitment of MSCs [4, 69–71]. MSCs are multipotent stem cells that are capable of differentiating into various cell types, such as OBs, adipocytes, and chondroblasts [72, 73]. In the bone marrow, MSCs are located around sinusoids and the perivascular network in the stroma [74, 75]. During bone remodeling and fractured-bone regeneration, MSCs migrate to the bone surface or fracture site and then differentiate into OBs to reconstruct the bone [76], subsequent to the

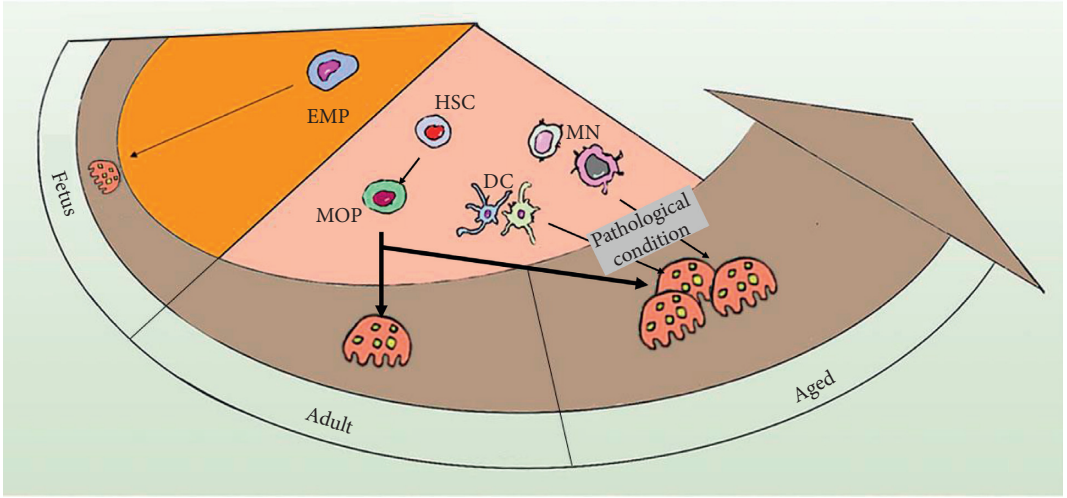

Figure 1: Origins of osteoclasts in an age-dependent manner [21]. Osteoclasts (OCs) differentiate from the embryonic erythro-myeloid progenitor (EMP) lineage during the embryonic and postnatal period. In adulthood, bone marrow myeloid/monocyte/macrophages (BMMs) derived from hematopoietic stem cells (HSCs) are the main origin of osteoclasts. Moreover, monocytes (MNs) and dendritic cells (DCs) are also important origins of osteoclasts in aged or pathological conditions. MOP: macrophage/osteoclast progenitor.

osteoclastic resorptive phase. It has been well demonstrated that local growth factors and signals play important roles in the recruitment and commitment of MSCs [77], such as the bone morphogenetic protein (BMP) family [78], insulin-like growth factor (IGF) [79, 80], TGF-β [68, 81], fibroblast growth factor 2 (FGF-2) [82], vascular endothelial growth factors (VEGF) [78], and platelet-derived growth factors (PDGFs) [83, 84]. Moreover, emerging evidence showed that many of these local factors are associated with the viability and activity of OCs [17, 20, 54].

4.1. Osteoclastic Resorption Releases Bone Matrix Embedded Factors and Recruits Mesenchymal Stem Cells. Factors released from the bone matrix during bone resorption may be the first signal from OCs that has been found to affect MSCs. The bone matrix contains many latent growth factors that are deposited by OBs during matrix construction and then released by osteoclastic resorption on the bone surface [85, 86]. Howard et al. [87] firstly proposed that the release of coupling factors embedded in the bone matrix may positively affect MSC-derived osteogenesis. To date, several matrix-derived factors have been identified as potential factors involved in bone remodeling, such as TGF-β [85, 88], IGF-1 [69], bone morphogenetic protein (BMP)-2 [89, 90], and vascular endothelial growth factor (VEGF) [91]. In particular, matrix-derived TGF-β1 and IGF-1 have shown definite effects linking bone resorption to MSC recruitment and differentiation based on genetically manipulated mice data. Tang et al. [88] demonstrated that TGF-β1 released during OCs culture on bone slices *in vitro* induces the migration of MSCs. They also found high levels of active TGF-β1 in the bone resorption-conditioned media (BRCM) when functional OCs were cultured with bone slices *in vitro*, whereas active TGF-β1 was barely detectable in the conditioned media prepared without bone slices. Moreover, BRCM prepared using OCs generated from normal mice and bone slices prepared from TGF-β1 1 knockout (TGF-β1−/−) mice was significantly less effective in promoting the migration of BMSCs [88], demonstrating that matrix-derived TGF-β1 plays a key role in recruiting MSCs. Similarly, it has also been well demonstrated that IGF-1 released from the bone matrix by functioning OCs stimulated OB differentiation of MSCs by activating the mammalian target of rapamycin (mTOR) through the PI3K-Akt pathway [69].

4.2. Osteoclast-Secreted Factors Recruit Mesenchymal Stem Cells and Promote Osteoblast Differentiation. Besides the matrix-derived factors, increasing data also suggest that factors directly secreted by OC lineage cells play a crucial role in coupling osteoclastic bone resorption with osteoblastic bone formation. Henriksen et al. [55] performed a research to address the anabolic effect of OC linage cells in different stages. They collected the conditioned medium (CM) from macrophages, pre-OCs, and mature functional or nonresorbing OCs and tested their effects on osteogenesis *in vitro*. Their results suggested that CM from macrophages did not induce bone formation, while CM from mature OCs promoted osteogenesis, both dependent on and independent of their resorptive activity. Kim et al. [56] also conducted a research to explore when the coupling factors are taking effect during osteoclastogenesis. They found that CM from OCs in the early stage of differentiation predominantly enhanced the migration of osteoblastic lineage cells, confirming that OCs play an important role in the coupling by stimulating pre-OBs migration.

To date, increasing studies have identified numerous secreted molecules from OCs and explored their potential roles in bone remodeling. In Table 1, we have summarized the OC-secreted factors and their effects on MSC migration, OB differentiation *in vitro*, or bone formation *in vivo*. Among them, factors including Afamin [56], CXCL16 [98],

Table 1: Summary of osteoclast-secreted factors on bone remodeling.

Factor secreted by osteoclasts		Effect on bone remodeling	Reference
Osteoclast-derived enhancing factors of bone formation			
Afamin	Afamin	Afamin secreted by osteoclasts in the early stage of differentiation stimulates preosteoblasts migration *in vitro* via the Akt-signaling pathway Afamin can prevent Wnt proteins from aggregating and deliver Wnt ligands to its receptors on the cell surface, which plays an important role in osteogenesis	[56, 92]
BMP6	Bone morphogenic protein 6	Synthesis of BMPs has been confirmed in osteoclasts using immunocytochemistry and in situ hybridization BMP6 promotes osteoblast differentiation	[93, 94]
C3a	Complement component 3a	C3 gene expression increases during osteoclastogenesis, and the cleavage product C3a is detected in the conditioned medium of osteoclasts C3a promotes osteoblast differentiation	[95]
CT-1	Cardiotrophin-1	CT-1 promotes osteoblast differentiation Neonatal Ct-1−/− mice have decreased osteoblast numbers and BV/TV	[96]
CTHCR1	Collagen triple repeat containing1	CTHCR1 is secreted by mature bone-resorbing osteoclasts CTHCR1 stimulates osteoblast differentiation Osteoclast-specific deletion of CTHCR1 in mice resulted in osteopenia due to reduced bone formation	[97]
CXCL16	Chemokine (C-X-C motif) ligand 16	TGF-β1 released from the bone matrix during bone resorption induces CXCL16 production in osteoclasts, which promotes migration of osteoblast progenitors in bone remodeling	[98]
HGF	Hepatocyte growth factor	Osteoclasts can synthesize and secrete biologically active HGF, which promotes osteoblast proliferation and increases osteopontin expression in osteoblasts	[99, 100]
PDGF-BB	Platelet-derived growth factor BB	PDGF-BB induces MSC migration, but it inhibits osteoblast differentiation	[53, 101–103]
S1P	Sphingosine-1-phosphate	S1P stimulates MSC migration and promotes osteoblast differentiation Raising S1P levels in adult mice markedly increased bone formation	[104–106]
SLIT3	slit guidance ligand 3	Osteoclast-secreted SLIT3 synchronously inhibits bone resorption and stimulates bone formation SLIT3 injection in mice markedly rescued bone loss after ovariectomy surgery	[107]
TRAP	Tartrate-resistant acid phosphatase	TRAP promotes osteoblast differentiation TRAP overexpressing transgenic mice have an increased rate of bone turnover	[108, 109]
Vesicular RANK	Vesicular TNF receptor superfamily member 11A	Mature OCs secrete vesicular RANK, which binds osteoblastic RANKL and promotes bone formation via triggering RANKL reverse signaling	[110]
Wnt10b	Wnt family member 10b	Wnt10b expression increases during osteoclastogenesis Wnt10b promotes mineralization	[104]
Osteoclast-derived inhibiting factors of bone formation			
LIF	Leukemia inhibitor factor	LIF inhibits TGFb1-induced osteoblast migration	[98]
Sema4D	Semaphorin 4D	Sema4D suppresses bone formation by inhibiting IGF-1 signaling Sema4d-/- mice show an osteosclerotic phenotype due to augmented bone formation	[111]
SOST	Sclerostin	SOST is expressed in osteoclasts from aged mice and inhibits osteoclast-mediated stimulation of mineralization	[112]
Exosomal miR-214-3p	Exosomal miR-214-3p	miR-214-3p reduces bone formation in elderly women with fractures and in ovariectomized mice	[113, 114]

PDGF-BB [101, 102], and S1P [104, 105] secreted by OCs can promote the migration of MSC or OB progenitors, and factors such as BMP6 [98], C3a [95], CT-1 [96], CTHCR1 [97], HGF [99, 100], SLIT3 [107], Trap [108, 109], and vesicular RANK [110] exhibit enhancing effects on OB differentiation *in vitro* or bone formation *in vivo*. However, some other factors such as Sema4D [111], sclerostin [112], and exosomal miR-214-3p [113] show an inhibiting effect on bone formation. These factors may act as a "fine-tuning" mediator of the bone remodeling process in the BMUs, by inhibiting the remodeling process under some special conditions. Besides, these factors are often highly expressed in OCs from aged or ovariectomized mice, suggesting that they may play a role in bone remodeling disorders during aging. Overall, on the basis of the current findings, most OC-secreted factors show enhancing effects on MSC recruitment or OB differentiation, indicating an essential role of OCs in maintaining normal bone formation during the remodeling process.

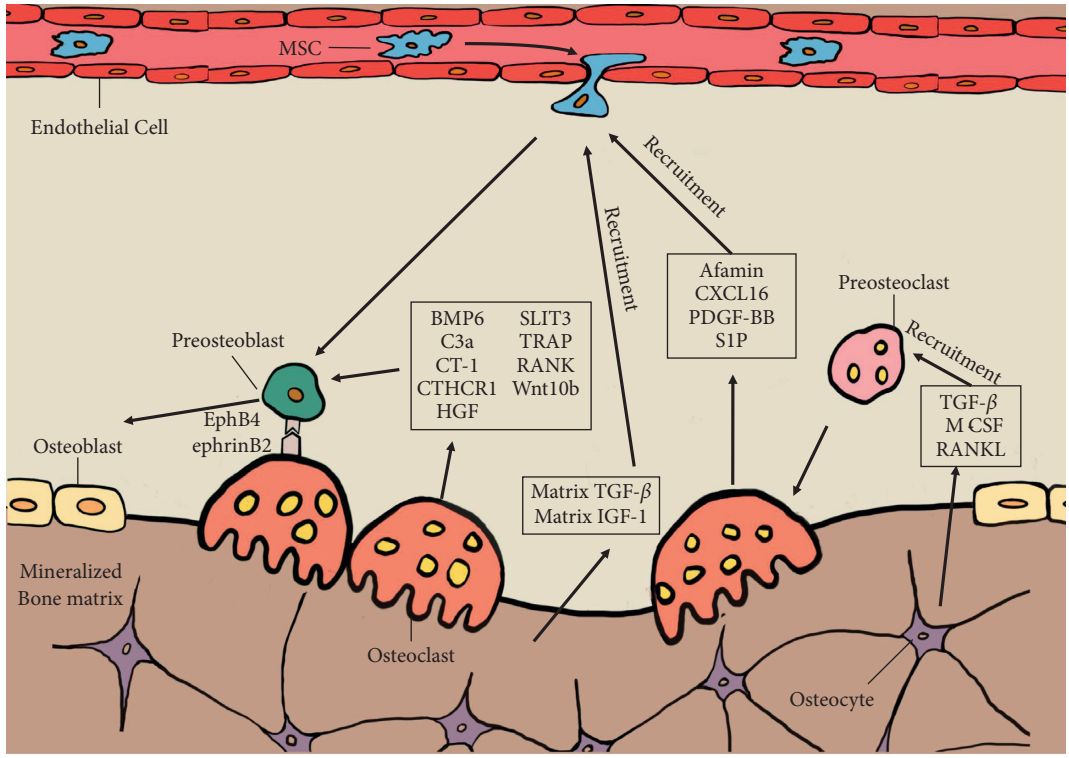

Figure 2: Schematic illustration of the interaction between osteoclast (OC) and osteoblast (OB) lineage cells in bone remodeling. OC precursors are activated by TGF-β, M-CSF, and RANKL secreted by osteocytes and attracted to prospective resorption sites. Once attached to the bone matrix, OC precursors can differentiate into mature OCs. Mature OCs will further acidify and resorb the mineralized bone matrix by pumping hydrogen ions into resorptive captivity through their ruffled border structure. During bone resorption, OC can release several coupling factors, such as matrix-derived TGF-β, matrix-derived IGF-1, Afamin, CXCL16, PDGF-BB, and S1P et al., which recruit circulated mesenchymal stem cells (MSCs) to the resorption area. Besides, OC also secretes some other coupling factors, such as BMP6, SLIT3, C3a, TRAP, CT-1, and RANK et al., which further promote the differentiation from MSCs towards OBs. Additionally, the ephrinB2/ephB4 interaction between OC and OB precursors suppresses the bone resorption activity of OCs, whereas such interaction could trigger OB differentiation of OB precursors and enhance bone formation.

4.3. Osteoclast and Osteoblast Cell-Cell Contact: A Potential Mechanism of Transition in Bone Remodeling. OCs and OB lineage cells can also communicate through cell-cell contact to achieve the coupling of bone resorption and formation. Traditionally, it has been thought that OCs and OBs do not occur simultaneously at the same BMUs, and direct contact between mature OBs and functioning OCs is relatively rare [115]. In recent years, direct OC–OB contact *in vivo* has been detected using transmission electron microscopy [31] and intravital two-photon imaging [116]. Furuya et al. demonstrated that mature OCs became nonresorptive when they made contact with mature OBs, and intermittent administration of the parathyroid hormone (PTH) led to an increase in cell-cell contact between OCs and OBs, which causes bone anabolic effects [116].

How does the cell-cell contact cause bidirectional effects between OCs and OBs? EphrinB2/EphB4 interaction between OCs and OBs plays a role in the transition from bone resorption to the formation. Ephrin/Eph family members are local mediators of cell function through contact-dependent manner during various developmental processes [117, 118]. Interaction between ephrin-expressing and Eph-expressing cells leads to bidirectional signal transduction. Mature OCs express ephrinB2, whereas OB precursors express EphB4 (Figure 2). Forward signaling through the EphB4 receptor into OB precursors enhances osteogenic differentiation by reducing RhoA activity, while reverse signaling through ephrinB2 ligand into OCs suppresses OC function by inhibiting the osteoclastogenic c-Fos-NFATc1 cascade [119]. However, it has also been suggested that mice lacking ephrinB2 showed no skeletal abnormalities [119]. Thus, the role of ephrinB2/EphB4 interaction between OCs and OBs in the transition from bone resorption to formation needs further confirmation.

5. Summary and Perspectives

The skeletal system provides mechanical support, protects vital organs, and controls mineral homeostasis in the human body. It is the constant bone remodeling throughout one's life that removes the old and damaged bone, keeping the skeletal system healthy. During the recent decade, many studies have demonstrated mechanisms for how osteoclastic bone resorption contributes to the subsequent bone formation in bone remodeling (Figure 2) and provided a well-rounded understanding of the roles of OCs in maintaining proper bone remodeling.

Osteoporosis, the most prevalent disorder of bone remodeling by far, is characterized by the heightened activity of OCs [6, 7]. Currently, the available treatments of osteoporosis comprise antiresorptive agents, such as bisphosphonate and denosumab, and anabolic treatments such as PTH [6, 13]. However, most antiresorptive agents that suppress OC differentiation will concomitantly impair bone formation because of the coupling effect, leading to an unsatisfactory long-term effect and potentially increasing the likelihood of long-term adverse events, such as osteonecrosis of the jaw [120]. Thus, new agents under development for osteoporosis may try to retain the OC coupling factors while inhibiting OC functions. Odanacatib, a small-molecule inhibitor of CTSK, can decrease bone resorption without affecting OBs and appears to promote bone formation [106, 121, 122], probably because of the suppression on OC activity rather than the inhibition on OC viability, thus allowing continuous crosstalk between OCs and OBs. Unfortunately, because of the unforeseen cerebrovascular events, the clinical development of odanacatib was terminated. The side effects may result from the off-target effects of CTSK inhibitors on other members of the cathepsin family, such as cathepsins B, L, and S. Nonetheless, the experience learned from the underlying biology of CTSK inhibitors could guide future therapeutic approaches for osteoporosis: dissociating the inhibition of bone resorption from the coupled reduction in bone formation. This may be a promising strategy in the development of a new drug and we believe that a better outcome will be achieved when proper strategies are made to coordinate OCs and OBs in managing bone remodeling disorders.

Conflicts of Interest

All authors declare that there are no conflicts of interest.

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Review Article

Dyslipidemia Might Be Associated with an Increased Risk of Osteoarthritis

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Background. According to several studies, the autoimmune response may lead to osteoarthritis and dyslipidemia and may affect the homeostasis of the human body's internal environment and then cause its own immune regulation. Consequently, the risk of osteoarthritis might be increased by dyslipidemia, but this association is not universally acknowledged. Therefore, a systematic review and meta-analysis was conducted to study the relationship between dyslipidemia and the risk of osteoarthritis. *Methods*. In this study, PubMed, EMBASE, and the ISI Web of Science were used to identify related studies published before July 2018. The relationship between dyslipidemia and the risk of osteoarthritis was evaluated on the basis of relative risk (RR) values and the corresponding 95% confidence intervals (CIs). To further investigate this relationship, we also employed the random effects model proposed by DerSimonian and Laird. *Results*. A total of nine studies were included to study the effect of dyslipidemia on the risk of osteoarthritis, including four cohort, three case-control, and two cross-sectional studies. Among these studies, six stated data for knee osteoarthritis, two reported on hand osteoarthritis, and one reported on hip osteoarthritis. A total of 53,955 participants were included in the meta-analysis, comprising 22,501 patients with OA (19,733 hand OA, 2,679 knee OA, and 89 hip OA). Based on the meta-analysis of case-control and cross-sectional studies, osteoarthritis was clearly higher in those with dyslipidemia compared to those who did not suffer from dyslipidemia (case-control: OR = 1.37; 95%CI = 1.27–1.46; cross-sectional: OR = 1.33; 95%CI = 1.21-1.46). In addition, the meta-analysis of cohort studies did not present any relationship between dyslipidemia and OA (RR = 1.00; 95%CI = 0.85–1.14). *Conclusions*. Even though our meta-analysis of case-control and cross-sectional studies suggested a strong relationship between dyslipidemia and osteoarthritis; this relationship was not validated by our meta-analysis of only cohort studies. As a result, further investigation needs to be conducted on the relationship between dyslipidemia and osteoarthritis, considering the significant public health relevance of the topic.

1. Introduction

Osteoarthritis (OA) refers to a chronic degenerative disease that involves the cartilage, as well as its surrounding tissues [1]. OA is considered the most common joint disease, and nearly 10-12% of the population suffers from OA [2]. In addition, it is expected that this number will increase dramatically due to the quickly increasing aging population combined with the growing prevalence of obesity [3]. Consequently, osteoarthritis is considered to have a negative influence on the health economy [4]. It can be forecast that by the year 2032, an additional 26,000 per million patients over the age of 45 will present to their general practitioner with osteoarthritis compared to 2012 [5]. OA is associated with age, female gender, obesity, joint injury, and career, as well as a high level of physical activity [5]. In addition, the autoimmune response of the synovium plays an important role in rheumatoid arthritis. In recent years, the immunological pathogenesis of synovium in osteoarthritis has attracted the attention of many researchers. Whether the immune

mechanism and inflammatory mediators are involved in the occurrence and development of osteoarthritis deserves further discussion. This may provide a new research idea for the pathogenesis of osteoarthritis, to improve our understanding of the development of this disease and change the way of treatment. In recent years, research has shown that metabolic syndrome is closely associated with OA, which is even a part of generalized metabolic disorder. Metabolic syndrome is composed of a bundle of interrelated metabolic risk factors, including diabetes, obesity, dyslipoproteinemia, and hypertension [6]. Furthermore, the incidence of metabolic syndrome is very high; it has been estimated to be as high as 26.7% in industrialized countries [6]. In the context of musculoskeletal disorders, metabolic syndrome has increasingly gained more attention because of its relationship with knee OA [7]. Obesity, the main feature in metabolic syndrome, is overwhelmingly related to degenerative joint changes in regard to mechanical load [8]. Alternatively, obesity-related OA can afflict nonweight-bearing joints (e.g., the hands), signifying a role of adipokines (circulating mediators released by adipose tissue), such as leptin. Thus, OA may have a systemic metabolic element [9]. In addition, OA can be categorized into three phenotypes: metabolic OA, age-related OA, and injury-related OA [10]. Nevertheless, as one of the components of metabolic syndrome, the role of dyslipidemia in the pathogenesis of OA is not completely understood. Dyslipidemia may affect the homeostasis of the human body's internal environment and then cause its own immune regulation. Dyslipidemia is related to chronic low-grade inflammation and oxidative stress, likely increasing the development of OA [11, 12]. A survey carried out by Ghandehari concluded that approximately 51.4 million US adults presented with high cholesterol and triglycerides, in addition to 36.1 million with elevated low-density lipoproteins [13]. As a result, we chose to conduct a systematic review and meta-analysis of the published observational studies to better comprehend the relationship between dyslipidemia and the risk of OA.

2. Materials and Methods

This research was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement [14] and the Meta-analysis of Observational Studies in Epidemiology (MOOSE) guidelines [15].

2.1. Data Sources and Search Strategy. Published studies in PubMed, EMBASE, and the Web of Science were searched based on the following keywords: ("hyperlipidaemia" OR "dyslipidemia" OR "triglyceride" OR "cholesterol" OR "lipoprotein" OR "lipid" OR "metabolic syndrome") and ("OA"). No restrictions on language or the date of publication were placed. Additionally, this study also searched the reference lists. Unpublished studies and original data were not included.

2.2. Eligibility Criteria for Study Selection. The eligibility criteria were as follows: study design (randomized controlled trials and cohort, case-control, or cross-sectional studies); an exposure factor of blood lipid levels and an outcome of OA; availability of the odds ratio (OR)/risk ratio (RR) values and corresponding 95% confidence intervals (CIs) for dyslipidemia patients and the general population; or the availability of sufficient information to measure these variables. The most recent all-inclusive study was searched under the condition that two studies used the same population. The definition of dyslipidemia was in line with the US National Cholesterol Education Program Adult Treatment Panel III guidelines. In accordance with the National Cholesterol Education Program, the definition of dyslipidemia was high-density lipoprotein cholesterol (HDL-C) < 40 mg/dL, as well as total cholesterol, low-density lipoprotein cholesterol (LDL-C), and TG levels of ≥200, ≥130, and ≥130 mg/dL, respectively [5]. The definition of osteoarthritis was in line with the American College of Rheumatology (ACR) clinical and clinical plus radiographic criteria [6]. The ACR classification criteria for (OA) permits the categorization of individuals for hand, knee, and hip OA [6]. We strictly abided by this classification standard.

2.3. Data Abstraction and Quality Assessment. Two scholars (J.X. and J.L.) obtained the essential information from the chosen studies according to the standard. The following information was gathered: name of the first author, publication year, country in which the research was carried out, study design, number of participants, period of follow-up, sources of controls, potential adjusted confounding variables, OR/RR values, and 95% CIs.

To date, no available common scale has been proposed to evaluate the quality of all kinds of observational studies. As a result, two authors individually employ the modified Newcastle-Ottawa Scale (NOS) [16] as reported by Zhu et al. [17] to assess the quality of the included studies. Quality types were allocated in accordance with the scores of each study, consisting of high quality (score 7-9), medium quality (score 4-6), and low quality (score less than 4) [18]. The maximum total score could reach 9 points, and discrepancies were solved by mutual agreement.

2.4. Statistical Analysis. The random effects model put forward by DerSimonian and Laird was applied to investigate the relationship between dyslipidemia and the risk of OA among the cohort studies [19]. The I^2 statistic was employed to evaluate heterogeneity between the studies. Low, medium, and high heterogeneities were categorized as 25%, 50%, and 75%, respectively [20]. Definite heterogeneity was assumed if the p value was less than 0.1. Sensitivity analyses were conducted by altering the pooling model [21]. In addition, a sensitivity analysis was carried out to evaluate the influence of each individual study on the summarized estimate by means of successively excluding one research study at a time. Publication bias was assessed using Begg's [22] and Egger's [23] tests. No testing for funnel plot asymmetry was carried out due to the limited number of studies included in the analysis ($n < 10$) [24].

Furthermore, we conducted a meta-analysis of the case-control and cross-sectional studies in regard to the influence of dyslipidemia on the risk of OA and expressed the results as

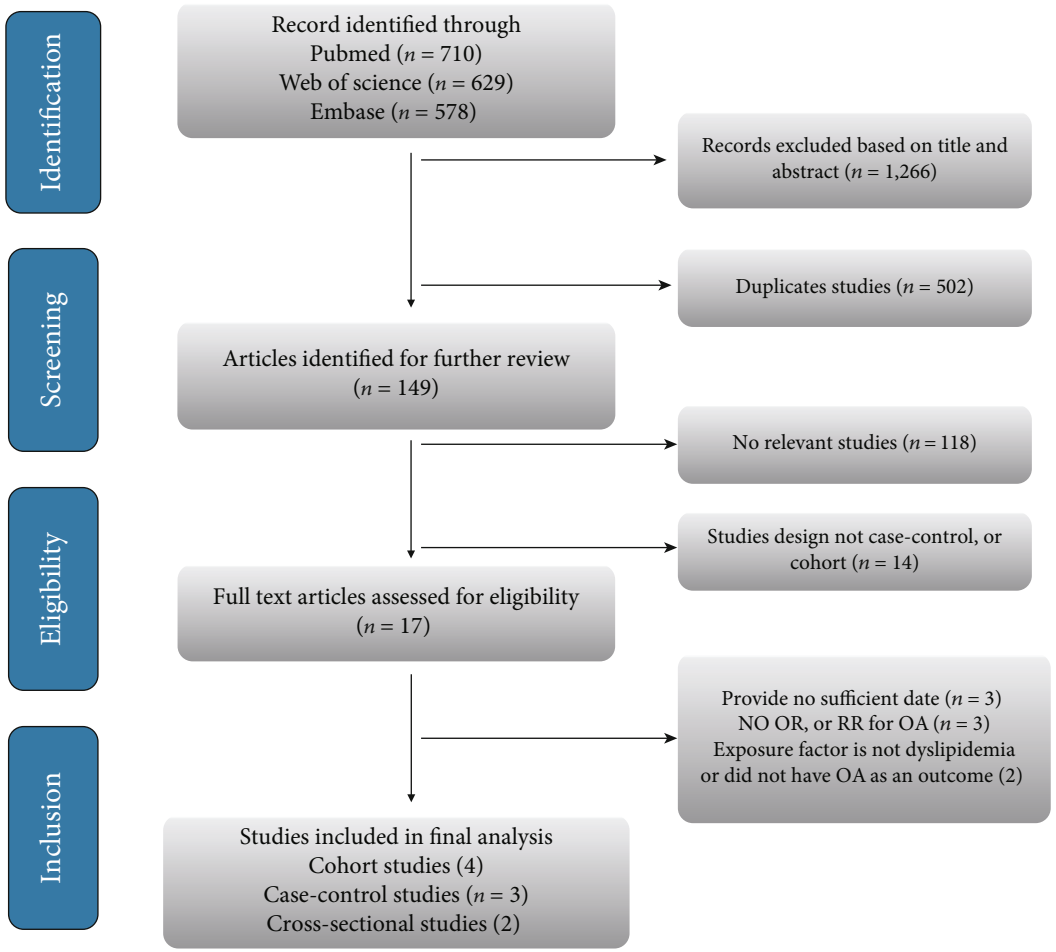

Figure 1: Study selection process for the meta-analysis.

pooled risk ratios with 95% CIs with the application of a random effects model.

STATA version 12.0 (Stata) was carried out to perform all statistical analyses.

3. Results

3.1. Study Selection and Study Characteristics. The process of study selection for the meta-analysis can be found in Figure 1. In total, 1,917 articles were obtained through the initial search, and 502 were duplicates. An additional 1,266 studies were removed based on the title and abstract. Eventually, after evaluation of the full texts, eight studies were excluded for the reason that they did not satisfy our inclusion criteria: three studies offered inadequate information [25–27], three studies did not offer ORs or RRs for OA or adequate information to calculate these variables [28–30], and two studies were removed that either did not have dyslipidemia as an exposure or did not have OA as an outcome [31, 32]. Ultimately, nine available observational articles were recognized for our meta-analysis [33–41].

Table 1 presents the principal features of the studies included in the meta-analysis, all of which were observational studies. The studies were conducted in the following countries: two in China, one in the UK, one in Germany, one in Switzerland, one in Sweden, one in Australia, one in Japan, and one in Korea. Four studies were cohort studies, three were case-control studies, and two were cross-sectional studies. All the studies included the levels of serum TC and TGs, as well as LDL and HDL cholesterol as exposures. A total of 53,955 participants were included in the meta-analysis, comprising 22,754 patients with OA (19,733 hand OA, 2,679 knee OA, and 89 hip OA). The mean age of patients ranged from 46.5 to 81.2 years, and the presence of hypertension and diabetes mellitus was described by 17.5% to 79.3% and 7.1% to 48.5% of patients, respectively. Data from 1988 to 2014 were collected. The follow-up period was within the range of 1 to 13 years. The modified NOS scores for the included studies ranged from 5 to 9, including seven high-quality studies and two medium-quality studies (Table 2). A large proportion of the studies offered risk estimates that were adjusted for age (6 studies), smoking (6 studies), gender (4 studies), physical activity (4 studies), and alcohol consumption (3 studies). Fewer studies were adjusted for lipid-lowering agents/statins (2 studies) and BMI (1 study) (Table 1).

3.2. Cohort Studies. Reports from four studies permitted the calculation of effect estimates for OA [34–36, 38]. All of the cohort studies were population based, and the follow-up

Table 1: Main characteristics of the included studies. RR, relative risk; OR, odds ratio; CI, confidence interval; BMI: body mass index; CHD, coronary heart disease; COPD, chronic obstructive pulmonary disease; NR: not reported.

Author/year of publication	Country	No. of case/control	Follow-up period	Source of controls	Site of OA	Study subtype	Adjusted factors	Adjusted OR/RR (95% CI)
Zhou/2017	China	281/3066	2008-2013	Hospital	Knee	Cross-sectional	Age (as a continuous variable), WHR (as a continuous variable), gender, physical workload, physical exercise, smoking, and drinking	1.34 (1.15–1.55)
Frey/2017	Switzerland	19,590/19,590	1995-2014	Population	Hand	Case-control	Smoking, alcohol consumption, diabetes mellitus, hypertension, COPD, hand fractures, hormone replacement therapy, osteoporosis, and statin use	1.37 (1.28-1.47)
Xie/2017	China	1669/4095	2013-2014	Hospital	Knee	Cross-sectional	Age, gender, activity level, smoking status, alcohol drinking status, and educational background	1.33 (1.18–1.50)
Gil/2017	UK	143/707	1988-1989	Population	Hand	Cohort	Age, any current medication, diabetes medication, statin use, hormone replacement therapy (HRT), previous CVD, menopause, smoking, body mass index (BMI), and systolic and diastolic blood pressure	1.75 (0.82–3.70)
Engstrom/2009	Sweden	89/5082	1991-1994	Population	Knee and hip	Cohort	Age, gender, smoking, physical activity, and CRP	0.9 (0.5-1.4)
Han/2013	Korea	270/1964	2008-2009	Population	Knee	Cohort	Age, height, exercise, alcohol intake, and smoking	1.04 (0.74, 1.47)
Inoue/2011	Japan	52/243	1995-2005	Population	Knee	Case-control	NR	1.21 (0.85-1.65)
Hussain/2014	Australia	660/19,208	2003-2007	Population	Knee	Cohort	Age, gender, country of birth, level of education, physical activity and BMI	0.99 (0.83–1.18)
Sturmer/1998	Germany	809/809	NR	Hospital	Knee	Case-control	NR	1.61 (1.06-2.47)

Table 2: Modified Newcastle-Ottawa Scale scores for the included studies. The asterisks represent a score (number of stars).

Author/year of publication	Fully defined cases	Defines the study design	Selection of controls	Describes the general characteristics	Controlled for important factors or confounding factors	Lists of inclusion and exclusion criteria for all participants	Provides enrollment duration for all participants	Indicates study period and follow-up duration	Total score
Zhou/2017	*	*	*	*	**	*	*	*	9
Frey/2017	*	*		*	**	*	*	*	8
Xie/2017	*	*	*	*	**	*	*		8
Gil/2017	*		*	*	**	*		*	7
Engstrom/2009	*	*		*	*	*	*	*	7
Han/2013	*	*	*	*	*	*	*	*	8
Inoue/2011	*	*		*			*	*	5
Hussain/2014	*	*	*		*	*	*	*	7
Sturmer/1998	*	*	*			*	*		5

was in the range of 1 to 3 years. Among them, two studies reported data for knee OA, one study reported on hand OA, and one study reported on hip and knee OA. In the pooled analysis, dyslipidemia exerted a null influence on the risk of OA (RR = 1.00; 95%CI = 0.85–1.14; $I^2 = 0\%$) (Figure 2). In the sensitivity analysis, the general results for the relationship between dyslipidemia and OA kept steady with the changes of the pooling model (fixed: RR = 1.00; 95%CI = 0.85–1.14). In addition, when we successively removed each study in turn to evaluate the stability of the results, we found that no study likely influenced the pooled risk estimate (Figure 3). No test for funnel plot asymmetry was carried out due to the limited number of included studies ($n < 10$). Nevertheless, Begg's ($p = 0.697$) and Egger's ($p = 0.465$) tests failed to identify substantial publication bias.

3.3. Case-Control Studies. The relationship between dyslipidemia and the risk of OA was investigated by three case-control studies [33, 40, 41]. Two case-control studies were population-based, and one case-control study was hospital based. According to Frey et al. [33] and Sturmer et al. [41], there exists an increase in the risk of knee OA among dyslipidemia patients. Nevertheless, the results obtained by Inoue et al. [40] suggested that dyslipidemia does not obviously change the risk of hand OA. There exist significant pooled estimates of the effect of cirrhosis without any considerable heterogeneity (OR = 1.37; 95%CI = 1.27–1.46; $I^2 = 0\%$) (Figure 2). Based on the general results, the risk of OA was obviously higher among dyslipidemia patients in comparison with the general population. In accordance with the sensitivity analysis, despite excluding studies where the sources of controls were not hospital-based, the relationship between dyslipidemia and OA remained steady. In addition, the general results regarding the relationship between dyslipidemia and OA were kept under the condition that the pooling model was changed (fixed effects model: OR = 1.37; 95%CI = 1.27–1.46; random effects model: OR = 1.37; 95%CI = 1.27–1.46). Finally, when we successively removed each study in turn to evaluate the stability of the results, no research likely influenced the pooled risk estimate.

3.4. Cross-Sectional. Two hospital-based, cross-sectional studies reported the influence of dyslipidemia on the risk of OA [37, 39]. Both studies reported data on knee OA, and both reported an obvious connection of dyslipidemia with OA. The random effects meta-analysis also demonstrated that dyslipidemia considerably increased the risk of OA, without heterogeneity (OR = 1.33; 95%CI = 1.21-1.46; $I^2 = 0\%$) (Figure 2).

4. Discussion

Admittedly, this is the first meta-analysis to investigate the relationship between dyslipidemia and the risk of OA. Even though a meta-analysis of cohort studies did not present any connection between dyslipidemia and OA, a meta-analysis of case-control and cross-sectional studies demonstrated that the risk of OA was obviously higher among those suffering from dyslipidemia compared to those without dyslipidemia.

The potential pathophysiological mechanisms accounting for these results remain unknown. Changed expression of cholesterol influx genes in human osteoarthritic chondrocytes and in the cartilage of patients with OA has been depicted [42]. Moreover, in vivo studies have demonstrated that decreased high-density lipoprotein cholesterol plays an important role in the pathogenesis of OA. Based on early research, dietary cholesterol intake has been shown to increase spontaneous cartilage damage in mice [43]. Correspondingly, high LDL levels stimulate synovial inflammation, as well as ectopic bone formation, in mouse OA models [43]. LDL could be involved in the development and progression of OA through the stimulation of synovial cells and chondrocytes [44].

There are several strengths of our study. First, this is the first meta-analysis to investigate the relationship between dyslipidemia and the risk of OA with a large sample size (22,754 cases of OA and 53,955 participants), probably providing a reference in regard to this relationship. Clinically, the results indicate that patients with dyslipidemia are required to focus more on their risk of OA in comparison

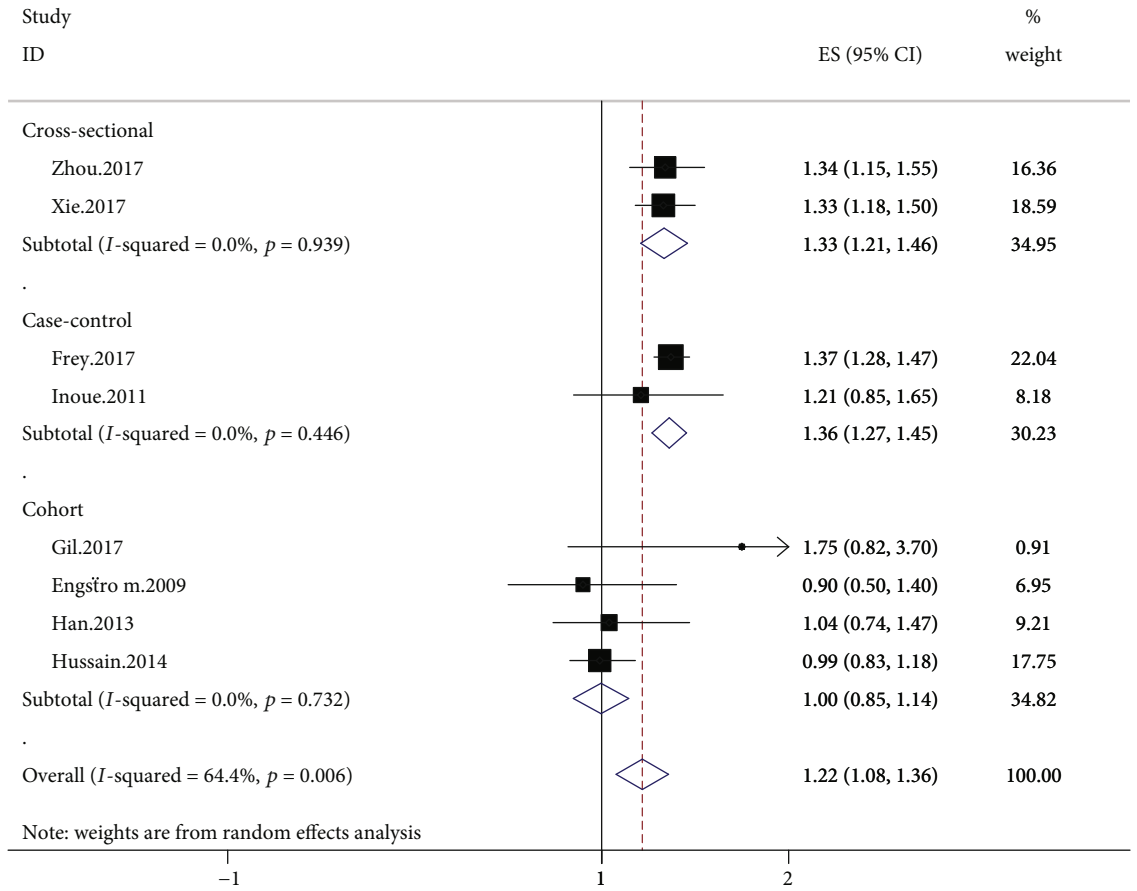

Figure 2: Forrest plot showing the relationship between dyslipidemia and the risk of OA, using a random effects model and depicted on a logarithmic scale. Squares represent the risk estimates for each individual study. Horizontal lines represent the 95% confidence intervals, and diamonds represent the summary risk estimates with 95% confidence intervals. CI: confidence interval; ES: effect size.

with the general population. Second, a comprehensive literature search was conducted using PubMed, EMBASE, and the Web of Science to recognize potential studies from which to examine the relationship between dyslipidemia and the risk of OA. Additionally, most of the studies included were high-quality studies. All of these features enhance the reliability of our study.

However, this study still has some limitations. First, a meta-analysis of the different locations of OA, such as the knee, hip, and hand, was not conducted due to the limited number of included studies. The effect of dyslipidemia on the risk of knee vs. hip OA may have differences because of the diverse susceptibility of these two joints to metabolic factors. The knee may have more dependence on soft tissue and neuromuscular control for its stability. Comparatively, in the hip, the bony shape and joint congruence seem to have greater significance on the development of hip OA, which makes the hip less vulnerable to the effects of inflammation. Second, the observed outcome was an association, which is considered a confounding bias. Seven studies reported data on knee or hip osteoarthritis, but not all of the studies were adjusted for BMI or physical activity. Zhou et al. [39], Xie et al. [37], Engstrom et al. [35], Han et al. [36], and Monira Hussain et al. [34] reported on knee or hip osteoarthritis adjusted for physical activity (activity level). Hussain et al. [34] reported on knee osteoarthritis adjusted for BMI. Two studies (Inoue et al. [40] and Sturmer et al. [41]) that reported data on knee osteoarthritis did not adjust for BMI or physical activity. There were few data on knee OA patients compared with normal participants, because the studies by Inoue et al. [40] and Sturmer et al. [41] are aimed at investigating the data of the general population. Even though most studies were adjusted for recognized risk factors for OA, such as age, alcohol consumption, and smoking, many potential adjustment factors remained unclear, such as cardiovascular disease and obesity, showing a close association with the development of OA. Only two studies in the meta-analysis adjusted the OR/RR with lipid-lowering agents/statins. Additionally, we did not succeed in obtaining information concerning medication use, especially regarding lipid-lowering agents such as statins, which could have an influence on the development of OA. It has been indicated that statins might play a protective role in developing OA, likely caused by pleiotropic anti-inflammatory properties or by enhancing chondrogenesis [45, 46]. Third, a meta-analysis of the effects of various kinds of dyslipidemia, such as low-density lipoprotein levels, triglyceride levels, and cholesterol levels, was not carried out due to the limited number of included studies.

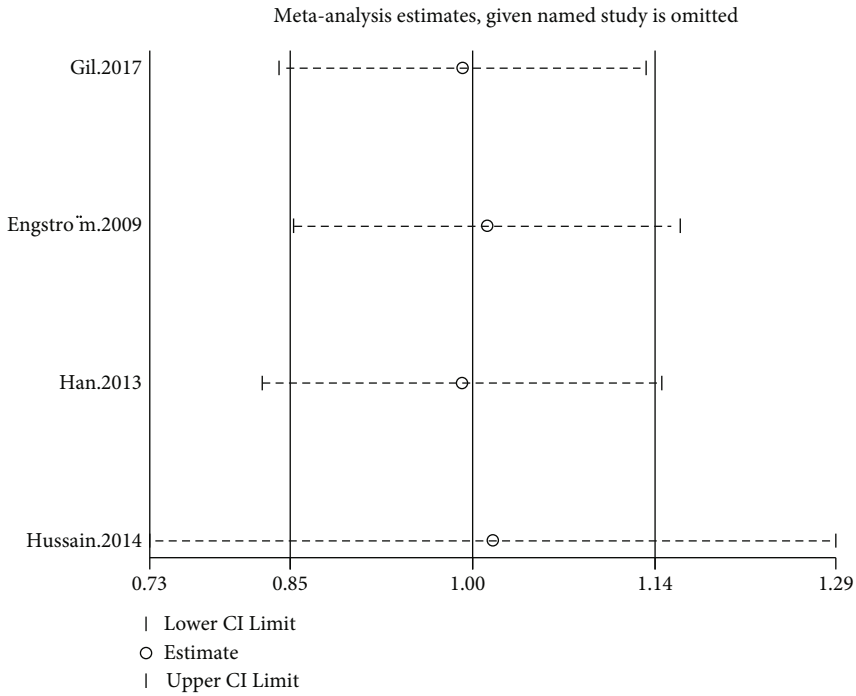

Figure 3: Sensitivity analysis of the association between dyslipidemia and the risk of OA (cohort study).

Ultimately, a large proportion of the included studies were cohort studies, yet we also included two cross-sectional studies and three case-control studies. Case-control studies tend to have recall and selection biases, and cross-sectional studies are insufficient for assessing the relationship between cirrhosis and the risk of stroke. It must also be admitted that a meta-analysis cannot deal with the limitations of the included studies. Finally, it is difficult to determine the causality of this relationship on the basis of these observational studies alone given the heterogeneity of the results and the limited data from prospective studies, which were restricted to 4 studies that, respectively, included 89, 270, 143, and 660 cases of OA, as well as the lack of information about time-related factors. Although we were not always able to ascertain the source of the heterogeneity, we performed several sensitivity and subgroup analyses to address this issue.

In conclusion, considering there was a lack of evidence from the cohort studies but that strong connections were noted in the case-control and cross-sectional studies, the current systematic review and meta-analysis offers inadequate but fascinating evidence of the effects of dyslipidemia on the risk of OA. In general, the findings indicate that dyslipidemia might exert a significant pathogenic role in the development of OA, as well as offers a rationale for the shared care of patients by metabolic physicians. To be specific, structured clinical trials with predefined criteria for patient selection are still required to investigate the role of lipid-lowering therapies in the management of OA.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

J.X. designed the study and completed the manuscript. J.X. and J.L. searched the databases and reviewed the studies. X.C. and J.X. collected and analyzed the data. Y.L. and H.S. coordinated and provided financial the support for the proposed study. The final manuscript was read and approved by all the authors. Y.L. is the guarantor for the current study. Thanks are due to Simon C. Robson of Harvard Medical School Liver Center and Transplant Institute for helping us modify the language of the manuscript. Jianping Xiong, Junyu Long, and Xi Chen contributed equally to this work.

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Research Article

Protective Effect of Genistein on Condylar Cartilage through Downregulating NF-κB Expression in Experimentally Created Osteoarthritis Rats

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Temporomandibular joint osteoarthrosis (TMJOA) is characterised by chronic inflammatory changes, with subsequent gradual loss of joint cartilage. NF-κB is a crucial transcription factor in the course of inflammatory and immune responses, which are involved in OA pathology activated by proinflammatory cytokines. Genistein is known to have anti-inflammation and modulation of metabolic pathways through repression of the NF-κB signaling pathway in inflammatory disease. But so far, studies on the effects of genistein on TMJOA are very limited. So, the purpose of this study is to investigate the protective effect of genistein against experimentally induced condylar cartilage degradation through downregulating NF-κB expression in created osteoarthritis rats *in vivo*. Male SD rats were created as temporomandibular joint osteoarthritis models and administered through oral gavage with low and high dosage genistein (30 mg/kg and 180 mg/kg, respectively) daily for 4 weeks. The morphological changes of the condylar cartilage were studied with HE and Masson staining. The expressions of p65 and inflammatory cytokines (IL-1β and TNFα) were detected using immunohistochemistry and real-time PCR. The results showed that experimentally created osteoarthritis reduced the condylar cartilage thickness of rats and increased the gene expression of cytokines (IL-1β and TNFα) and positive cells of p65. Genistein treatment had positive effects on the condylar cartilage renovation, while high dose genistein treatment had more significant effects on the reversing of OA changes and reduction of the expression of p65 and inflammatory cytokines (IL-1β and TNFα). The results indicated that high dose genistein treatment had obvious therapeutic effects on condyle cartilage damages of OA rats. The mechanism may be that genistein suppresses the NF-κB expression activated by inflammatory cytokines.

1. Introduction

Temporomandibular joint osteoarthrosis (TMJOA), which is characterised by chronic inflammatory changes, with subsequent gradual loss of joint cartilage, mostly occurred in condyle [1–3]. Although condyle degradation can cause many clinical symptoms such as arthralgia, joint clicking, mandibular deviation, or retraction, it can hardly be radically cured because the exact etiologies of TMJOA were not fully understood [4, 5]. However, inflammatory pathways were verified as a "triggering factor" that activated the immune responses in OA pathogenesis [6]. Proinflammatory cytokines including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) played a significant role in the development of OA. Some studies showed that IL-1β and TNF-α contributed to the pathogenesis of osteoarthrosis and induced the inflammation and destruction of the joints [7,8]. IL-1β was reported to involve in the activation of the inflammatory pathways in synovitis and cartilage degradation. Meanwhile, TNF-α is also a potent proinflammatory cytokine which had higher expression in the OA joints and participated in host immune response [7, 8].

Nuclear factor κB (NF-κB) has been considered as a central mediator in diverse cellular responses, particularly in the inflammatory process and immune response [9]. Many studies reported that IL-1β and TNF-α induced the nuclear

translocation of NF-κB [10–14]. Then, NF-κB activated the downstream Bcl-2 family proteins to regulate apoptosis [15, 16]. Bcl-2 and Bax are both important members in the Bcl-2 family, which involved in the mechanism of apoptotic cell death [17].

Genistein is an isoflavone compound extracted from plants, well known for its anti-inflammatory, antioxidant, and anticancer effects, is widely used for the treatment of inflammatory diseases [18]. Some study has demonstrated that genistein has a bone-protective effect in rat models of osteoporosis in knee joints [19]. However, the knee and hip joints are composed of hyaline cartilage, while mandibular condyle is covered by fibrocartilage [20]. Up to now, the studies about its therapeutic effect on TMJOA is limited.

In this study, we investigated the therapeutic effect and the possible mechanism of genistein on OA of TMJ in rats and presumed that genistein has restorative effects on cartilage destruction by inhibition of NF-kB signaling pathways.

2. Materials and Methods

2.1. Model Building. Twenty-four male rats (8-week old, 232.3 ± 2.5 g) were purchased from the animal center of Zhejiang University. The rats were divided into a normal control group (NC), four-week OA group (OA), low-dose GE group (GE1), and high-dose GE group (GE2). The setup of the TMJOA model was referred to previous studies [21,22]. Collagenase (0.05 ml; Sigma Biochemical, St. Louis) was injected in the upper cavity of TMJ in OA, GE1, and GE2 groups, and normal saline (0.05 ml) was injected in the NC group. All animals were given substitute diet with corn oil replacing soybean oil to avoid the interference of GE in soybean [22]. Daily treatment was performed with genistein (99.5% pure; Winherb Med., China) and sterile saline by intragastric administration in GE1 (30 mg/kg), GE2 (180 mg/kg), NC (2 ml saline), and OA (2 ml saline) groups, respectively. Figure 1 showed the chemical structure of genistein [23].

2.2. Measurement of Cytokines by RT-PCR. Synovial fluid samples were collected from the superior joint cavity using diluted aspiration as described by Uehara et al. [24]. Briefly, 2% thiopental sodium anaesthesia (35 mg/kg) was injected after disinfection. 0.2 ml saline was repeatedly injected and withdrawn in the upper joint cavity to aspirate synovial fluids. The mean diluted synovial fluid was 0.20 ml (range: 0.16–0.24 ml). The aspirates were centrifuged at $3000 \times g$ for 15 min at room temperature, and the supernatant was kept for RT-PCR. Total RNA was extracted using an RNA mini kit (BIO-RAD, USA) according to the manufacturer. The cDNA of various groups were synthesized according to the GeneAmp PCR kit (ABI, USA). RT-PCR was performed by using a SYBR green RT-qPCR kit (TOYOBO Corporation). All PCR reactions were performed using iCycler iQTM (Bio-Rad, Hercules, CA, USA). The cycling conditions were 10 min at 95°C, followed by 40 cycles: denaturation at 94°C for 15 s, annealing for 30 s at 57°C, and extension at 72°C for 30 s. The primers for IL-1β were F: 5′-CAC CTT CTT TTC CTT CAT CTT TG-3′ and R: 5′-GTC GTT GCT TGT CTC TCC TTG TA-3′, the product length is 241, and the GenBank Accession Number is NM_031512.2; for TNF-α were F: 5′-ACT GAA CTT CGG GGT GAT TG-3′ and R: 5′-GCT TGG TGG TTT GCT ACG AC-3′, the product length is 153, and the GenBank Accession Number is XM_008772775.2; and for GAPDH were F: 5′-GTA TTG GGC GCC TGG TCA CC-3′ and R: 5′-CGC TCC TGG AAG ATG GTG ATG G-3′, the product length is 202, and the GenBank Accession Number is XM_017593963.1.

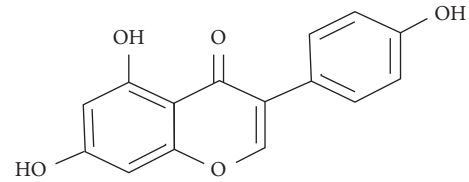

Figure 1: Chemical structure of genistein.

2.3. Histopathology and Immunohistochemistry. The haematoxylin and eosin (HE) and Masson staining were operated according to previous studies [21]. After synovial fluid collection, all the rats were sacrificed. Condyles were dissected carefully, fixed in 4% PFA, then decalcified in 10% EDTA for 4 weeks. After dehydration, the samples were incubated in paraffin overnight. The left condyles were prepared for sagittal sections and stained with HE and Masson, respectively. The destruction of the joint cartilage was evaluated by the modified Mankin score system. Three independent location of samples were chosen for assessment [25]. The description for histological evaluation included 4 aspects: structural integrity (0–6 points)(HE), matrix staining (0–4 points)(Masson), cellularity (0–3 points)(HE), and tidemark integrity (0–2 points)(HE) [25]. The right condyles were prepared for immunohistochemistry of p65. After dewaxing by immersing in xylene, the samples were incubated with primary monoclonal mouse anti-p65 antibody (Abcam, USA; dilution 1 : 40) for 1 h at 37°C. The percentage of 65 positive chondrocytes was calculated by the number of immune positive cell divided by total cell number. All sections ($n = 3$) were analyzed under the microscope (Olympus IX71, Japan).

2.4. Statistical Analysis. The data are expressed as mean ± SD. SPSS 16.0 was used for the statistical analysis. Also, p values less than 0.05 were considered to have a statistically significant difference. One-way ANOVA was used for multiple comparisons among various groups.

3. Results and Discussion

3.1. Results

3.1.1. Structural Changes in the Condyle Cartilage. HE staining of the condyle cartilage in various groups is shown in Figure 2. Compared with the NC group, significant

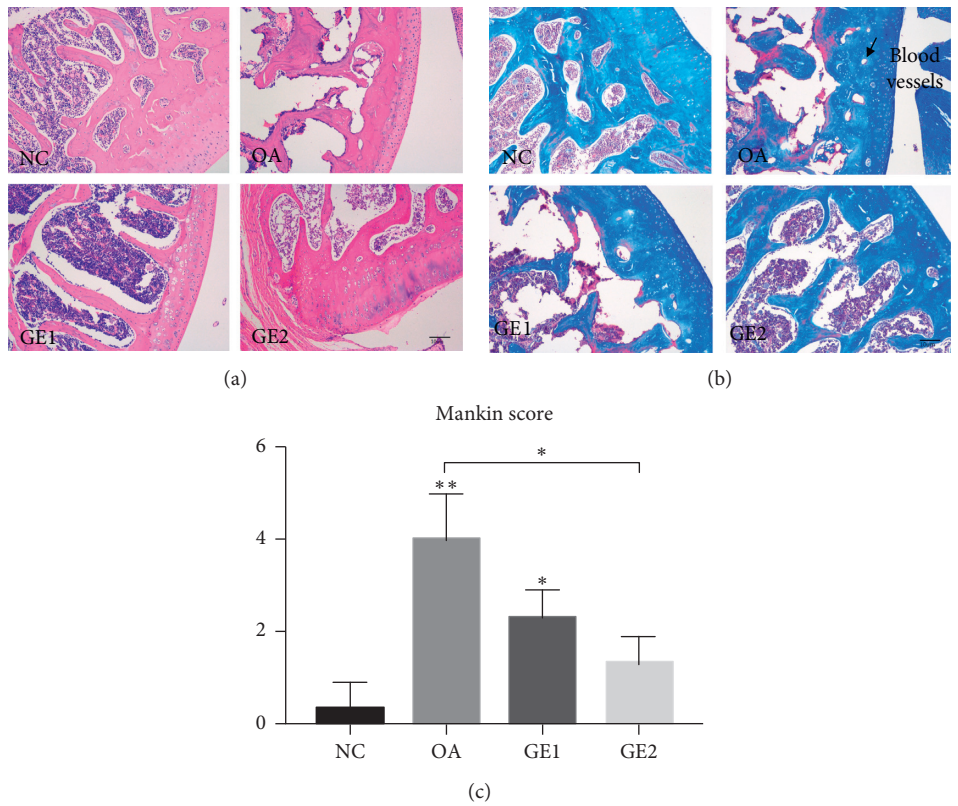

Figure 2: Treatment effects of GE on condyle cartilage changes in the rat model of TMJOA. (a) Representative histology changes in the condyle cartilage for the OA and GE treatment animals at 4 weeks after injection by HE staining. (b) Demonstration of matrix changes by Masson staining. (c) Quantitation of histology changes by the modified Mankin score system. The histological evaluation included 4 aspects. HE staining for the evaluation of structural integrity, cellularity, and tidemark integrity and Masson-stained sections for the evaluation of the cartilage matrix. Quantitative data are shown as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different when compared with NC and OA groups, respectively, $n = 6$.

changes of the cartilage were found in the OA group. The cartilage thickness of OA rats reduced obviously compared with the NC group. Also, multilevel cartilage tidemarks appeared in the OA group. There were also some changes in the bone trabecula and marrow cavity. OA animals had narrowed bone trabecula and bigger marrow cavity, while GE treatment had restorative effects on condylar cartilage repairment. High-dose genistein treatment showed more obvious improvement on cartilage repairment than low-dose treatment. Masson staining showed the same tendency of structural changes with HE staining. In OA animals, several blood vessels and cell mass appeared, while GE treatment could reduce this kind of changes. The modified Mankin scores system was used to evaluate the structural changes of the condyle cartilage in the four groups. A statistically significant difference in the Mankin score was observed in the NC, OA, and GE treatment groups. The score of OA was higher than that of NC and GE treatment groups. High-dose genistein treatment showed more improvement on cartilage repairment.

3.1.2. Immunohistochemistry Analysis of p65 in Condyle Cartilage. p65 immunoreactivity can be found in all positive sections of the cartilage cells throughout the condylar cartilage (Figure 3(a)). Positive cells were identified as tissue with brown staining. Comparative intensity of p65 in various groups is shown in Figure 3(b), which displayed marked variation in various groups. Positive signals of p65 in the OA group was much more than that in the NC group ($p < 0.0001$), while high-dose GE treatment significantly reduced the intensity of p65 ($p < 0.0001$).

3.1.3. Expression of IL-1β and TNF-α in the Condylar Cartilage. The results of the real-time PCR analysis are shown in Figure 4. In the OA group, the expression of IL-1β was much higher than that in the NC group ($p < 0.001$), and the expression of TNF-α had the same tendency with IL-1β ($p < 0.01$). Meanwhile, low-dose GE treatment could decrease the expression of IL-1β and TNF-α, but significantly statistical difference was not achieved ($p > 0.05$), while the high-dose GE treatment significantly reduced the expression of inflammatory factors ($p < 0.01$).

4. Discussion

This is the first study to investigate the effect of genistein on cartilage repairment of temporomandibular joint osteoarthritis models *in vivo*. In this study, the TMJOA model was

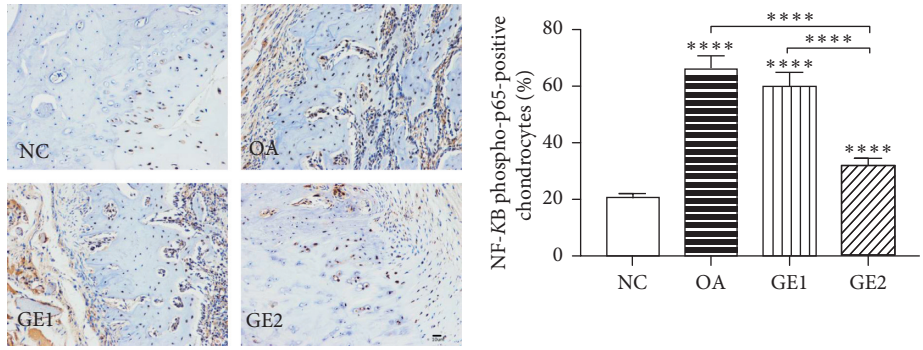

Figure 3: Effects of GE on P65 immunoreactivity in TMJOA rats. (a) GE inhibits p65 immunoreactivity in the condylar cartilage of OA rats. (b) Comparative intensity of p65 in various groups. Quantitative data are shown as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, significantly different when compared with NC and OA groups, respectively, $n = 6$.

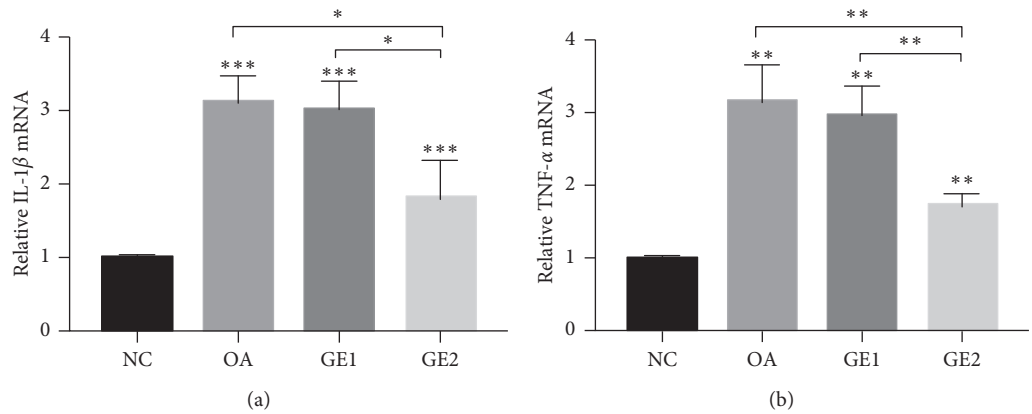

Figure 4: Effects of GE on the gene expression of IL-1β and TNF-α. (a) IL-1β expression in OA animals increased significantly compared with the NC group, while high dose of GE could significantly inhibit IL-1β expression. (b) TNF-α expression increased significantly in OA animals, and high dose of GE treatment could significantly inhibit TNF-α expression. Quantitative data are shown as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, significantly different when compared with NC and OA groups, respectively, $n = 6$.

set up on rats by injection of collagenase [21, 22], which causes histological changes including erosion of the cartilage surface, decreased thickness of the cartilage, and even changes in the bone trabecula and marrow cavity. In the GE treatment groups, the high-dose GE had better therapeutic effects compared with low GE groups, which are as indicated by Mankin scores. Masson staining of the present study showed the same tendency of structural changes with HE staining in various groups, which verified the therapeutic effects of GE.

The destruction of the extracellular matrix is usually accompanied by an increase of proinflammatory cytokines, such as IL-1β and TNF-α and these cytokines are also key mediators of the intracapsular pathological conditions of the temporomandibular joint [7]. IL-1β plays a critical role in OA pathogenesis, which was verified independently or combining with other mediators to induce inflammatory reactions and catabolic effect in the course of OA [12, 13]. Many studies demonstrated that patients with OA have an elevated level of IL-1β than normal people [13], and blocking IL-1β production could counteract the degradative mechanisms associated with OA pathology [26]. Our result agreed with the previous publications. The expression of IL-1β was higher in OA rats than that of normal rats. Meanwhile, TNFα is another key inflammatory cytokine in the pathophysiological processes of OA. Many studies reported that synovial fluids of OA joints showed higher concentration of TNFα than that of normal joints [7, 24, 27], while high TNFα expression was detected in local arthritic tissues, which was related to the autoimmune reaction and can cause aggressive cartilage destruction of joints by suppressing the synthesis of the cartilage matrix [24]. The present result showed that the expression of TNF-α was higher in OA rats than that of normal rats, which had the same tendency with IL-1β.

NF-κB is a crucial transcription factor that participates in the development of inflammatory and immune diseases activated by inflammatory responses [12, 28]. In an inactive state, NF-κB binds to the I-κB member. Some inflammatory cytokines could activate I-κBα, and then p65 protein separates from I-κBα and translocates from cytoplasm to the nucleus to bind related inflammation genes to induce

inflammation [18]. Many studies provide evidence for the role of NF-κB in mediating enhancement of apoptosis [14, 15]. Apoptosis, or programmed cell death, has been suggested to have a close relationship with OA progression [29,30]. In the adult of TMJ dysfunction, apoptosis has been found in the course of joint remodeling [31]. Emerging evidence suggests apoptosis and NF-κB signaling are highly activated in OA pathology which can be triggered by proinflammatory cytokines [13, 14, 29]. These inflammatory mediators were verified to activate NF-κB signaling pathways abnormally to induce apoptosis in OA chondrocytes [12]. The present study showed that positive signals of p65 in various groups have the same tendency with the expressions of inflammatory mediators, which indicated that NF-κB phosphorylation may be induced by IL-1β and TNF-α in the TMJOA model.

Genistein, which is known to have multiple molecular effects, such as the inhibition of inflammation, promotion of apoptosis, and modulation of metabolic pathways, plays an important role in preventing and treating common disorders [23]. Studies on positive effects of genistein have been reported in inflammatory disease. Genistein had positive effect on the treatment of psoriasis by inhibiting TNF-α-induced I-kBα phosphorylation and p65 nuclear translocation [18]. Genistein was also shown to suppress the production of COX-2 and NO in primary human chondrocytes [32]. Some study indicates that genistein could reverse ox-LDL-induced inflammation through repression of the NF-κB signaling pathway in HUVECs [33]. But so far, studies on the effects of genistein on TMJOA are very limited. The present study found that high-dose GE treatment had obviously therapeutic effects on condyle cartilage damage of OA rats. Furthermore, high-dose GE treatment could reduce positive signals of p65 and the expression of inflammatory mediators in TMJOA rats, which indicated that the effects of genistein on repairing cartilage damage may be through the repression of IL-1β-TNF-α-mediated NF-κB signaling pathway in TMJOA models. It is recognized that Bcl-2 and Bax play a crucial role in the regulation of the apoptotic process [20, 31]. The effect of genistein on cartilage repairment may be through repression of the NF-κB signaling pathway, which in turn decreased the ratio of Bax/Bcl-2. Another mechanism may be related to hypoxia-inducible factor-2 alpha (HIF-2α) which was regulated by NF-κB before and during inflammation [34]. HIF-2α downstream degradation factors, such as MMP-3,13 and ADAMTs-4,5, played essential roles in the degradation of cartilage aggrecan and had been recognized as one of the most primary targets for therapeutic intervention in osteoarthritis [21]. However, further investigation on the underlying mechanism of the effect of genistein on the cartilage repairment of TMJOA models is still needed.

5. Conclusions

This is the first study to investigate the protective effect of genistein on cartilage repairment of temporomandibular joint osteoarthritis models *in vivo*. This study revealed that high-dose GE treatment had obviously therapeutic effects on cartilage repairment and downregulated the expression of p65 and the inflammatory mediators. It was indicated that therapeutic effects of genistein may be through repression of the NF-κB signaling pathway.

Data Availability

The data that support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Research Article

PTEN Inhibits Inflammatory Bone Loss in Ligature-Induced Periodontitis via IL1 and TNF-α

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Phosphatase and tensin homolog (PTEN) is a critical regulator of tumorigenesis and bone remodeling, which is also found expressed in the periodontal tissues. Periodontitis is one of the most common oral diseases and associated with alveolar bone resorption and tooth loosening in adults. However, the functional relevance of PTEN in periodontitis remains unclear. Here, we report that PTEN plays an essential role in periodontitis. The in vivo results of our study showed a significant decrease of PTEN in the ligature-induced mouse periodontitis model. The function of PTEN in the macrophages was shown to be associated with inflammatory factors interleukin 1 (IL1) and tumor necrosis factor (TNF-α) by using overexpression and silence methods. Further mechanistic studies indicated lack of PTEN-activated IL1 and TNF-α, which increased the number of osteoclasts and led to alveolar bone erosion and loss. Moreover, PTEN nanoparticles could directly inhibit the inflammatory process and bone erosion, suggesting a controlling role of PTEN during bone remodeling. All these data identified the novel function of PTEN as a key factor in periodontitis and bone remodeling.

1. Introduction

Inflammatory osteolysis is a critical pathological feature of clinical inflammatory diseases such as periodontitis which is one of the most common oral diseases and associated with alveolar bone resorption and tooth loosening in adults [1]. The therapeutics for periodontitis are intended to block inflammation locally, but not enough for inflammatory bone destruction [2]. However, many patients still suffer from bone loss under the anti-inflammation treatments [3]. Therefore, more effective and economic treatments to reduce the inflammation and bone destruction are urgently needed. In the pathogenesis of periodontitis, osteolysis is predominantly caused by increased osteoclasts number and activity [3, 4]. The most determined cytokine involved in osteoclastogenesis is the receptor activator of nuclear factor κB ligand (RANKL) [5]. Presence of RANKL could be modulated by several proinflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin 1 (IL1), and interleukin 6 (IL6) [6]. Thus, an ideal therapeutic strategy to prevent inflammatory bone destruction is to suppress proinflammatory cytokines activation during osteoclastogenesis.

The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a specific tumor suppressor gene which can efficiently limit phosphoinositide 3-kinase (PI3K) activity and downstream serine/threonine kinase Akt (also known as protein kinase B or PKB) signaling [7]. Several studies indicate that the PI3K/PTEN pathway regulates the inflammatory response [8, 9]. The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway is the classical proinflammatory signaling pathway based on the role of NF-κB in proinflammatory gene expressions. The study has shown that Toll-like receptor (TLR)-induced PI3K / Akt activation is phosphorylated, thereby inhibiting downstream glycogen synthase kinase (GSK) 3β, leading to a diminished expression of NF-κB-driven proinflammatory genes in monocytes [10]. PTEN also plays an important role

in varieties of cellular regulation, including survival, apoptosis, autophagy, migration, and proliferation [11]. However, its role during inflammatory bone loss remains unclear.

In the present study, ligature-induced periodontitis in wild type (C57BL/6J) mice were used as in vivo models and RAW264.7 cells were used for in vitro studies. The protective effects of PTEN on ligature-induced periodontitis and the underlying mechanisms associated with inflammatory factors regulation were investigated. Our study provides a novel insight into understanding the protective effects of PTEN on inflammation and bone remodeling in periodontitis and proposes that PTEN can be used as an adjuvant therapy for inflammatory diseases.

2. Materials and Methods

2.1. Animals. All mice were maintained in C57BL/6J background and housed under pathogen-free conditions with a 12-hour light/dark cycle and fed regular chow and sterile water throughout the experimental period. Our study was approved by the Ethics Committee of Shandong Provincial Hospital affiliated to Shandong University. All experiments were performed according to the Regulations and Guidelines approved by the Shandong Provincial Hospital Animal Care and Use Committee.

2.2. Generation of Ligature-Induced Periodontitis Model. Ligature-induced periodontitis model was performed as described previously with slight modification [12]. A 5-0 silk ligature (Roboz Surgical Instrument Co., MD, USA) was tied gently around the left maxillary second molar under anesthesia to induce periodontitis, which was maintained for 10 days, and the contralateral molar was served as a control. The ligatures remained in place in all mice throughout the experimental period, monitored every 3 days and retied if the ligature was loose or gone. Furthermore, if the ligature was not present at the termination of the experiment, the sample was removed from the analysis.

2.3. Nanoparticle Therapy. To prepare nanoparticle for in vivo treatment, 10 μg of recombinant PTEN plasmid (pcDNA3.1-PTEN) or pcDNA3.1 (control) was mixed with 20 μl of nanoparticle in vivo DNA transfection reagent (Engreen Biosystem Co. Ltd., Beijing, China) according to previous studies and the manufacturer's protocol [13, 14]. Mice (10 weeks old) were given local injection of nanoparticles containing 10 μg plasmid at the left maxillary second molar periodontal tissues every 3 to 4 days during the two weeks of therapy.

2.4. Histology and Histological Scoring. Histological studies were performed as previously described [15]. Briefly, samples were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 4 weeks. Specimens were optimal cutting temperature compound- (OCT-) embedded, cut into 7 μm-thick sections in the coronal plane using a microtome, and stained with hematoxylin and eosin (H&E) (Abcam, UK). In addition, samples were further stained with tartrate-resistant acid phosphatase (TRAP) (Sigma-Aldrich, MO, USA) to evaluate the number of osteoclasts [16].

All specimens were blindly analyzed by an experienced examiner and scored for inflammation and bone destruction [17–19]: Score 0: no inflammation and bone destruction, normal gingiva; Score 1: mild inflammation, sparse mononuclear cells, osteoclast activation; Score 2: moderate inflammation, monocyte infiltration, and/or sparse eosinophils or neutrophils, osteoclasts lacunas; Score 3: severe inflammation, eosinophils or polymorphonuclear neutrophils infiltration, with abscess areas, signs of bone erosion; Score 4: severe inflammation with severe alveolar bone erosion and resorption.

2.5. Plasmid or siRNA Transfection. Plasmids were constructed by cloning the PTEN gene open-reading frame (ORF) into pcDNA3.1-Flag. For siRNA transfection, RAW264.7 cells were transfected with siRNA targeting PTEN (#1 sense strand: CGTTAGCAGAAACAAAAGGAG and #2 sense strand: GATCTTGACCAATGGCTAA) using Lipofectamine™ 2000 reagent (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. A final concentration of 100 nM siRNA was used. After transfection for 48 hours, quantitative real-time PCR analysis was used to assess the mRNA content.

2.6. RNA Analysis. RNA analysis was performed as described with a minor modification [20]. Message RNA from mouse molar periodontal tissues was isolated using Trizol reagent (Life Technologies, USA). 2 μg of RNA was reverse transcribed into complementary deoxyribonucleic acid (cDNA) using the Reverse Transcription Kit (TAKARA, Japan). The reaction mixture we prepared contained primers, the cDNA template, and the double stranded DNA-specific dye SYBR Mix (Bimake, USA). The primers' sequences are shown in Table 1.

2.7. Statistical Analysis. All data were expressed as the mean ± SEM ($n \geq 5$). To determine the difference between two groups, a two-tailed Student's t-test was performed. To compare more than two groups, a two-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test with a 95% confidence interval (Graphpad Prism, USA) was used. p values <0.05 were considered to be statistically significant.

3. Results

3.1. Ligature-Induced Periodontitis and Inflammatory Bone Loss in Mice. To assess the changes in inflammation and bone mass, histological analysis was performed. Ligature-induced periodontitis mice showed a significant gingival swelling, a greater soft tissue thickness, and severe bone resorption compared to the unligated group (Figure 1(a)). In addition, a wide range of inflammatory cells were shown around the root of the second molar (Figures 1(a) and 1(b))

Table 1: List of primers sequence for qPCR.

Target (Genbank no.)	Forward primer (5′–3′) and reverse primer (5′–3′)	Product size (bp)
GAPDH (NM_001289726.1)	AGGTCGGTGTGAACGGATTTG and TGTAGACCATGTAGTTGAGGTCA	123
PTEN (NM_008960.2)	ACACCGCCAAATTTAACTGC and TACACCAGTCCGTCCCTTTC	170
IL1 (NM_008361.4)	GCCCATCCTCTGTGACTCAT and AGGCCACAGGTATTTTGTCG	230
IL6 (NM_001314054.1)	AGTTGCCTTCTTGGGACTGA and TCCACGATTTCCCAGAGAAC	159
TNF-α (NM_001278601.1)	CGTCAGCCGATTTGCTATCT and CGGACTCCGCAAAGTCTAAG	206
TRAP (NM_001102405.1)	CAGCAGCCAAGGAGGACTAC and ACATAGCCCACACCGTTCTC	190
Cathepsin K (NM_007802.4)	CCAGTGGGAGCTATGGAAGA and AAGTGGTTCATGGCCAGTTC	162
Osteocalcin (OC) (NM_001032298.3)	AAGCAGGAGGGCAATAAGGT and TTTGTAGGCGGTCTTCAAGC	156
ALP (NM_007431.3)	GCTGATCATTCCCACGTTTT and CTGGGCCTGGTAGTTGTTGT	204

and the inflammation score was significantly increased in ligature-induced periodontitis mice (Figure 1(c)). Ten days after ligature, there was a significant difference in bone mass between the ligature-induced periodontitis group and the unligated group. The bone loss around the ligature was significantly increased in mice compared to unligated controls (Figure 1(a)). To assess osteoclast activity, samples were further analyzed by TRAP staining (Figures 1(d) and 1(e)). The result showed that TRAP-positive multinucleated cells increased significantly in the ligature-induced periodontitis group compared to the unligated group (Figure 1(f)).

3.2. Gingival mRNA Expressions of PTEN and Inflammatory Cytokines. PTEN, as a regulator of the mitogen-activated protein kinase (MAPK) pathway and inflammatory cytokines, was found decreased in ligature-induced periodontitis (Figure 2(a)). And the inflammatory cytokines such as IL1, IL6, and TNF-α mRNA expression levels were increased in the ligature-induced periodontitis group, especially the expression of IL1 and TNF-α (Figures 2(b)–2(d)). Inflammation was shown to cause excessive bone resorption as well as impaired bone formation. Thus, we determined the bone formation gene markers, osteocalcin (OC) and alkaline phosphatase (ALP), and bone erosion markers, tartrate-resistant acid phosphatase (TRAP) and cathepsin K (Figure 3). The results showed that the gene expressions of bone erosion makers TRAP and cathepsin K were significantly increased in the ligature-induced periodontitis (Figures 3(a) and 3(b)), rather than bone formation markers OC and ALP (Figures 3(c), and 3(d)).

3.3. PTEN Regulated the Expression of Inflammatory Cytokines. Macrophages play important roles in the pathogenesis of periodontitis by regulating the immune response and regulating tissue repair and bone loss [21, 22]. We first silence the PTEN expression in RAW264.7 cells through siRNA and found the same results as those of in vivo experiment; the expressions of inflammatory cytokines IL1 and TNF-α were significantly increased (Figure 4(a)). To further confirm this, we then forced overexpression of PTEN in RAW264.7 cells. The results showed that overexpression of PTEN reduced the expression of IL1 and TNF-α (Figure 4(b)).

3.4. Overexpression of PTEN Inhibits the Inflammation In Vivo. To further study the anti-inflammatory effects of PTEN, we made PTEN nanoparticles by mixing the 10 μg PTEN plasmids with 20 μl nanoparticles and injected the mixture into the gingiva three times/week for two weeks (Figure 5(a)). After injection, we found that PTEN gene expression was increased (Figure 5(b)) while the inflammatory factors such as IL1, IL6, and TNF-α were decreased, especially the expression of IL1 and TNF-α (Figures 5(c)–5(e)). Moreover, osteoclast markers TRAP and cathepsin K were downregulated after exogenous PTEN injection (Figures 5(f) and 5(g)). Conclusively, our results showed that nanoparticle-packaged PTEN can alleviate inflammatory osteolysis.

4. Discussion

Our in vitro and in vivo data highlight the anti-inflammatory role of PTEN presentation. The schematic structure of the predicted PTEN protein has been reported [23]. The phosphoinositide 3-kinases can be inhibited by the protein and lipid phosphatase activity of PTEN [24]. Here, we found the gene expression level of PTEN is responsible for inflammation regulation. However, we cannot neglect the effect of protein and its postmodification; thus, further study will focus on the protein and modification level.

In our study, inhibition of PTEN expression with siRNA results in upregulation of IL1 and TNF-α, whereas the expression of IL6 was not significantly altered, suggesting that PTEN may exert anti-inflammatory effects in mouse periodontitis by modulating the expression of IL1 and TNF-α. NF-κB is generally considered to be the major regulatory pathway of genes encoding proinflammatory proteins including IL1, IL6, and TNF-α [25]. The expression of TNF-stimulated NF-κB-dependent genes can be blocked by PTEN [26], which supports our hypothesis that PTEN can play a role in regulating the expression of proinflammatory proteins IL1 and TNF-α. In addition, PTEN can regulate NF-κB via the PI3K/Akt pathway [9]. Under physiological conditions, PI3K/Akt/PTEN signaling plays a key role in inflammation [27]. As the common downstream molecules of NF-κB pathway, IL1, IL6, and TNF-α play a role in maintaining the cell homeostasis [28–30], further suggesting that PTEN may regulate the inflammatory response through the PI3K/Akt/NF-κB pathway.

PTEN enforced overexpression in RAW 264.7 cells inhibited inflammation and osteoclast in our mouse model,

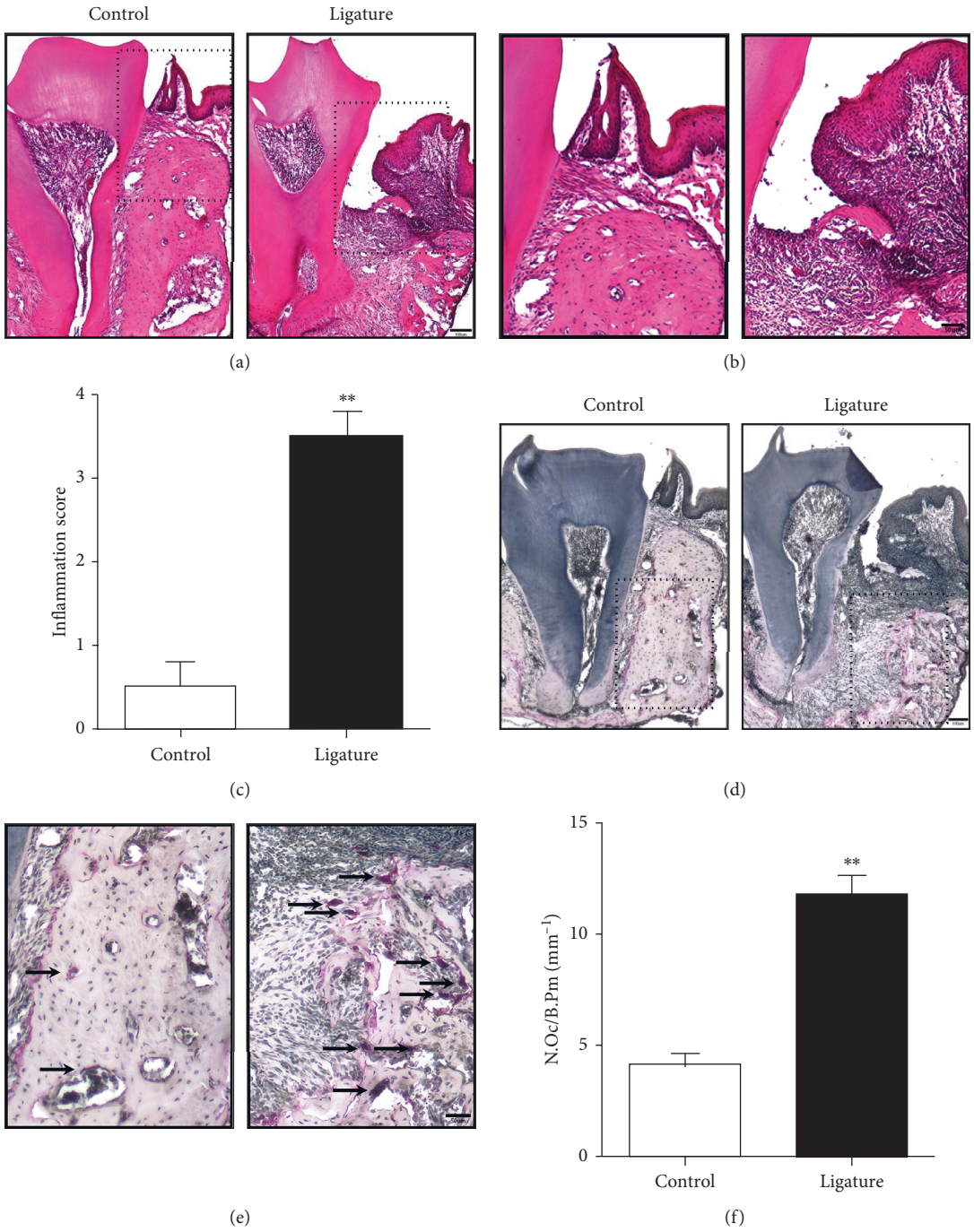


Figure 1: Ligature induced the periodontitis and inflammatory bone loss. (a) Histologic changes in periodontal tissue were monitored by hematoxylin and eosin (H&E) staining. Noticeable inflammatory reaction and damage to organizational structures were observed in ligature-induced periodontitis. Scale bars, 100 μm. (b) Higher magnification of (a). Scale bars, 50 μm. (c) Quantitative analysis of inflammation score of maxillary second molar periodontal tissues. Values are the mean ± SEM. Asterisks (**) denote significant differences ($p < 0.01$), $n = 5$ biological replicates. (d) TRAP staining showed a significant increase of TRAP-positive multinucleated osteoclasts in mouse maxilla in the ligature-induced periodontitis group. Scale bars, 100 μm. (e) Higher magnification of (d). Scale bars, 50 μm. (f) Quantitative analysis of TRAP-positive multinucleated osteoclasts showed a significantly increased number of osteoclasts in the ligature-induced periodontitis group compared to unligated group. N.Oc/B.Pm (mm^{-1}): number of osteoclasts per bone perimeter. Values are the mean ± SEM. $^{**}p < 0.01$, $n = 5$ biological replicates.

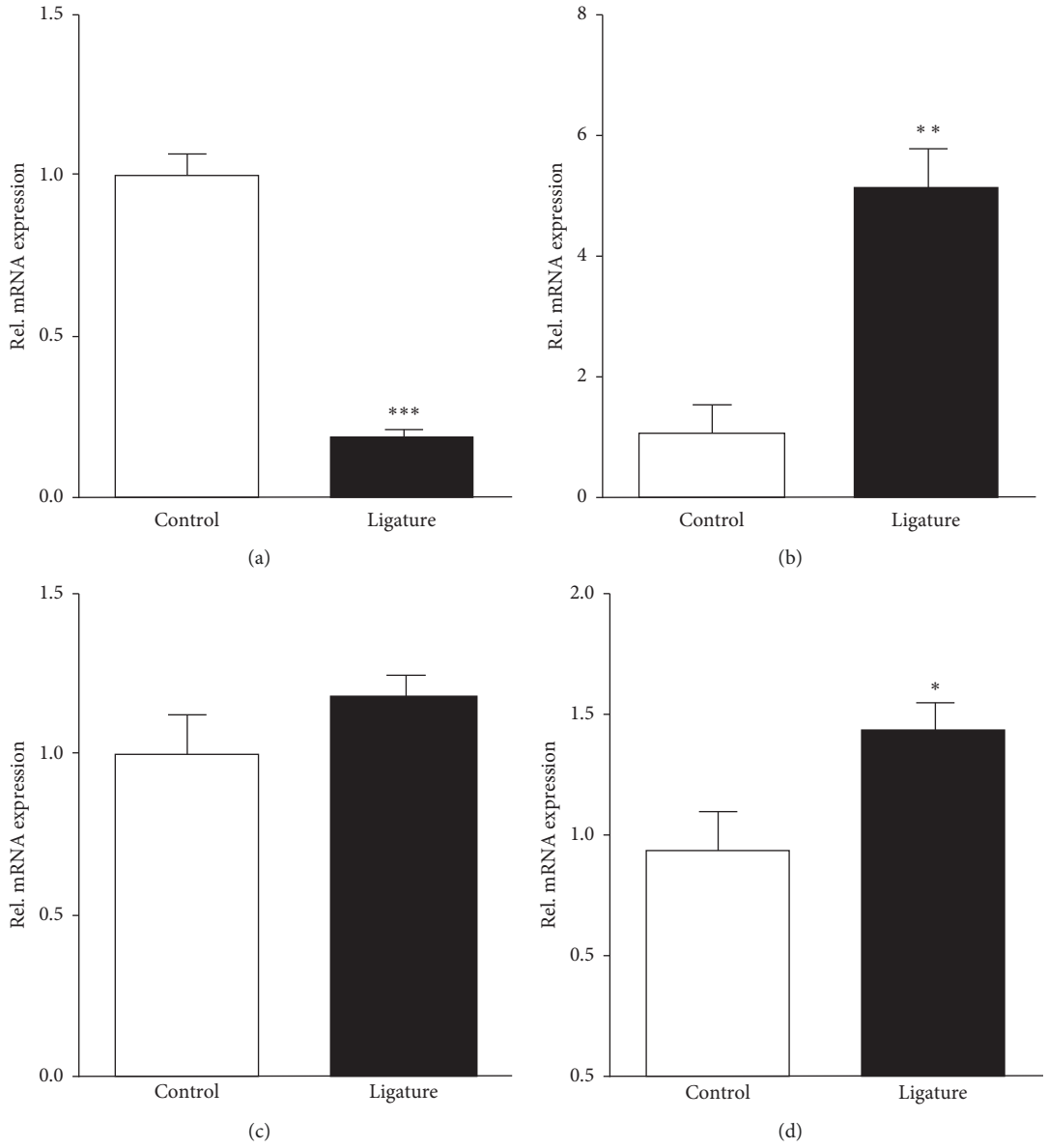


Figure 2: PTEN mRNA were reduced and inflammatory factor mRNA was increased by ligature. (a) Quantitative real-time PCR analysis of PTEN mRNA transcripts from ligature-induced and control mice. Each sample was standardized to GAPDH levels and run in triplicate. Data represent PTEN expression in periodontal tissue relative to control. $n = 5$ biological replicates. Inflammatory factors (b) IL1, (c) IL6, and (d) TNF-α were measured as described in Materials and Methods from periodontal tissues. $n = 5$ biological replicates. Values are the mean ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $n = 5$ biological replicates.

indicating that PTEN may act as a critical regulator between inflammation and bone remodeling. Some relative studies have shown that downregulating PTEN increased phosphorylation of Akt which can promote osteoclastogenesis [31–33]. We herein found that PTEN can regulate osteoclast activity through inflammatory factors, particularly IL1.

Numerous studies have demonstrated that IL1 can stimulate osteoclast differentiation and activation [34–37]. In addition, inhibiting IL1 significantly reduces bone erosions and cartilage degradation, whereas blocking TNF-α decreases inflammation [35, 38, 39]. IL1 may increase multinucleated cell formation through direct stimulation [40]. IL1 is capable of inducing CSF-GM production and can stimulate CSF-GM production in the bone marrow. CSF-GM stimulates the proliferation and differentiation of CFU-GM, the probable progenitor for osteoclast [41, 42]. We therefore speculate that PTEN regulate the bone remodeling mainly through activation of IL1. However, no significant changes were found in osteoblast marker genes, suggesting that PTEN primarily regulates osteoclasts rather than osteoblasts during bone remodeling.

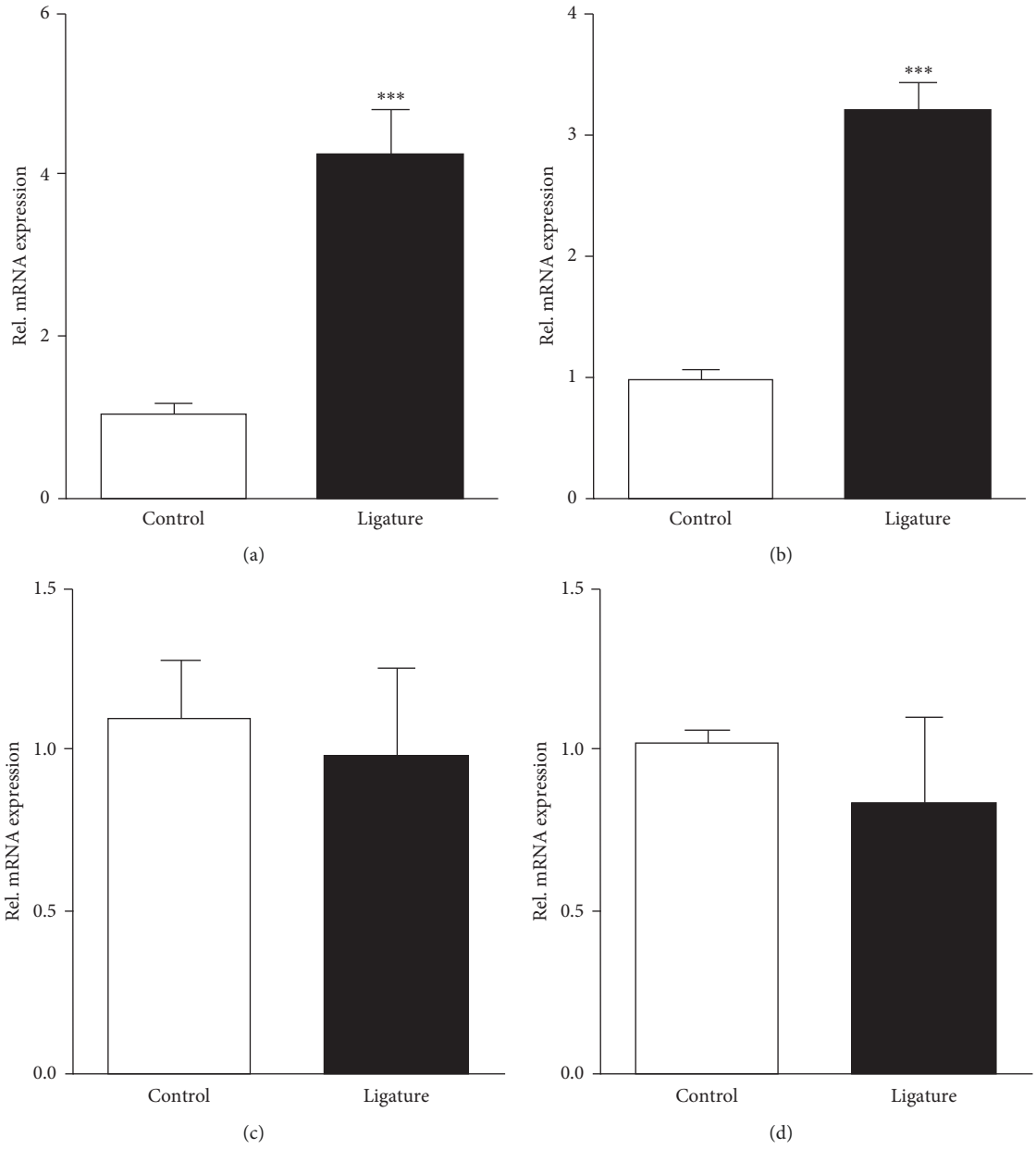
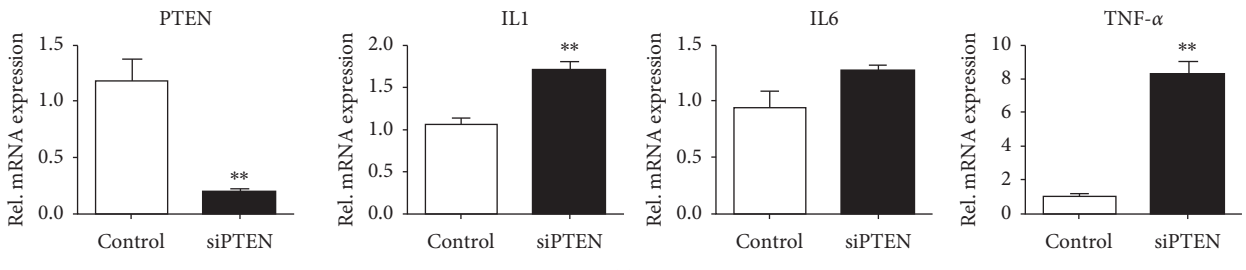


Figure 3: Ligature-induced periodontitis enhanced bone erosion but not bone formation. Levels of expression of mRNA for (a) osteoclast TRAP and (b) cathepsin K in tissue extracted from periodontal tissue obtained on day 10 from control and ligature mice. Data were normalized for GAPDH expression. Values are the mean ± SEM. $^{*}p < 0.05$; $^{***}p < 0.001$; $n = 5$ biological replicates. Levels of expression of mRNA for the (c) osteoblast marker OC and (d) ALP in tissue extracts from periodontal tissue obtained on day 10 from control and ligature mice as in (a). Values are the mean and SEM of 5 mice per group.



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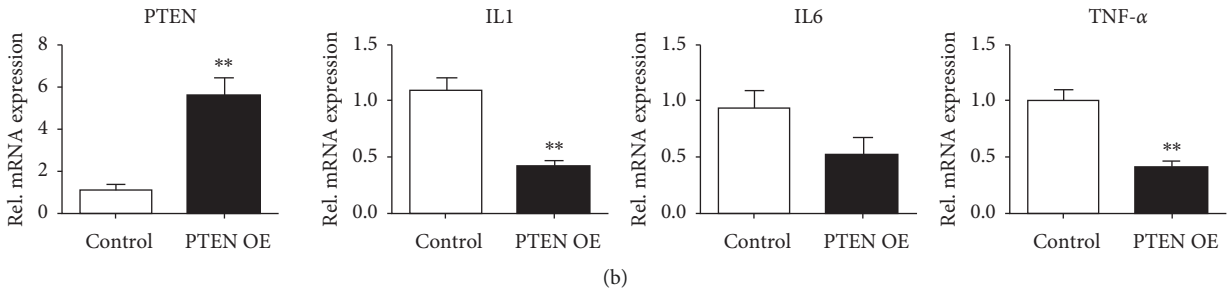

Figure 4: PTEN could positively regulate inflammatory factors. PTEN affects the expression of inflammatory factors. RAW264.7 cells were treated with control siRNA (MOCK) and siPTEN (a) or transfected with pcDNA3.1 (MOCK) and pcDNA3.1-PTEN plasmids (PTEN overexpression; PTEN OE) (b) for 48 h and used to measure PTEN, IL1, IL6, and TNF-α mRNA levels by quantitative real-time PCR. The results showed that overexpression of PTEN inhibits inflammatory factors, while knockdown of PTEN promotes inflammatory factors (IL1, IL6, and TNF-α). Values are the mean ± SEM. $^{**}p < 0.01$; $n = 5$ biological replicates.

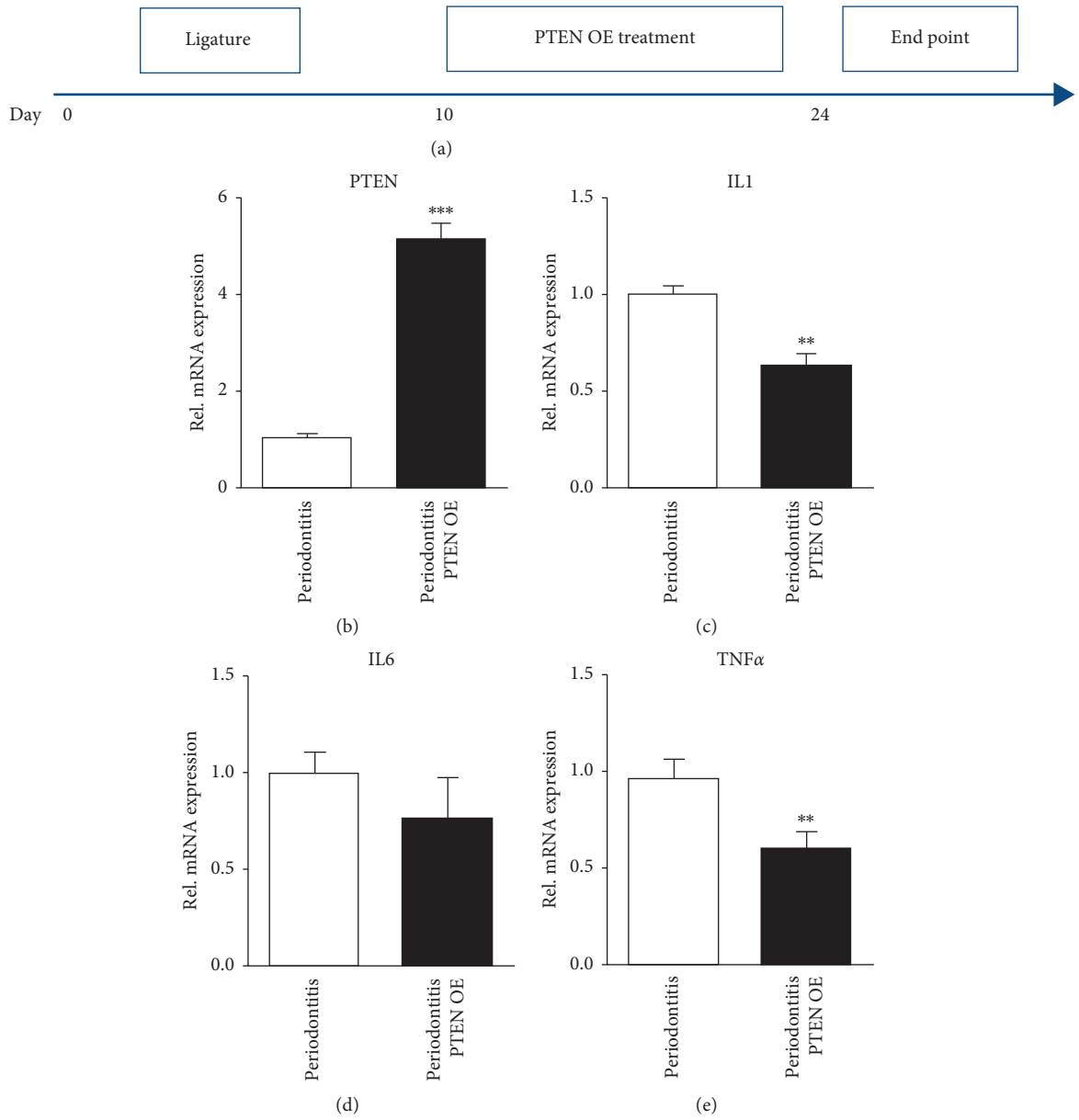

Figure 5: Continued.

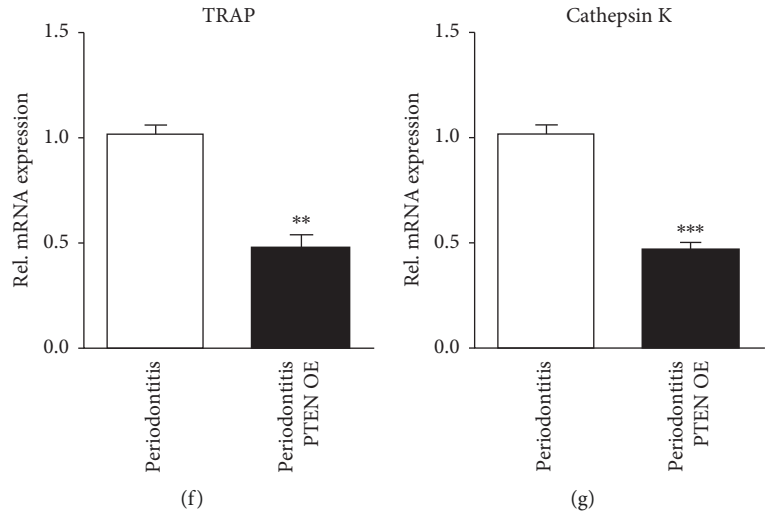

Figure 5: Forced overexpression of PTEN could inhibit inflammation and bone erosion. (a) Schematic figure shows PTEN nanoparticles (10 μg PTEN plasmids with nanoparticles) treated with the ligature on day 10 for two weeks, three times per week. (b) Levels of expression of mRNA for PTEN in periodontal tissue extracts obtained on day 24 from ligature and ligature with PTEN nanoparticle treatment mice. Data were normalized for GAPDH expression. Values are the mean ± SEM. $^{***}p < 0.001$; $n = 5$ biological replicates. Relative mRNA expression levels for the inflammatory marker genes (c) IL1, (d) IL6, and (e) TNF-α in tissue extracts from periodontal tissue obtained as in (b). Values are the mean ± SEM. $^{**}p < 0.01$; $n = 5$ biological replicates. Relative mRNA expression levels for the osteoclast marker genes (f) TRAP and (g) cathepsin K in tissue extracts from periodontal tissue obtained as in (b). Note that osteoclast marker genes decreased when treated with PTEN nanoparticles. Values are the mean ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 5$ biological replicates.

5. Conclusions

In summary, we report here for the first time that activation of PTEN is necessary for inhibition of inflammation and alveolar bone loss, which suppresses both processes via inhibiting the IL1 and TNF-α pathways in periodontitis.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

All authors declare there are no conflicts of interest.

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Research Article

Magnesium Enhances Osteogenesis of BMSCs by Tuning Osteoimmunomodulation

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In the process of bone tissue engineering, the osteoimmunomodulatory property of biomaterials is very important for osteogenic differentiation of stem cells, which determines the outcome of bone regeneration. Magnesium (Mg) is a biodegradable, biocompatible metal that has osteoconductive properties and has been regarded as a promising bone biomaterial. However, the high degradation rate of Mg leads to excessive inflammation, thereby restricting its application in bone tissue engineering. Importantly, different coatings or magnesium alloys have been utilized to lower the rate of degradation. In fact, a prior study proved that β-TCP coating of Mg scaffolds can modulate the osteoimmunomodulatory properties of Mg-based biomaterials and create a favorable immune microenvironment for osteogenesis. However, the osteoimmunomodulatory properties of Mg ions themselves have not been explored yet. In this study, the osteoimmunomodulatory properties of Mg ions with involvement of macrophages and bone marrow stem cells (BMSCs) were systematically investigated. Microscale Mg ions (100 mg/L) were found to possess osteoimmunomodulatory properties that favor bone formation. Specifically, microscale Mg ions induced M2 phenotype changes of macrophages and the release of anti-inflammatory cytokines by inhibiting the TLR-NF-κB signaling pathway. Microscale Mg ions also stimulated the expression of osteoinductive molecules in macrophages while Mg ions/macrophage-conditioned medium promoted osteogenesis of BMSCs through the BMP/SMAD signaling pathway. These findings indicate that manipulating Mg ion concentration can endow the Mg biomaterial with favorable osteoimmunomodulatory properties, thereby providing fundamental evidence for improving and modifying the effect of Mg-based bone biomaterials.

1. Introduction

Foreign materials for repairing bone defects have a great influence on osteogenesis and osteoclasts, forming the basis for the study of osteoimmunology. Osteoimmunology aims to understand the interaction and related mechanism between the skeletal system and immune system [1]. When an implant is placed into a host, immune response around the implant is triggered. Following the start of immune response, phenotype switching of macrophages and adhesion of interleukin- (IL-) 10, IL-1ra, and other inflammatory factors occur, which also have an influence on cells associated with osteogenesis and osteoclasts [1, 2]. As there is a strong relationship between the immune system and the skeletal system, an ideal bone biomaterial in the host should be able to accelerate osteogenesis in the bone defect area through local immune response. Immunomodulatory properties of bone substitute materials are suggested to be of great importance for the success of bone tissue engineering [1, 2].

Magnesium (Mg) is an essential inorganic component in bone tissue and plays an important role in skeletal development. Mg has mechanical properties similar to those of bone tissue and displays antibacterial activity, excellent biocompatibility, and biodegradability [3, 4]. Studies have shown that Mg ion supplementation improved the adhesion of osteoblasts to biomaterials, mediated by integrin [5]. In addition, Mg ions act as the nuclei for hydroxyapatite

formation to promote bone matrix mineralization [6, 7]. Mg-incorporation of mesoporous TiO_2 coatings showed better surface, osteoconductive ability, and elevated expression of osteogenic genes [8]. However, there is still a great challenge that must be addressed before Mg can be utilized clinically. The active chemical character of Mg will not only produce a great amount of air, which reduces the contact between bone and material, but also result in an inflammatory reaction due to rapid degradation. Notably, different coatings or magnesium alloys have been utilized to lower the rate of degradation. A prior study showed that β-TCP coating of Mg scaffolds can modulate the scaffold's osteoimmunomodulatory properties and shift the immune microenvironment toward one that favors osteogenesis over osteoclastogenesis [9]. However, the osteoimmunomodulatory properties of Mg ion itself have not yet been explored.

Macrophages play an important role in human immune defense and osteoimmunology [10, 11]. There are two typical phenotypes of macrophages. The classically activated M1 phenotype mainly participates in T helper cell 1- (Th1-) type inflammation, which is involved in defense against foreign harmful substances, but can sometimes cause excessive inflammatory response in host. Additionally, the alternatively activated M2 phenotype is involved in Th2-type inflammation which reduces inflammation response and improves impairment [12, 13]. These two phenotypes can switch to each other in response to biomaterials or microbes. Furthermore, following phenotype switching of macrophages, osteoinductive molecules such as bone morphogenetic protein 2 (BMP-2) and transforming growth factor-β (TGF-β) can be secreted to promote osteogenesis [14, 15]. Given their important roles in bone remodeling, the response of macrophages was applied to evaluate the osteoimmunomodulatory properties of biomaterials [16].

In the present study, the osteoimmunomodulatory properties of microscale Mg ions were extensively investigated by using a biomimicking condition comprising Mg ions, bone marrow stem cells (BMSCs), and macrophages. First, the phenotype changes of macrophages in response to Mg ions and inflammatory/anti-inflammatory cytokines were evaluated to assess the immune environment. Thereafter, the important inflammatory signaling pathway factor, nuclear factor kappa B (NF-κB), was studied to explore the molecular mechanism of Mg ions in macrophages. The osteogenic differentiation of BMSCs mediated by the Mg ions was then investigated under the influence of macrophages, to prove whether the regulated immune environment by Mg ion could promote osteogenesis. The aim of this study was to determine whether microscale Mg ions possess osteoimmunomodulatory properties and whether this regulated immune environment could positively influence osteogenesis, ultimately providing the fundamental evidence of utilizing Mg-based biomaterial as bone scaffold.

2. Materials and Methods

2.1. Cell Culture. The murine-derived macrophage cell line, RAW 264.7 (RAW), was cultured in Dulbecco's modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco) and 1% (v/v) penicillin/streptomycin (Sigma, USA) at 37°C in a humidified CO_2 incubator. Growing cells were expanded for two passages before use in this study. Mice BMSCs were isolated and cultured following protocols from previous studies [9]. Bone marrow was briefly isolated from SD mice (5–6 weeks old). Under aseptic conditions, bilateral femurs and tibias of rats were isolated and removed. Bone marrow was rinsed with DMEM solution and centrifuged at 1000 rpm for 5 min. The supernatant was discarded, and the precipitate was resuspended with culture medium containing DMEM, 15% FBS, and 1% (v/v) penicillin/streptomycin. Cells were then seeded in tissue culture flasks and incubated at 37°C in a humidified CO_2 incubator. The culture medium was first changed within 24 h and then every 3 days. The attached cells were expanded and early passages (p3) were used in the following study.

2.2. Effect of Mg Ions on RAW264.7 Cells

2.2.1. Proliferation of RAW Cells Stimulated with Mg Ions. Cell culture medium consisted of DMEM without Mg ions (HyClone, USA), 10% FBS, and 1% (v/v) penicillin/streptomycin. Based on the molecular weights of Mg, S, and O, $MgSO_4$ was added to the culture medium to a final concentration of 0, 5, 10, 25, 50, 100, 250, and 500 mg/L Mg ions. Additionally, the effects of Mg ions on RAW proliferation were investigated using Cell Counting Kit-8 Assay (CCK-8, Dojindo, Japan). RAW cells were seeded in 96-well microplates at a density of 2×10^3 cells/well and were allowed to adhere and spread for 24 h. RAW cells were then treated with various concentrations of Mg ions (0, 5, 10, 25, 50, 100, 250, and 500 mg/L) for 1, 3, and 7 days. Thereafter, cells were incubated with the CCK-8 solution for 2 h. The absorbance was measured at 450 nm by using a UV spectrophotometer.

2.2.2. Phenotype Switches and Expression of Inflammatory Genes in RAW Cells. Gene expression of macrophage surface markers (CCR7 and CD206) and inflammatory-related cytokines (IL-1ra, IL-10, IL-1β, IL-6, IL-18, and TNF-α) were detected by RT-PCR to observe the phenotype changes and pro/anti-inflammation ability of RAW cells. These cells were also seeded on 6-well plates at a density of 1×10^6 cells/well. LPS (1 μg/mL) was added to the media when it reached 80% confluence to activate RAW cells for 2 h. Cells were then stimulated with different concentrations of Mg ions (0, 10, 100, and 500 mg/L) for 6 h. Thereafter, total RNA was extracted by using TRIzol reagent (Invitrogen, USA), and the RNA concentrations were quantified with a Nanodrop protein/nucleic acid spectrophotometer (Thermo-Fisher, USA). Notably, first strand cDNA was synthesized using the RNA reverse transcription kit (Takara, Japan), and qRT-PCR was performed using a SYBR Green I Master kit (Takara) in LightCycle 96 RT-PCR (Roche, Switzerland). Primers for the target genes are listed in Table 1.

Table 1: Primer pairs used in the qRT-PCR.

Gene	Primer sequences
CD206	Forward: 5′-AGACGAAATCCCTGCTACTG-3′ Reverse: 5′-CACCCATTCGAAGGCATTC-3′
CCR7	Forward: 5′-ATGACGTCACCTACAGCCTG-3′ Reverse: 5′-CAGCCCAAGTCCTTGAAGAG-3′
IL-1ra	Forward: 5′-CTCCAGCTGGAGGAAGTTAAC-3′ Reverse: 5′-CTGACTCAAAGCTGGTGGTG-3′
IL-10	Forward: 5′-GAGAAGCATGGCCCAGAAATC-3′ Reverse: 5′-GAGAAATCGATGACAGCGCC-3′
IL-1β	Forward: 5′-TGGAGAGTGTGGATCCCAAG-3′ Reverse: 5′-GGTGCTGATGTACCAGTTGG-3′
IL-6	Forward: 5′-ATAGTCCTTCCTACCCCAATTTCC-3′ Reverse: 5′-GATGAATTGGATGGTCTTGGTCC-3′
IL-18	Forward: 5′-TGGCCGACTTCACTGTACAAC-3′ Reverse: 5′-TGGGGTTCACTGGCACTTTG-3′
TNF-α	Forward: 5′-CTGAACTTCGGGGTGATCGG-3′ Reverse: 5′-GGCTTGTCACTCGAATTTTGAGA-3′
Myd88	Forward: 5′-AGGTAAGCAGCAGAACCAGG-3′ Reverse: 5′-TGTCCTAGGGGGTCATCAAGG-3′
Ticam1	Forward: 5′-AGATGGTTCAGCTGGGTGTC-3′ Reverse: 5′-TGGAGTCTCAAGAAGGGGTTC-3′
Ticam2	Forward: 5′-CTTGGCGCTGCAAACCATC-3′ Reverse: 5′-GCCTCTCAAATACAGACTCCCG-3′
TGF-β1	Forward: 5′-GTGGAAATCAACGGGATCAGC-3′ Reverse: 5′-CAGCAGTTCTTCTCTGTGGAGC-3′
TGF-β3	Forward: 5′-CAACACCCTGAACCCAGAG-3′ Reverse: 5′-CTT CACCACCATGTTGGACAG-3′
BMP-2	Forward: 5′-GCTCCACAAACGAGAAAAGC-3′ Reverse: 5′-AGCAAGGGGAAAAGGACACT-3′
BMP-6	Forward: 5′-TGGCAGGACTGGATCATTGC-3′ Reverse: 5′-ACCAAGGTCTGTACAATGGCG-3′
VEGF	Forward: 5′-GTCCCATGAAGTGATCAAGTTC-3′ Reverse: 5′-TCTGCATGGTGATGTTGCTCTCTG-3′
GAPDH (mouse)	Forward: 5′-TGACCACAGTCCATGCCATC-3′ Reverse: 5′-GACGGACACATTGGGGGTAG-3′
Runx-2	Forward: 5′-TCTTTTGGGATCCGAGCACC-3′ Reverse: 5′-ATCTCCACCATGGTGCGGTT-3′
ALP	Forward: 5′-CCA TTT CAG CCT CAG GAT CG-3′ Reverse: 5′-TGG CCA CGT TGG TGT TGA GT-3′
OPN	Forward: 5′-CCAAGCGTGGAAACACACAGCC-3′ Reverse: 5′-GGCTTTGGAACTCGCCTGACTG-3′
OCN	Forward: 5′-GCCCTGACTGCATTCTGCCTCT-3′ Reverse: 5′-TCACCACCTTACTGCCCTCCTG-3′
SMAD4	Forward: 5′- TACCACCATAACAGCACTAC-3′ Reverse: 5′-GAACACCAATATTCAGGAGC-3′
SMAD5	Forward: 5′-GTACTATGAACTGAACAACGG-3′ Reverse: 5′-TATAGATGGACACCTTTCCC-3′
SMAD1	Forward: 5′-GAGATCAATAGAGGAGATGTTC -3′ Reverse: 5′-TCGGTTCTTATTGTTGGAAG-3′
BMPR1A	Forward: 5′-GACACGTGCGAATTGGACAATG-3′ Reverse: 5′-CGTCTGATTTCATACCAGTAC-3′
GAPDH (rat)	Forward: 5′-TCAGCAATGCCTCCTGCAC-3′ Reverse: 5′-TCTGGGTGGCAGTGATGGC-3′

2.2.3. Activation of Toll-Like Receptor (TLR) and NF-κB Signaling Pathways in RAW Cells. The TLR and NF-κB pathways were analyzed to explore the molecular mechanisms that underlie the macrophage gene changes. RAW cells were seeded on 6-well plates at a density of 1×10^6 cells per well and grew to 80% confluence. RAW cells were first activated by LPS (1 μg/mL) for 2 h and then stimulated with different concentrations of Mg ions for 6 h. Total RNA was collected for gene detection of myeloid differentiation factor 88 (MyD88) and TIR domain-containing adapter molecule 1 (Ticam1) and Ticam2 by RT-PCR, using the same method described in Section 2.2.2.

Whole cell lysates were also collected after 6 h of stimulation by Mg ions, and protein expression of NF-κB p65 and inhibitor protein kappa B (I-κB) were determined by western blot. In addition, total protein from RAW cells was extracted using the total protein extraction reagent kit (Beyotime Institute, Shanghai, China) and protein concentration was measured using the BCA assay. Equal amounts of protein (20 μg) were prepared and separated using 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto

PVDF membranes. Membranes were blocked in TBST containing 50 g/L skim milk powder for 2 h and incubated with primary antibodies overnight at 4°C. The primary antibodies included rabbit against mice anti-p-I-κB polyclonal antibody (Bioss Corporation, Beijing, China), anti-NF-κB p65 monoclonal antibody (Santa Cruz, USA), and anti-GAPDH (Abcam, UK). Membranes were then washed 3 times and probed with the secondary antibody, anti-rabbit IgG (Bioteke Corporation, Beijing, China). The results were detected with the ECL detection kit, and the relative intensity of protein bands was quantified using the Image J software. Levels of I-κB and NF-κB expression were calculated relative to GADPH.

2.2.4. Expression of Osteogenesis-Related Cytokines in RAW Cells. RAW cells were seeded on 6-well plates, activated by LPS for 2 h, and stimulated with different concentrations of Mg ions for 6 h as described in Section 2.2.3. Samples were collected and subjected to RT-PCR for the detection of BMP-2, BMP-6, TGF-β1, TGF-β3, and vascular endothelial growth factor (VEGF), using the method described in Section 2.2.2.

2.3. Effects of Mg Ions/RAW Cells-Conditioned Media on the Osteogenic Differentiation of BMSCs

2.3.1. ALP Activity Test. RAW cells were stimulated with different concentrations of Mg ions (0, 10, 100, and 500 mg/L) for 6 h. Culture media were then collected and marked as Mg ions/RAW264.7 cells-conditioned media. Alkaline phosphatase (ALP) activity of BMSCs in the condition medium was measured using the Alkaline Phosphatase Assay Kit (BioAssaySystems, USA). BMSCs were seeded on 24-well plates at a density of 5×10^4 cells per well with the complete culture medium. After 80% confluence, cells were stimulated with Mg ions/RAW264.7 cells-conditioned media for 7 days. Cells were then lysed in 1% Triton X-100. The supernatant of the medium was then harvested for ALP assay, and optical density (OD) was detected at 405 nm with a spectrophotometer. ALP activity is presented as OD values divided by the reaction time and total protein amount.

2.3.2. Osteogenic Gene Expression of BMSCs. BMSCs were seeded on 24-well plates at a density of 5×10^4 cells per well with the complete culture medium. BMSCs were also stimulated with Mg ions/RAW264.7 cells-conditioned media for 1 day and 3 days. Samples were collected and subjected to RT-PCR for the detection of the osteogenic genes, Runx-2, ALP, OPN, and OCN, with the method described in Section 2.2.2.

2.3.3. Activation of BMP/SMAD Signaling Pathway in BMSCs. BMSCs were stimulated with Mg ions/RAW264.7 cells-conditioned media for 1 and 3 days, and total RNA was extracted to study the activation of BMP/SMAD pathway by RT-PCR as described in Section 2.2.2. Pathway-related genes included mothers against decapentaplegic homolog 1/4/5 (SMAD1/4/5) and bone morphogenetic protein receptor type IA (BMPR1A). Protein levels of SMAD4 and BMPR1A were further confirmed by western blot on days 3 and 7. The detailed methods are described in Section 2.2.3.

2.3.4. Statistical Analysis. All data were presented as mean ± standard deviation (SD). Statistical analyses were performed with SPSS 22.0. One-way analysis of variance (ANOVA) was used to analyze the statistical difference when more than 2 groups were compared. Student's t-test was used to compare experimental groups and control group. A P value <0.05 was considered to be statistically significant.

3. Results

3.1. Effect of Mg Ions on Cell Proliferation of Macrophages. To identify the cytotoxic effects of Mg ions, macrophages were treated with different concentrations of Mg ions (5, 10, 25, 50, 250, and 500 mg/L) for 1, 3, and 5 days (Figure 1). The CCK-8 assay showed that 100 mg/L (<100 mg/L) Mg ions had no obvious influence on the proliferation of RAW264.7 cells compared to control ($P > 0.05$). However, Mg ions at a concentration of 100 mg/L significantly increased the proliferation of RAW264.7 on days 3 and 5 ($P < 0.05$). On day 5, cell proliferation also significantly increased at a concentration of 250 mg/L ($P < 0.05$).

3.2. Surface Marker Changes and Inflammatory Gene Expression of RAW Cells in response to Mg Ions. Stimulation of RAW cells with Mg ions (100 mg/L) revealed the increased gene expression of the M2 surface marker, CD206, relative to the control group ($P < 0.05$, Figure 2(a)), thereby indicating a shift toward the M2 phenotype in response to Mg ions. In contrast, stimulation with Mg ions resulted in reduced gene expression of the M1 phenotype marker, CCR7, compared to the control group ($P < 0.05$, Figure 2(b)).

The gene expression of anti-inflammatory and inflammatory cytokines was detected in RAW264.7 cells after exposure to Mg ions for 6 h. The expression level of anti-inflammatory genes (IL-10 and IL-1ra) was upregulated at all concentrations of Mg ions compared to the control ($P < 0.05$, Figure 3(a)). In contrast, the expression of the inflammatory cytokine, TNF-α, was significantly downregulated at concentrations of 10 mg/L and 100 mg/L of Mg ions ($P < 0.05$, Figure 3(b)). Furthermore, the expression of other inflammatory cytokines increased slightly. For instance, the expression of IL-6 and IL-1β increased at 500 mg/L Mg ions and that of IL-18 increased at 10 mg/L Mg ions ($P < 0.05$, Figure 3(b)). However, the fold changes of inflammatory cytokines (IL-6, IL-1β, and IL-18) were obviously less than that of anti-inflammatory cytokines (IL-10 and IL-1ra).

3.3. Effect of Mg Ions on TLRs and NF-κB Signaling Pathway in RAW 264.7 Cells. To explore the molecular mechanisms of the inflammation-related gene alterations, the TLRs and NF-κB signaling pathways were examined in RAW cells.

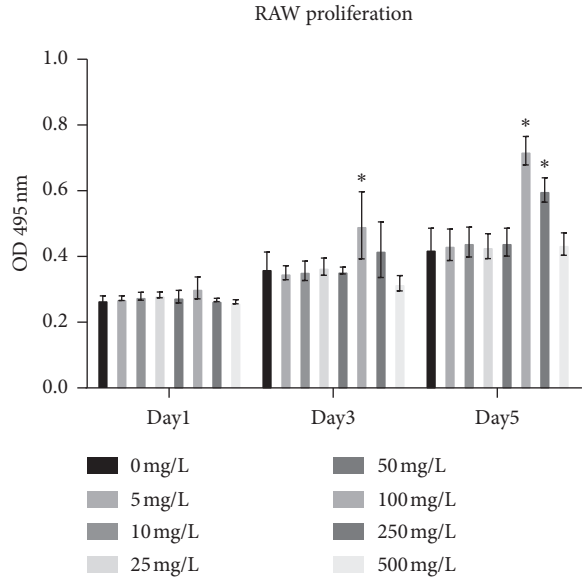

Figure 1: Effect of Mg ions on the proliferation of RAW264.7 cells. $^{*}P < 0.05$ versus the control without Mg ions.

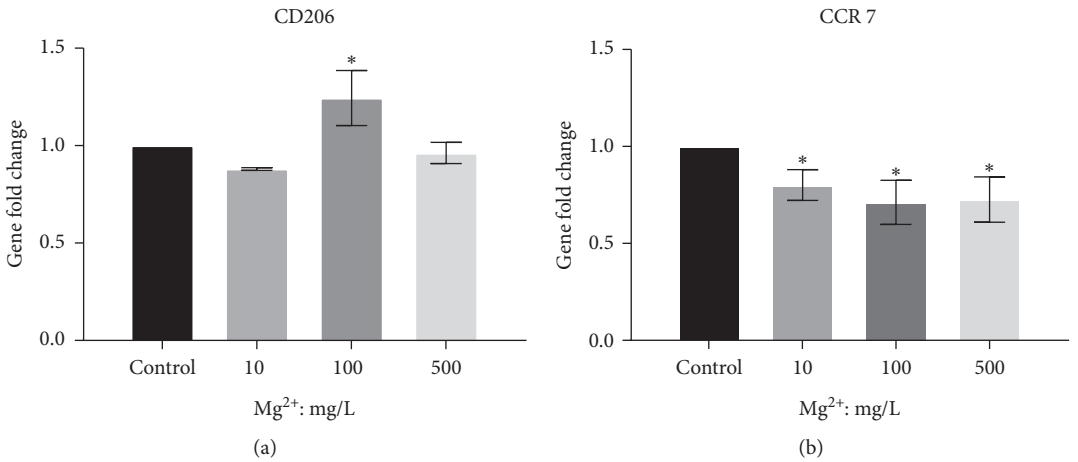

Figure 2: Effect of Mg ions on RAW264.7 phenotype transformation. (a) Gene expression of the M2 phenotype marker, CD206. (b) Gene expression of the M1 phenotype marker, CCR7. $^{*}P < 0.05$, compared to the control group without Mg ions.

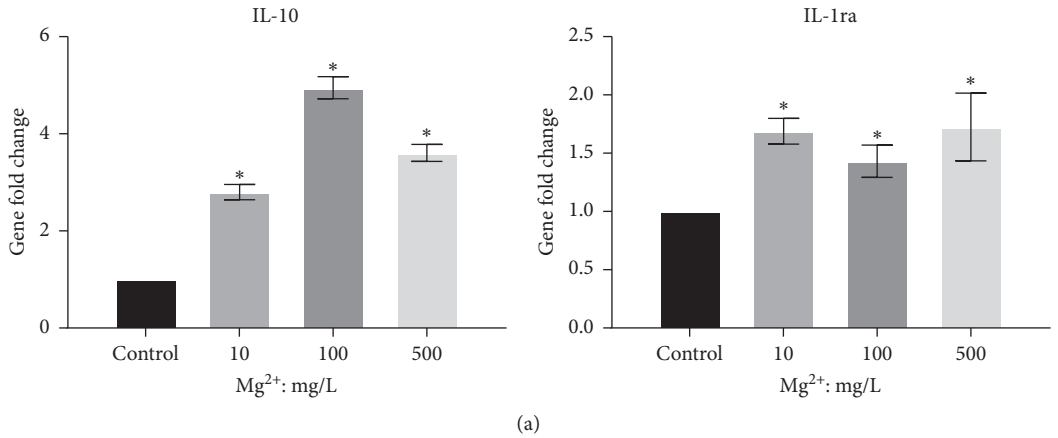

Figure 3: Continued.

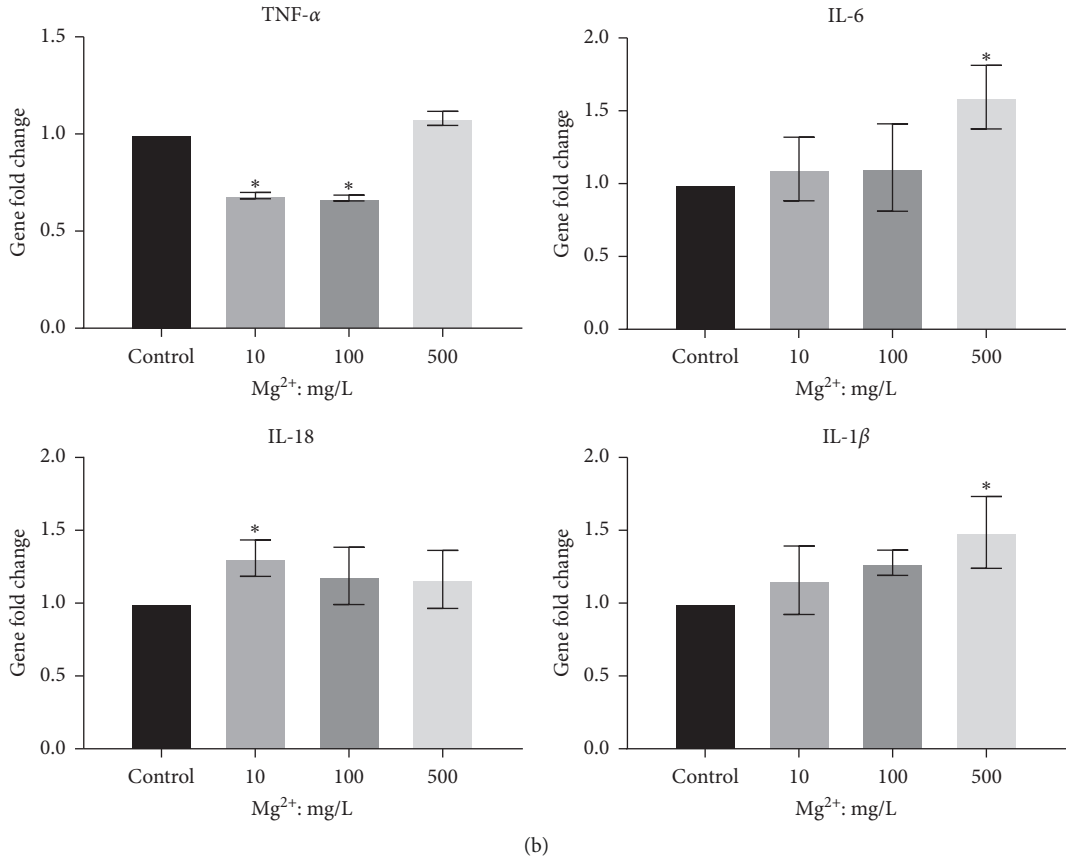

Figure 3: Effect of Mg ions on the gene expression of anti-inflammatory and inflammatory cytokines in RAW264.7 cells. (a) Gene expression of the anti-inflammatory cytokines, IL-10 and IL-1a. (b) Gene expression of the anti-inflammatory cytokines, TNF-α, IL-6, IL-18, and IL-1β. $^*P < 0.05$, compared to the control group without Mg ions.

Compared to the control group, gene expression of Myd88, Ticam1, and Ticam2 were downregulated in 100 and 500 mg/L Mg ion groups with significant differences ($P < 0.05$, Figure 4(a)). Western blot also showed that the protein expression of NF-κB p65 had no significant difference between Mg ion and control groups. In contrast, the downstream molecular IκB-α was upregulated at 100 and 500 mg/L Mg ions compared to the control ($P < 0.05$, Figure 4(b)), indicating the inhibition of the TLR-NF-κB signaling pathway.

3.4. Effect of Mg Ions on the Expression of Osteogenesis-Related Cytokines in RAW264.7 Cells. BMPs, the TGF-β family, and VEGF are all important osteogenesis-related factors. RT-PCR demonstrated that gene levels of BMP-2 and VEGF were significantly higher in 100 mg/L Mg ion groups than that of control ($P < 0.05$, Figure 5). In contrast, TGF-β3 gene expression level was slightly downregulated in Mg ion groups compared to the control group ($P < 0.05$). Gene expression of TGF-β1 and BMP-6 showed no obvious differences in each group.

3.5. Effects of Mg Ions/RAW264.7 Cells-Conditioned Media on the Osteogenic Differentiation of BMSCs. To clarify whether Mg ions influence osteogenesis of BMSCs through regulating macrophages, Mg ions/RAW264.7 cells-conditioned media were utilized for osteogenic differentiation of BMSCs. The results showed that when BMSCs were stimulated with conditioned media containing 100 and 500 mg/L Mg ions, ALP activity was significantly enhanced compared to control ($P < 0.05$, Figure 6(a)). Furthermore, osteogenic gene expression of BMSCs in conditioned media was explored by RT-PCR. On day 3, BMSCs stimulated with Mg ions/RAW264.7 cells-conditioned medium of all concentrations of Mg ions had a significantly upregulated expression of the osteogenic genes (Runx-2, ALP, OPN, and OCN) compared to the control group ($P < 0.05$, Figure 6(b)). In addition, on day 1, conditioned medium with 100 mg/L Mg ions significantly increased gene expression of Runx-2, ALP, and OCN in BMSCs ($P < 0.05$, Figure 6(b)).

3.6. Activation of the BMP/SMAD Signaling Pathway in BMSCs Stimulated with the Mg Ion/RAW264.7 Cells-Conditioned Media. To explore the molecular mechanisms of improved osteogenesis of BMSCs in Mg ions/RAW264.7 cells-conditioned media, the BMP/SMAD signaling pathway was studied. RT-PCR results showed that the gene expressions of SMAD4, SMAD5, and BMPR1A were increased significantly with all concentrations of Mg ions on day 3 ($P < 0.05$, Figure 7(a)). Furthermore, on day 1, the conditioned

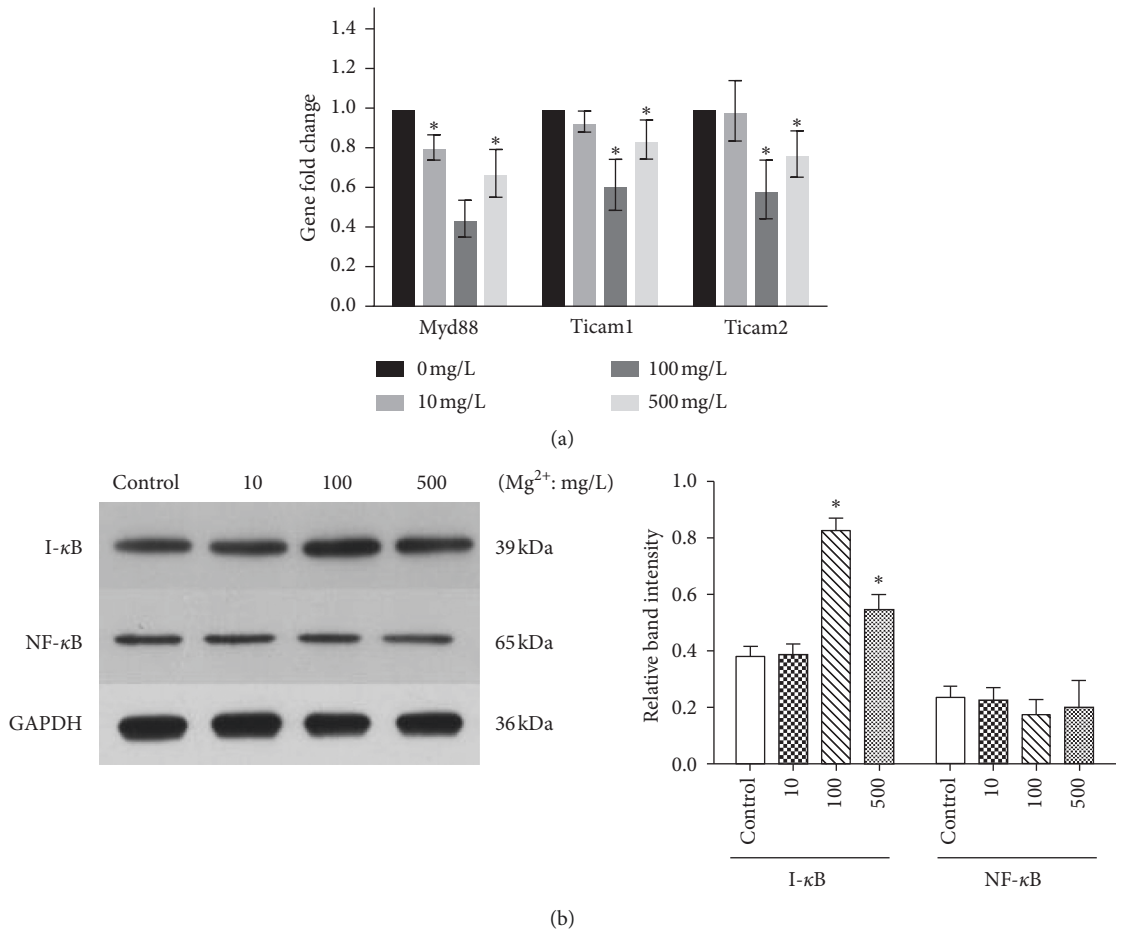

Figure 4: Effect of Mg ions on the TLR and NF-κB signaling pathways of RAW 264.7 cells. (a) Gene expression of the TLRs pathway markers, Myd88, Ticam1, and Ticam2 in RAW264.7 cells. (b) Protein expression of NF-κB p65 and I-κB in RAW 264.7 cells. $^*P < 0.05$, compared to the control group without Mg ions.

medium with 100 mg/L Mg ions caused a significant increase in the gene expression of SMAD4 and BMPR1A ($P < 0.05$, Figure 7(a)). However, gene expression of SMAD1 had no obvious change in all groups. The protein expressions of SMAD4 and BMPR1A were further confirmed by western blot. The result showed that the protein levels of SMAD4 and BMPR1A significantly enhanced in the conditioned medium with 10 and 100 mg/L Mg ions on day 3 ($P < 0.05$, Figure 7(b)). However, on day 7, 100 mg/L Mg ions/RAW264.7 cells-conditioned media also significantly upregulated the protein expression of SMAD4 and BMPR1A ($P < 0.05$, Figure 7(b)).

4. Discussion

In this study, the osteoimmunomodulatory properties of Mg ions with the involvement of macrophages and BMSCs were systematically investigated. Our results showed that microscale Mg ions (100 mg/L) possess the osteoimmunomodulatory property that favors bone formation. More specifically, microscale Mg ions induced the M2 phenotype changes of macrophages and release of anti-inflammatory cytokines by inhibiting the TLR-NF-κB signaling pathway. Mg ions stimulated the expression of osteoinductive molecules in macrophages, and Mg ions/macrophage-conditioned medium promoted osteogenesis of BMSCs, most likely through the BMP/SMAD signaling pathway. These findings indicated that manipulating Mg ion concentration can endow the Mg scaffold with favorable osteoimmunomodulatory properties, thereby providing the fundamental evidence for the development and modification of Mg-based bone biomaterials.

Mg scaffold is a promising bone substitute due to its excellent mechanical properties and biocompatibility [3, 4, 17]. However, Mg is a highly reactive metal and corrodes quickly, thereby causing massive inflammatory reaction *in vivo* [6]. We inferred that the ionic concentration of the Mg scaffold is a key factor that determines the osteoimmunomodulatory property of biomaterials. However, a previous study showed that coating of the Mg scaffolds with β-TCP greatly decreased the concentration of Mg ions in solution (195.4 ± 0.86 mg/L) compared to the Mg scaffolds (1021 ± 2.13 mg/L) [9]. Mg-β-TCP scaffold has been proven to induce macrophages expressing the M2 surface marker, CD163, and anti-inflammatory cytokines (IL-1ra) [9]. Therefore, we hypothesized that the anti-inflammatory

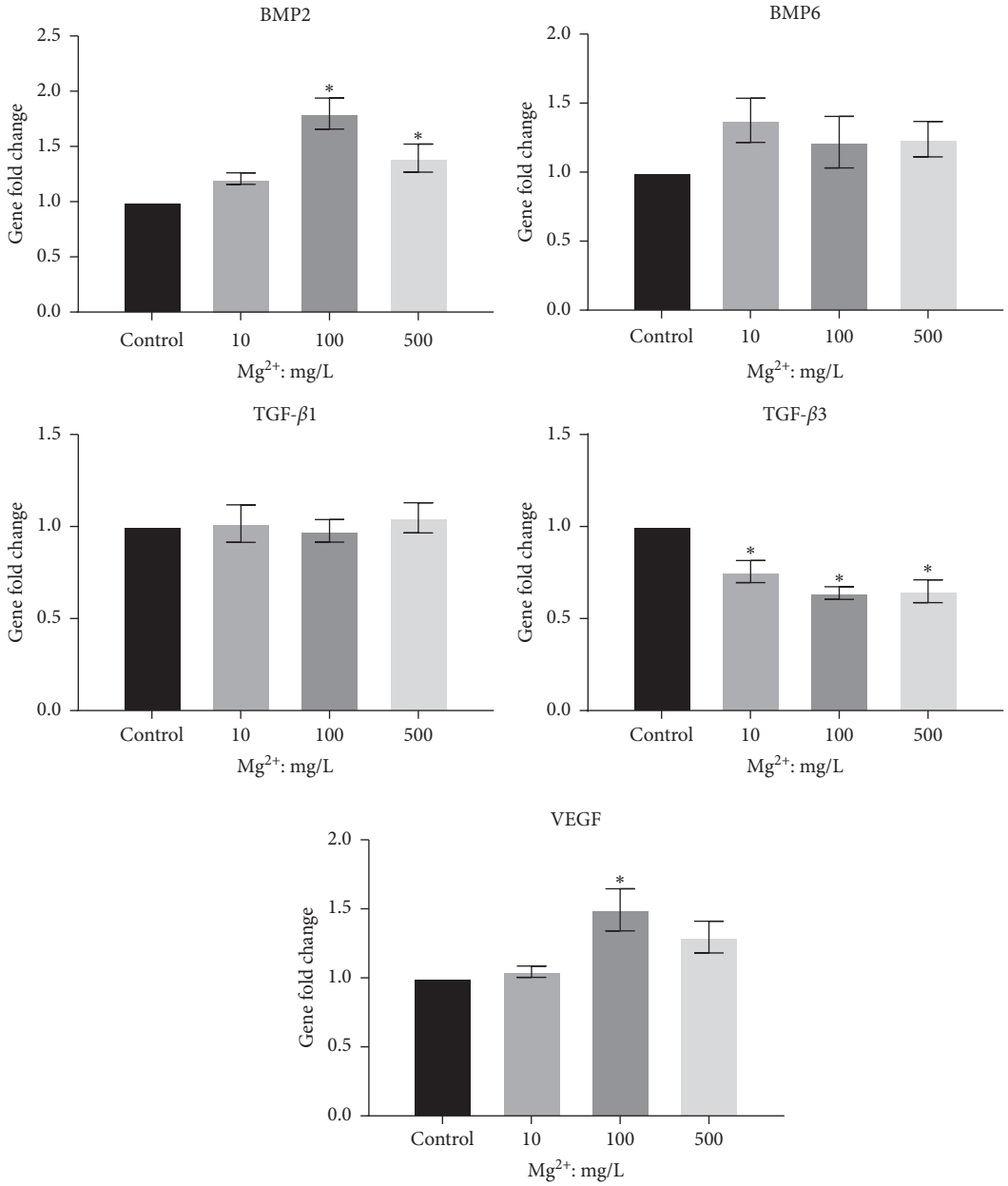

Figure 5: Effect of Mg ions on the gene expression of osteogenesis-related cytokines (BMP2, BMP6, TGF-β1, TGF-β3, and VEGF) in RAW264.7 cells. $^{*}P < 0.05$, compared to the control group without Mg ions.

effects of Mg-β-TCP are attributed to the lower concentration of Mg ions. Our present study however demonstrated that microscale Mg ions (100 mg/L) induce a shift toward the M2 phenotype of macrophage with increased gene expression of the surface marker, CD206, and reduced the M1 phenotype marker, CCR7. Microscale Mg ions (100 mg/L) also increased the gene expression of the anti-inflammatory cytokines, IL-10 and IL-1ra, and decreased the important inflammatory cytokine, TNF-α. Although the inflammatory cytokines of IL-6 and IL-1β increased slightly, it most likely occurred with the high concentration of Mg ions (500 mg/L). Consistently, a previous study by Sugimoto et al. showed that $MgSO_4$ at a concentration of 60 mg/L decreased inflammatory cytokine production of IL-6 and TNF-α by inhibiting the TLR receptor pathway [18], which is approximately the same concentration of Mg ion used in our study (100 mg/L). These findings indicate that the microscale Mg ions can induce macrophage polarization toward the M2 extremity and create an anti-inflammatory microenvironment for bone regeneration.

Notably, toll-like receptor (TLR) signaling is an essential pathway in the innate immune response, through which macrophages recognize foreign antibody and initiate antigen-specific adaptive immune response [19, 20]. Activation of TLR signaling is mediated by a unique interaction between TIR domain-containing cytosolic adapters which include MyD88 and TIR domain-containing adapter-inducing IFNb (TRIF) also known as toll-like receptor adapter

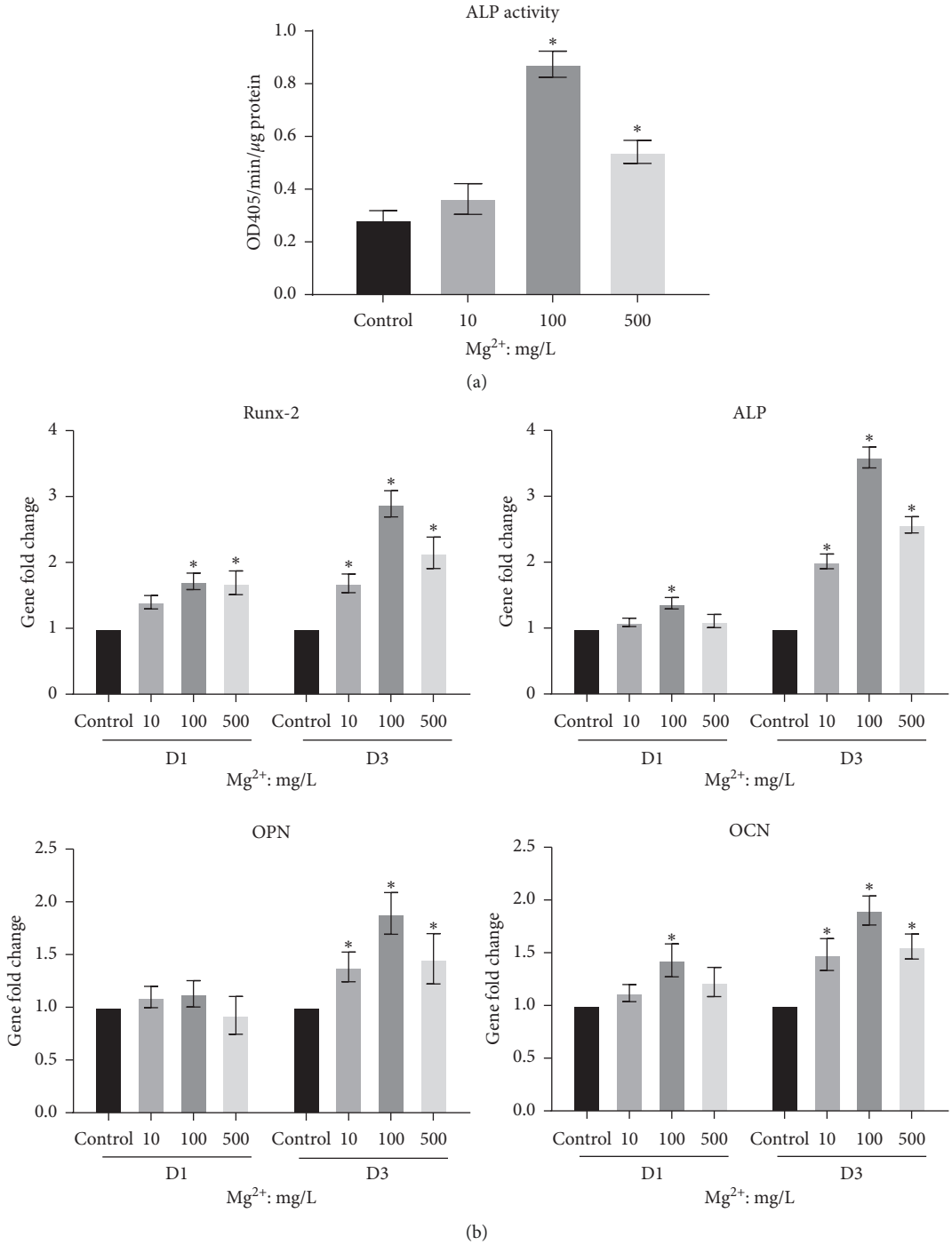

Figure 6: Effects of Mg ions/RAW264.7 cells-conditioned media on the osteogenic differentiation of BMSCs. (a) ALP activity of BMSCs in Mg ions/RAW264.7 cells-conditioned media. (b) Osteogenic gene expression of BMSCs in Mg ions/RAW264.7 cells-conditioned media. $^{*}P < 0.05$, compared to the control group.

molecule (Ticam) [20]. Importantly, upon ligand binding, TLR leads to the activation of NF-κB pathways to elicit the expression of inflammatory cytokines [21]. In most cell types, NF-κB is bound to its inhibitor, I-κB, and resides in the cytoplasm as an inactive NF-κB/I-κB complex [22]. However, the activated form of NF-κB is a heterodimer of the p65 subunit associated with p50 or p52 subunit, and p65/p50 or p65/p52 heterodimer migrates into the nucleus and initiates transcription of the inflammatory genes [22]. In the present study, the gene expression of Myd88, Ticam1, and Ticam2 were downregulated and the NF-κB inhibitor, IκB-α, was upregulated in the 100 and 500 mg/L Mg ion groups. Such finding would indicate that the microscale Mg ions dampen the inflammatory response potentially by inhibiting the TLR-NF-κB pathway. Similarly, a previous study by Sugimoto et al. showed that $MgSO_4$ decreases TLR-mediated

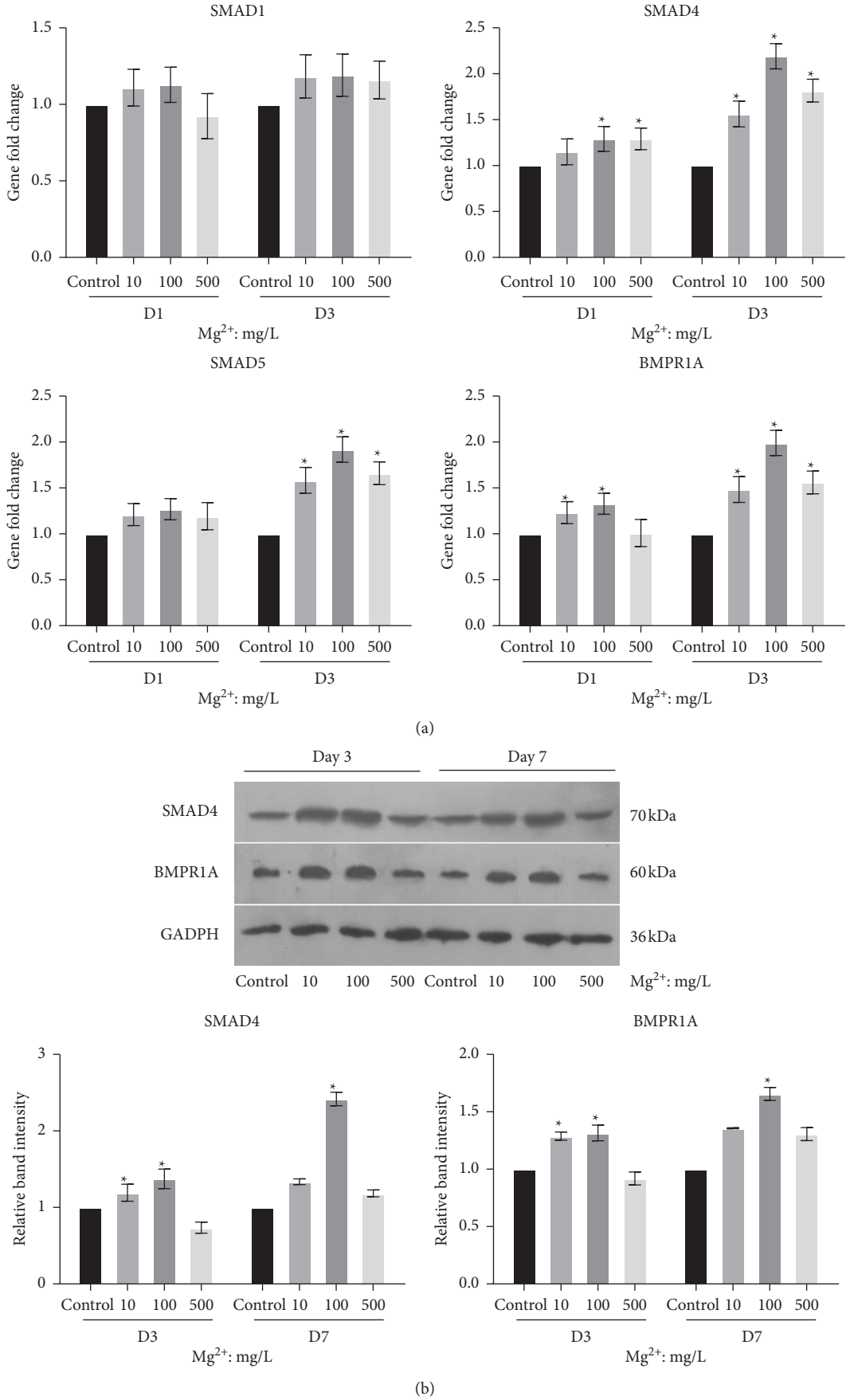

Figure 7: Activation of the BMP/SMAD signaling pathway in BMSCs stimulated with the Mg ions/RAW264.7 cells-conditioned media. (a) Gene expressions of the BMP/SMAD pathway markers SMAD1, SMAD4, SMAD5, and BMPR1A were demonstrated by RT-PCR. (b) Protein expressions of SMAD4 and BMPR1A were examined by western blot. $^{*}P < 0.05$, compared to the control group.

cytokine production in monocytes by increasing IκB-α levels and downregulating NF-κB p65 levels [18]. However, in our study, protein expression of NF-κB p65 showed no alteration in the Mg ion groups. Hence, we inferred that Mg ions might inhibit other components in the NF-κB pathway in macrophages, such as p50 or p52. This discrepancy might be due to the diverse inflammatory cell types and different experimental conditions.

Subsequently, we sought to clarify whether the modification of macrophages by Mg ions would influence osteogenesis of BMSCs. Therefore, Mg ions/RAW264.7 cell-conditioned media were utilized for osteogenic differentiation of BMSCs.

Importantly, RUNX2 is a key transcription factor of osteoblast differentiation [23]. ALP is a well-known marker for pre-osteoblast differentiation and osteoblast mineralization [24]. In addition, OPN and OCN are important genes in the process of mineral deposition [24]. The result showed that when stimulated with conditioned media of 100 mg/L Mg ions, BMSCs resulted in a significant enhancement in ALP activity and osteogenic genes (Runx-2, ALP, OPN, and OCN), which would indicate that Mg ions promote osteogenesis of BMSCs through macrophage regulation.

Although microscale Mg ions have been shown to transit macrophages phenotype into M2, the molecular mechanisms whereby M2 macrophage influences osteogenesis are yet to be established. We hypothesized that the M2 macrophage may promote osteogenesis of BMSCs through paracrine function. Notably, a previous study reported that the M2 phenotype secretes osteoinductive and osteogenic cytokines such as BMP-2 and VEGF [14, 25]. Among the BMP family members, BMP-2 is a potent osteoinductive agent [26–28] and VEGF is an important proangiogenic factor that binds to VEGFR and initiate angiogenic cascade [29]. In the process of bone formation, angiogenesis and osteogenesis are coupled with each other as the function of VEGF and BMP-2 has been found to be closely related and synergistic [30, 31]. Indeed, our study demonstrated that Mg ions upregulated the gene expression of BMP-2 and VEGF in macrophages. We inferred that the upregulation of BMP-2 might activate the BMP-2/SMAD signaling pathway in BMSCs which is the key pathway for osteogenic differentiation. In this pathway, BMP-2 binds with BMPR2 and then recruits BMPRA1 [32]. Subsequently, phosphorylation of SMAD1/5/8 is triggered, which sequentially causes dimer complex to form with SMAD4. The complexes translocate into the cell nucleus to induce transcription of the osteogenic gene, Runx2 [32]. The result however showed the activation of BMP-2/SMAD signaling in BMSCs as demonstrated by the upregulation of SMAD4 and BMPR1A at both gene and protein levels. Therefore, it is reasonable to infer that microscale Mg ions trigger the phenotype switches of macrophages into M2 by inhibiting the TLR-NF-κB signaling pathway and, as a result, causes the upregulation of anti-inflammatory cytokines (IL-10 and IL-1ra). Furthermore, the microscale Mg ions stimulate macrophages to upregulate VEGF and BMP-2 expression, which activate the BMP-2 pathway in BMSCs, thereby enhancing osteogenic differentiation of stem cells. The present study proposed that manipulating Mg concentration in bone biomaterial could regulate the immune environment that positively influences osteogenesis and avoids the destructive inflammatory reaction caused by the Mg-based biomaterial.

Apart from secretion of VEGF and BMP-2, excessive M2 macrophages have been reported to secrete fibrous agents, such as TGF-βs, resulting in pathological fibrosis, formation of scar tissue, or delayed wound healing [14, 25, 33]. TGF-β1 is a potent cytokine to promote fibroblast proliferation [34], and TGF-β3 induces the synthesis of extracellular matrix (ECM) protein, such as type I collagen, fibronectin, proteoglycans, and laminin [35]. In this study, we found the downregulation of TGF-β3 in Mg ion groups, which indicated that maybe stimulating macrophages with microscale Mg ions could not induce pathological fibrosis.

5. Conclusions

In summary, controlling the releasing concentration of Mg ions (approximately 100 mg/L) conquers the detrimental osteoimmunomodulatory properties of Mg-based biomaterials, causing them to be more favorable towards osteogenesis of BMSCs. Specifically, microscale Mg ions induced M2 macrophage phenotype switches and produced an anti-inflammatory environment most likely through the inhibition of the TLR-NF-κB signaling pathway. Microscale Mg ions stimulate macrophage expression of BMP-2 and activate the BMP-2 signaling pathway in BMSCs, thereby enhancing osteogenic differentiation. Therefore, manipulating the concentration of Mg ions in Mg-based bone scaffolds endows biomaterials with favorable osteoimmunomodulatory properties. The present study provides fundamental evidence and proposes novel strategies for the development or modification of advanced Mg-based bone biomaterials using stem cells.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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