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Research article

Peripheral inflammatory T cell subsets are effective predictive factors in the development of heterotopic ossification after posttraumatic elbow surgery

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ABSTRACT

Heterotopic ossification refers to the pathological formation of extra-skeletal bone. It is a common complication of trauma or surgery that can cause disability and has no definitive cure. Furthermore, the mechanisms underlying chronic inflammation during ossification remain unclear. Therefore, this study aimed to elucidate the systemic immune microenvironment status of heterotopic ossification and identify biomarkers of therapeutic efficacy and recurrence. A combination of stereoarthrolysis with prophylactic radiotherapy and non-steroidal anti-inflammatory drugs was used to treat patients with heterotopic ossification. Changes were observed in peripheral blood lymphocyte levels after treatment. The number of $IFN\gamma^{+}CD8^{+}$T cells (3.753 % vs 12.90 %, $P < 0.0001$) and $IL17^{+}CD4^{+}$T cells (3.420 % vs 5.560 %, $P = 0.0281$) were was higher in the peripheral blood of relapsed patients with heterotopic ossification than in that of non-relapsed patients. Similarly, the number of these cells was elevated in patients who developed heterotopic ossification after posttraumatic elbow surgery. Peripheral $CD8^{+}$T cells derived from patients with this pathology promoted osteogenesis through IFNγ expression *in vitro*. Our findings demonstrate that $IFN\gamma^{+}CD8^{+}$T cells and $IL17^{+}CD4^{+}$T cells are potential biomarkers of heterotopic ossification after posttraumatic elbow surgery. Furthermore, these cells can be used to predict therapeutic efficacy and relapse after combination therapy.

1. Introduction

Heterotopic ossification (HO) is a pathological state characterized by bone formation in soft tissues and joint spaces. It is widely thought to be a complication of trauma, burns, and orthopedic surgery [1]. Current evidence suggests that more than 10 % of patients

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who undergo aggressive operations can develop HO, and 65 % of severely wounded soldiers are affected [2]. The elbow joint is the preferred site of HO development. Thus, this pathology is a common complication after the surgical treatment of elbow fractures, with an incidence of approximately 7–62 % [2,3]. The incidence of ectopic ossification after elective and traumatic elbow replacement surgeries is 84 %; the proportion of patients in the trauma group (72 %) was considerably higher than that in the selective arthroplasty group (96 %) [4]. In addition, the occurrence rate of HO was 6.3 % in patients who underwent elbow arthroscopic surgery [5]. HO may cause chronic pain, joint swelling, deep venous thrombosis, motor dysfunction, and other complications that substantially affect patient quality of life [6]. The novel STEPHOP prediction model could effectively predict the occurrence of HO. The male sex, obesity, open wounds, dislocation, delayed surgical treatment, and lack of non-steroidal anti-inflammatory drug (NSAID) use are all adverse predictors of this pathology [7].

Current HO treatment approaches include surgery, bisphosphonates, glucocorticoids, radiotherapy, and NSAIDs [8]. However, 75 % of patients experience limited range of motion (ROM) after a single surgical intervention [9]. Furthermore, although radiotherapy is commonly used to treat HO, it is primarily used to treat tumors. Thus, surgery combined with prophylactic RT and NSAIDs presents a potential therapeutic approach [10]. No other treatment or prophylactic measures are effective in preventing this clinically devastating complication.

Although the pathogenesis of HO remains unclear, it has been established that trauma-induced HO occurs via endochondral ossification that begins with the aggregation of mesenchymal stem cells (MSCs), which then differentiate into chondrocytes [11]. Immunity plays an essential role in trauma-induced HO [12], with a significant emphasis placed on the relationships and interactions between osteoblasts, progenitor cells, and the immune system [13]. Several subsets of immune cells contribute to HO generation. These include neutrophils, which activate osteoclasts by upregulating RANKL expression under inflammatory conditions [14]. Moreover, macrophages stimulate progenitor cells to bony differentiation after tissue injury [15]. However, the cellular components of the adaptive immune system that contribute to the pathogenesis of HO or mediate its therapeutic response remain unclear. Therefore, this study aimed to elucidate the systemic immune microenvironment status of HO and identify predictive biomarkers of therapeutic efficacy and recurrence in the peripheral blood. The present study findings could enable the accurate prediction of therapeutic efficacy and relapse after combination therapy.

2. Materials and methods

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine. In addition, written informed consent was obtained from all study participants. (Approval number: 2022–1013).

2.1. Patients

Patients with posttraumatic HO of the elbow joint who received standard treatment were included in this study. The exclusion criteria were as follows: patients with posttraumatic HO of the elbow joint who did not receive standard treatment, HO of the non-

Table 1
Patient characteristics.

Variable	Result
Age at diagnosis median(range)	41(17–63)
Sex(Male: Female)	46:30
Site(Right: Left)	48:28
Types of trauma	N(%)
OF	8(10.5)
TT	10(13.2)
RHF	10(13.2)
HSF	6(7.9)
DHF	18(23.7)
ED	24(31.6)
Classification	
Class I	0(0)
Class II	67(88.2)
IIA	16(21.1)
IIB	0(0)
IIC	51(67.1)
Class III	9(11.8)
Time of HO diagnosis(monthes after trauma)	5(1–15)
Mayo elbow performace score	
pre-treatment	56.05
post-treatment	93.88
Follow Up time median(range)	33(23–54)
Recurrence(Yes: No)	5:71

OF: olecranal fracture; TT: terrible triad of the elbow; RHF: radial, head fracture; HSF: humerus shaft fracture; ED: elbow dislocation; DHF: distal humerus fracture.

elbow joint, and non-traumatic HO.

Patients diagnosed with HO of the elbow joint after trauma were treated with the combination therapy between January 2017 and December 2020. The combination therapy involved stereoarthrolysis of the elbow joint via arthroscopy or open surgery, combined with preoperative RT and NSAIDs for two weeks after surgery. The treatment was conducted at the Second Affiliated Hospital of Zhejiang University. Seventy-six patients diagnosed with posttraumatic HO of the elbow during the stipulated period were recruited for the study. Patient characteristics are shown in Table 1. With the patient's consent, we detected changes in peripheral blood immune cells at different time points before and after treatment in the patients. We actively followed up with and collected peripheral blood samples from patients who relapsed and those who did not after treatment. Furthermore, a validation group comprising 91 patients whose T cell subsets were also detected after posttraumatic elbow surgery was recruited for the study. The validation process sought to determine whether immunological intervention can prevent HO in specific patients. 4 of 91 developed HO at 1–3 months during follow-up.

2.2. Combination therapy

Preoperative RT (7–8 Gy, with 6 MV X-rays) of the elbow region was performed 24 h before the operation to prevent HO relapse. Contracture release and excision of the heterotopic bone were performed as previously described [16]. Additionally, patients received postoperative prophylactic treatment with NSAIDs for two weeks. All patients with HO were followed up.

2.3. Blood sample collection

Peripheral blood samples were collected the day before surgery (pre-treatment, pre) and 1, 3, 7, 15, and 30 days after surgery (POD1, 3, 7, 15, and 30, respectively). Approximately 10 mL of heparinized peripheral blood was collected from each patient at specific time points. Blood specimens were stratified using Ficoll-Paque (GE Healthcare) and centrifuged at 1500 rpm for 30 min. Mononuclear cell interfaces were collected and washed twice in phosphate-buffered saline (PBS). Peripheral blood mononuclear cells (PBMCs) were then stained with fluorescent-labeled antibodies.

2.4. Flow cytometry

To evaluate intracellular cytokine expression after cell culture, the cells were re-stimulated for 6 h in the presence of phorbol 12-myristate 13-acetate (PMA; 50 ng/mL; Sigma-Aldrich, St. Louis, MO, USA), ionomycin (750 ng/mL, Sigma-Aldrich), and GolgiStop (BD Biosciences) according to the manufacturers' instructions. Next, they were washed, permeabilized with 0.5 % saponin (Sigma-Aldrich), and intracellularly stained with various combinations of the following fluorescently conjugated antibodies (all from Bio-Legend): anti-CD3 PerCP Cy7, anti-CD8 APC Cy7, anti-CD4 FITC, anti-IL-17 PE, anti-IFNγ PerCP Cy5.5, and anti-TNFα APC. Stained cells were acquired using FACSCanto II or LSRFortessa (BD Biosciences). The CD20+/CD3+, CD8+T/CD3+T, CD4+T/CD3+T, TNFα+CD4+T/CD4+T, IL17 + CD4+T/CD4+T, IFNγ+CD4+T/CD4+T, TNFα+CD8+T/CD8+T, and IFNγ+CD8+T/CD8+T cell ratios from patients with HO, non-HO, relapse, and non-relapse patients were analyzed using flow cytometry. All flow cytometric data were analyzed using the FlowJo software (version 10.8.1, USA).

2.5. RNA sequencing

Total RNA was extracted from surgically removed HO tissues or PBMC using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). RNA transcriptome sequencing was subsequently performed by OEbiotech (Shanghai, China). Differentially expressed genes (DEGs) between groups were screened using the limma package in R with the following criteria: $|\text{fold change}| > 1.0$ and adjusted P value < 0.05. Volcano and heat map plots were created using the ggplot2 (v3.3.6) and pheatmap (v1.0.12) R packages, respectively. In addition, gene ontology (GO) enrichment analyses of the DEGs were performed using the ClusterProfiler R package (v4.2.1). GO terms with corrected P values < 0.05 were considered significantly enriched. Furthermore, ClusterProfiler (v4.2.1) was used to evaluate the statistical enrichment of DEGs in Kyoto Encyclopedia of Genes and Genome (KEGG) pathways.

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) at the National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human: HRA003596) and are publicly accessible at https://ngdc.cncb.ac.cn/gsa-human [17,18].

2.6. MSC isolation and culture

Bone marrow cells were obtained from healthy donors at our hospital. The mononuclear cell fraction was purified through Ficoll-Paque (1.077 g/mL) centrifugation and plated in growth medium (LG-Dulbecco's modified Eagle medium containing 10 % fetal bovine serum). Cells were cultured at 37 °C in a humidified atmosphere containing 5 % CO_2. The solution was changed after three days, and the suspended cells were discarded. Surface markers of human MSCs were characterized according to the guidelines of the Mesenchymal and Tissue Stem Cell Committee of the ISCT [19].

2.7. Co-culture experiments

MSCs were seeded at 8500 cells/cm^2 and co-cultured with CD8$^+$T cells sorted from PBMCs. An osteogenic differentiation medium (ODM) containing BMP-2 was used, with half of the medium changed every three days. The cells were lysed in 0.2 % (v/v) Triton X-100 in PBS for 30 min. An Alkaline Phosphatase (ALP) Activity Assay Kit (colorimetric) was subsequently used to measure the ALP activity of MSCs according to the manufacturer's instructions. OCN expression in MSCs was subsequently detected using RT-PCR on Day 7. The following primer sequences were used (Tsingke Biotechnology, Beijing, China): *OCN* (forward: 5′-GCAGCTTGGTGCACACCTAG-3′, reverse: 5′-ACCTTATTGCCCTCCTGCTT-3′) and *GAPDH* (forward: 5′-GAAGGTGAAGGTCGGAGTC-3′, reverse: 5′-GAA-GATGGTGATGGGATTTC-3′).

2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism (version 9; GraphPad Software, Boston, MA, USA). Data are shown as the mean ± standard error of the mean. Analyses were conducted using analysis of variance (ANOVA) with Bonferroni's post-hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. HO patient characteristics and treatment modalities

We retrospectively analyzed the data of 76 patients with HO and posttraumatic stiff elbow treated between June 2017 and December 2020. Combination therapy involving stereoarthrolysis of the elbow joint combined with preoperative RT and postoperative NSAIDs for two weeks was conducted at The Second Affiliated Hospital of Zhejiang University. As shown in Table 1, most of the patients were men (60.5 %, n = 46/76) with a median age of 41 years (age range: 17–63 years) at diagnosis and HO of the right elbow (63.2 %, n = 48/76). The initial trauma among patients with HO was olecranal fracture (OF, 10.5 %, 8/76), terrible triad injury of the elbow (TT, 13.2 %, 10/76), radial head fracture (RHF, 13.2 %, 10/76), humeral shaft fracture (HSF, 7.9 %, 6/76), distal humeral fracture (DHF, 23.7 %, 18/76), and elbow dislocation (ED, 31.6 %, 24/76). The average time to HO diagnosis was five months after trauma (range: 1–15 months). Patients with HO were followed up for a median of 33 months (range: 23–54 months), yielding a recurrence rate of 6.6 % (n = 5/76). We applied the Brooker scale, which classifies hip-associated HO into three classes of ascending severity (classes I–III), to determine the severity of elbow-associated HO [20]. All patients had a severity greater than Class I. Most patients had a Class II severity (88.2 %, 67/86) whereas only a small proportion showed Class III severity (11.8 %, 9/86), indicating ankylosis of the forearm, elbow, or both. The effectiveness of combination therapy in patients with HO characterized by posttraumatic

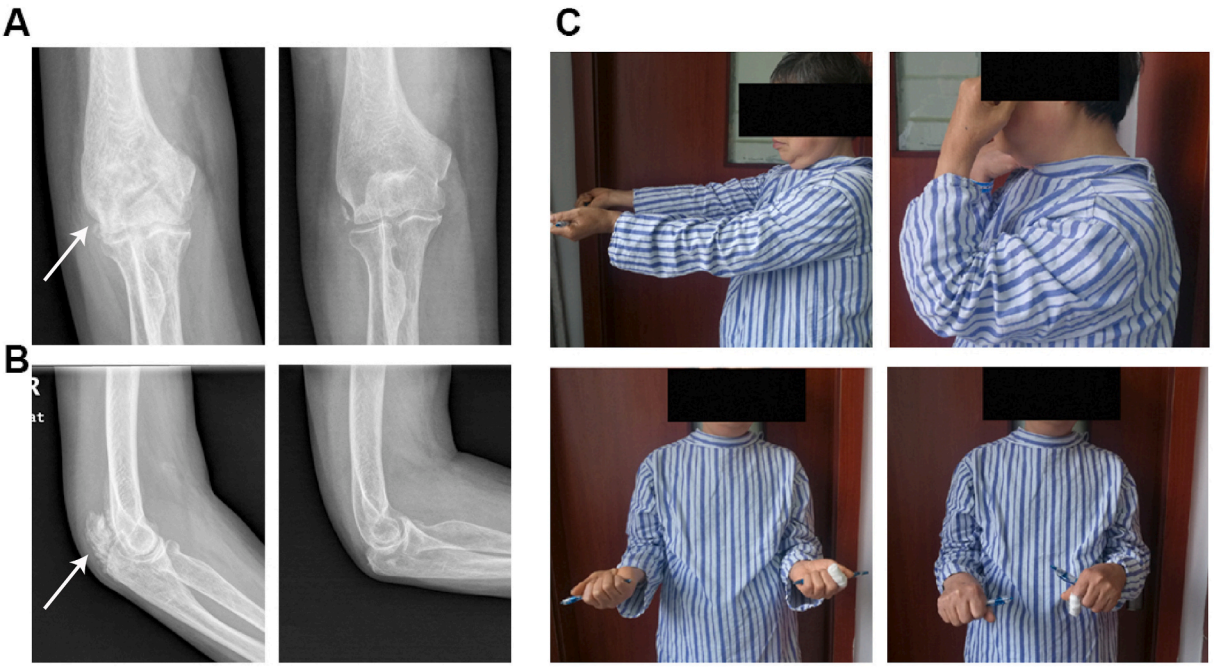

Fig. 1. Therapeutic effects on HO patients. (A) A radiograph of an elbow joint of a patient with HO in the lateral position. Left: before treatment; right: after treatment. (B) A radiograph of an elbow joint of a patient with HO in the anteroposterior position. Left: before treatment; right: after treatment. (C) The functionality of the elbow joint.

stiff elbow was analyzed using the radiological Brooker Score, and functional analysis was performed using the Mayo Elbow-Performance Score (MEPI) (Fig. 1). The MEPI is the primary functional score for evaluating elbow joint diseases, with a lower minimum detectable change, lower minimum clinically significant difference, and more considerable clinical benefits [21]. In the present study, our approach effectively treated HO and prevented relapse.

3.2. Immune cells in the peripheral blood and tissue of patients with HO

The immune system plays an important role in ectopic bone formation. In this study, RNA sequencing was conducted on the local tissues and peripheral blood of patients with HO who underwent posttraumatic elbow surgery to further explore the immunologic mechanism of HO occurrence. Immune cells were detected in HO tissue. Myeloid-derived cells, such as macrophages, were enriched following upregulation of the expression of markers such as MRC1, CD68, and NCAM1 in the local HO tissue. In contrast, lymphocytes

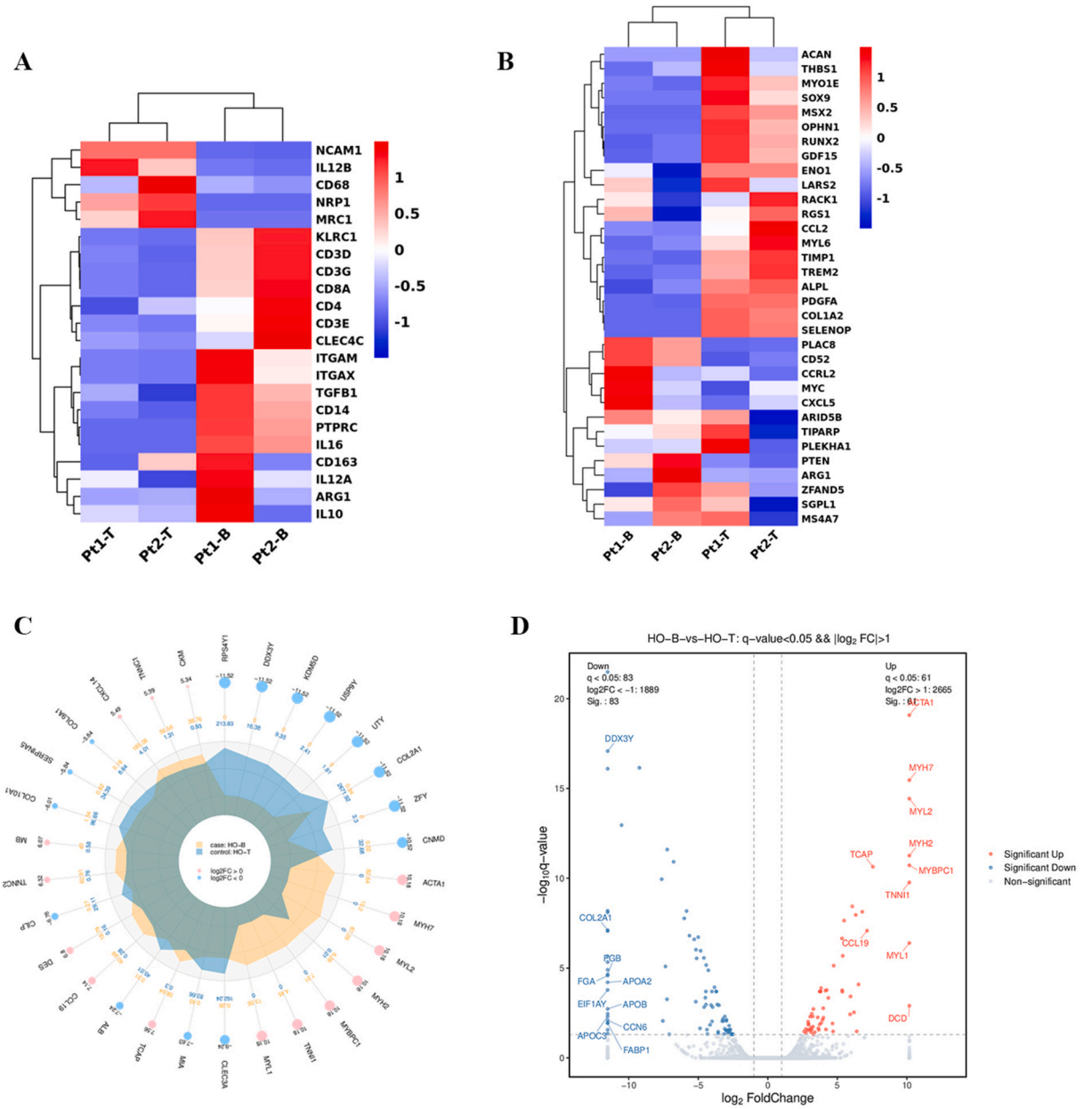

Fig. 2. Differentially expressed genes in the indicated group. (A) Heat maps of differentially expressed genes related to cell subpopulations in the indicated group. (B) Heat maps of differentially expressed genes related to osteoblastosis in the indicated group. (C, D) Differentially expressed genes between local tissue and the peripheral blood of a selected patient. Pt, patient; T, tissue; B, blood.

were enriched in peripheral blood with following upregulation of the expression of markers such as CD3, CD8, CD4, etc. KLRC1, also known as NKG2A, is mainly expressed on the surface of NK cells and CD8+T cell subsets and is significantly upregulated in peripheral blood. This marker has recently been recognized as a novel immune checkpoint [22]. (Fig. 2A and B). Most of the genes whose expression was upregulated in the local HO tissue were related to normal skeletal muscle movement. These genes included *ACTA1*,

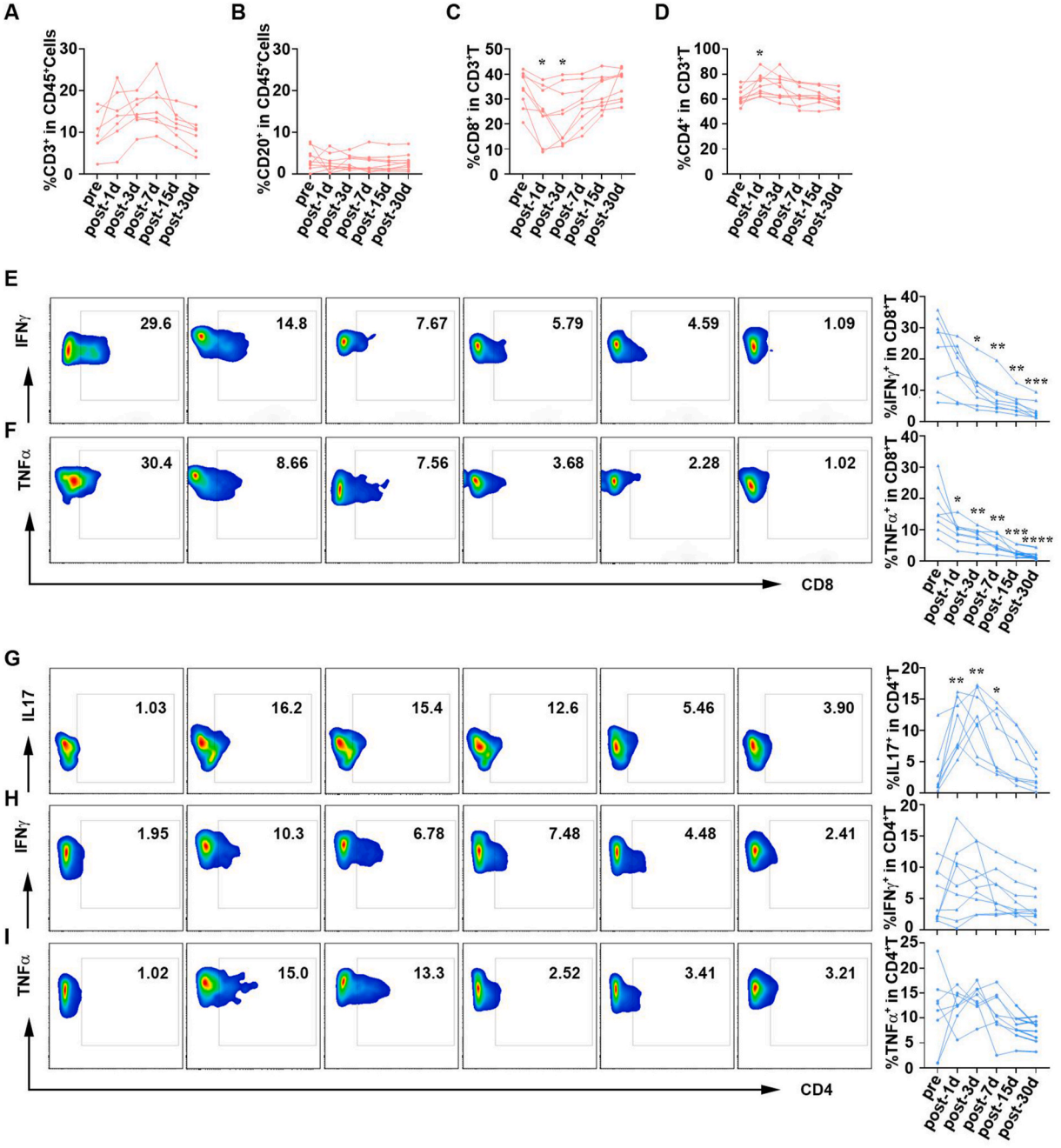

Fig. 3. Dynamic variation of lymphocytes in the peripheral blood of patients with HO during treatment with the Combination Therapy. PBMCs were harvested and labeled with fluorochrome-coupled antibodies on the day before treatment (pretreatment, pre), immediately the day after RT and operation (1, 3, 7, 15 and 30 days after surgery (termed POD1, 3, 7, 15 and 30, respectively). The percentages of $CD3^+$T cells in $CD45^+$ cells (A), $CD20^+$B cells in $CD45^+$ cells (B), $CD8^+$T cells in $CD3^+$T cells (C), $CD4^+$T cells in $CD3^+$T cells (D) were analyzed. (E–I) PBMCs were activated using a leukocyte activation cocktail for 6 h; cells were then collected and labeled with IL17, IFN-γ and TNF-α et al. The proportion of IFN-γ^+CD8^+T cells in $CD8^+$T cells (E), TNF-α^+CD8^+ T cells in $CD8^+$T cells (F), $IL17^+CD4^+$T cells in $CD4^+$T cells (G), IFNγ^+CD4^+T cells in $CD4^+$T cells (H), and TNFα^+CD4^+T cells in $CD4^+$T cells (I) were detected at the indicated times. All statistical analyses were performed using ANOVA of repeated measurement data followed by Tukey's test to compare to the pretreatment (Pre) results. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

MYH2, *MYH7*, *MYL2*, *TNNI1*, *MYL1*, and *MYBPC1*, etc. Mutations in these genes can lead to various muscle diseases characterized by muscle weakness. For example, missense mutation of MYH7 leads to Laing's distal myopathy, and missense mutation of MYBPC1 gene is associated with early-onset myopathy, characterized by muscle weakness, low muscle tone, and skeletal deformities [23,24]. In addition, the expression levels of chemokines such as CCL19, which recruits CCR7$^+$ T and dendritic cells (DCs), were elevated in local HO tissue (Fig. 2C and D). To identify immunologically related biomarkers in the peripheral blood during HO development, we first detected variations in immune cell subsets during HO treatment.

3.3. Dynamic monitoring of peripheral lymphocyte composition of patients with HO treated with combination therapy

T and B cells are essential components of the adaptive immune system during HO pathogenesis; however, their roles have not been fully characterized [25]. Therefore, we analyzed variations in the proportions of different peripheral lymphocyte subsets in patients with HO characterized by posttraumatic stiff elbow treated with combination therapy. Dynamic changes in the peripheral blood of eight patients who experienced good curative effects were analyzed during the treatment course, with six measurements taken at predefined time points. The levels of CD3$^+$T lymphocytes slightly increased after surgery and then gradually declined until 30 days

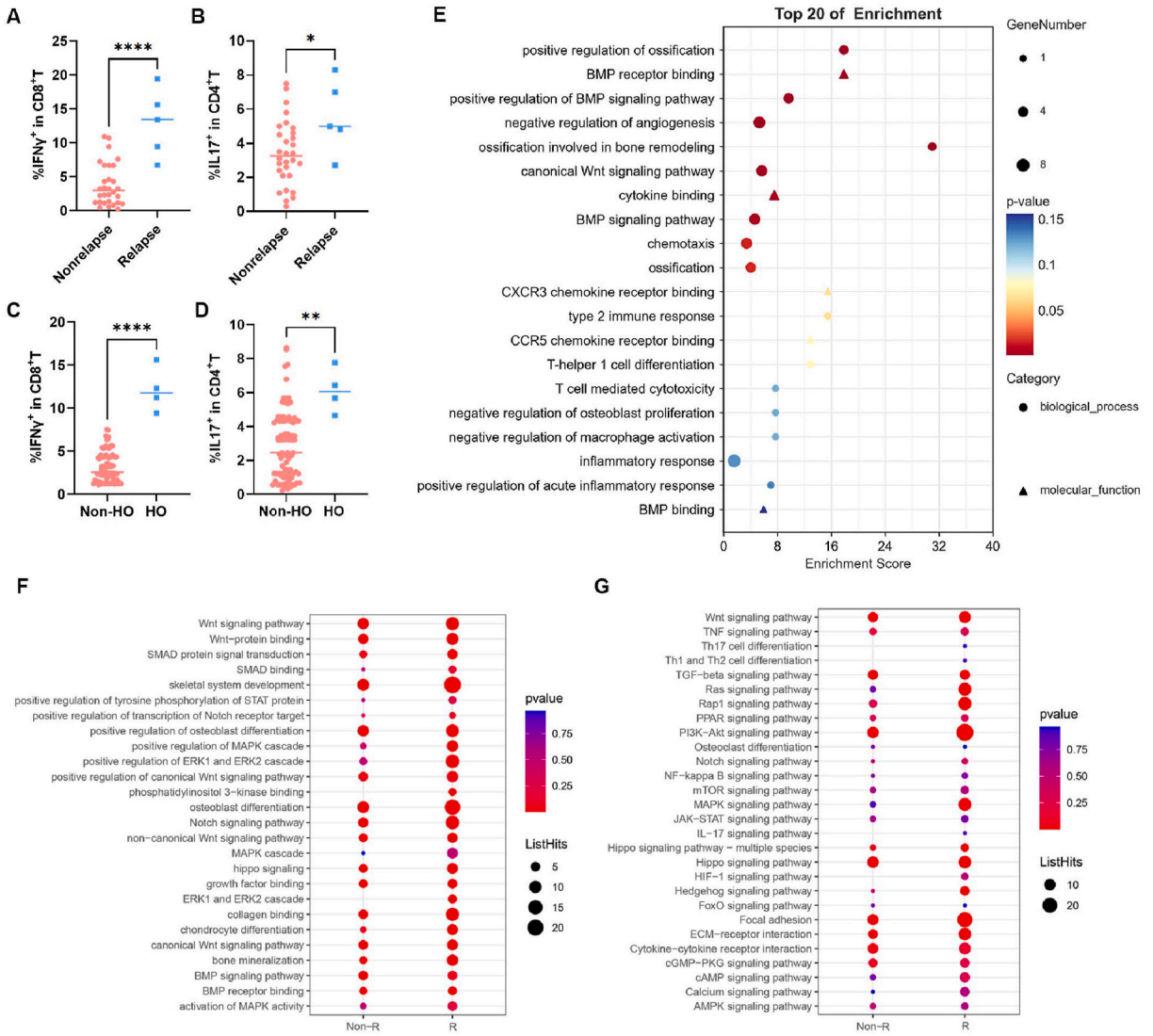

Fig. 4. Inflammatory T-cell subsets are predictive factors for HO. (A–B) The ratios of IFN-γ$^+$CD8$^+$T/CD8$^+$T (A) and IL17$^+$CD4$^+$T/CD4$^+$T (B) were calculated among patients with HO treated with combination therapy and observed for potential relapse. (C–D) The proportion of IFN-γ$^+$CD8$^+$T/CD8$^+$T (C) and IL17$^+$CD4$^+$T/CD4$^+$T (D) were quantified among patients who underwent posttraumatic elbow surgery and observed for potential HO development. (E) Bubble plot of top 20 enrichment pathways in the indicated groups. (F) Bubble plot of GO biological processes activated in the indicated groups. (G) Dot plot visualization showing module scoring of KEGG terms using gene lists shows overlapping enrichment of pathways in the indicated group. A two-tailed Student's t-test was performed to compare the two groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

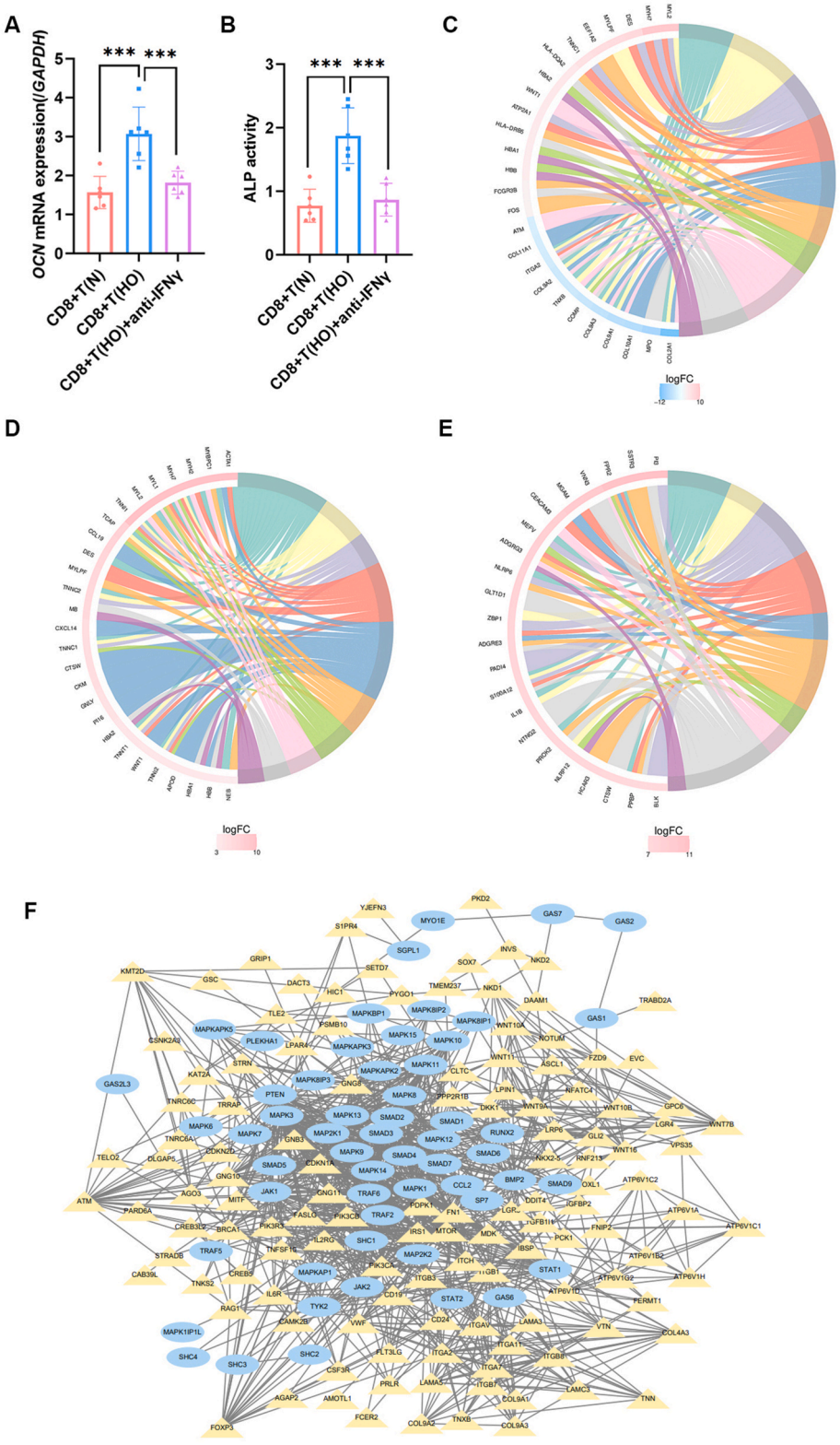

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Fig. 5. Inflammatory T-cell subsets promoted osteoblast differentiation. (A, B) MSCs were co-cultured with sorted $CD8^+$T cells directly with or without neutralized anti–IFN–γ antibody. *OCN* mRNA expression (A) and ALP activity (B) were quantified after 7 days. (C) KEGG analysis showed differential changed genes in the local tissues of patients with relapsed HO compared with those without recurrence. (D) Increased genes in the local tissues of patients with relapsed HO compared with those without recurrence. (E) Increased genes in the peripheral blood of patients with relapsed HO compared with those without recurrence. (F) Interaction analysis between the inflammatory molecule and osteogenesis in relapsed patients with HO. One-way ANOVA and Tukey's test were performed to compare more than two groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

after surgery (P = 0.0842, Fig. 3A). The percentage of $CD4^+$T cells among $CD3^+$T cells was upregulated after surgery and decreased to almost normal levels 30 days after surgery (P = 0.0078, Fig. 3D). In contrast, the $CD8^+$T/$CD3^+$T cell ratio declined after surgery but was restored to normal levels 30 days after surgery (P = 0.0149, Fig. 3C). A substantial proportion of B cells remained stable after treatment compared to that in untreated controls (P = 0.8935, Fig. 3B). These results demonstrate that dynamic changes in the percentage of lymphocyte subsets occurred in the peripheral blood of patients with HO during combination therapy. These results indicate that the proportion of $CD4^+$T cells increased, whereas the ratio of $CD8^+$T cells decreased after stereoarthrolysis but was restored to normal levels 30 days after surgery.

3.4. Peripheral Th17 cells were upregulated temporarily after HO treatment

Activated $CD4^+$ T-cells inhibit osteoblastic bone formation [26]. Furthermore, IFNγ-positive $CD4^+$T cells can lead to osteoclastogenesis via the expression of RANKL [27]. However, whether $CD4^+$T cells have an essential role in the immune environment of HO remains unclear. In this study, the level of $IFN\gamma^+CD4^+$ T cells was slightly upregulated after surgery but returned to pretreatment levels (P = 0.2874, Fig. 3H). Moreover, the percentage of $TNF\alpha^+CD4^+$T cells increased the day after therapy and declined to the levels of untreated controls (P = 0.0381, Fig. 3I). It is widely thought that Th17 cells confer osteoclastogenic properties through the production of inflammatory factors such as IL17 [28]. IL17 induced RANKL expression in mesenchymal cells and promoted osteoclastogenesis *in vitro* [29]. Therefore, we evaluated IL17 production in the peripheral $CD4^+$T cells of patients with HO after combination therapy. As shown in Fig. 3G, IL17 expression levels in $CD4^+$T cells were high on the first day after surgery, and the number of $CD4^+$T cells decreased, reaching a nadir 30 days after surgery (P = 0.0001). These results suggest that $CD4^+$T-cell subsets, such as Th17 cells, play an immunomodulatory role and may have a profound effect on HO.

3.5. Peripheral $CD8^+$T cell activation was downregulated after HO treatment

Current evidence suggests that $CD8^+$T cells inhibit osteoclastogenesis via OPG and RANKL expression. However, it is now generally accepted that the inhibition of OPG released by $CD8^+$T cells does not prevent the inhibition of osteoclastogenesis. This indicates that there are other mechanisms [30]. However, the role of $CD8^+$ T-cells in HO remains unclear. Therefore, we analyzed $CD8^+$T-cell subsets and characterized their specific roles in HO. We assessed the variations in $CD8^+$T cells during combination therapy in patients with HO by evaluating intracellular proteins such as TNFα, IFNγ, and IL17. As shown in Fig. 3, elevated levels of both $IFN\gamma^+CD8^+$T and $TNF\alpha^+CD8^+$T cells were observed in peripheral blood before therapy but dramatically declined the day after RT and stereoarthrolysis. The percentage of $IFN\gamma^+CD8^+$ T cells gradually decreased to normal levels and was rarely detected in the peripheral blood 30 days after surgery (P < 0.0001, Fig. 3E). Similarly, $TNF\alpha^+CD8^+$T cells exhibited markedly higher numbers in untreated patients with HO and gradually decreased to nearly 1 % (P < 0.0001, Fig. 3F). Furthermore, $IFN\gamma^+CD8^+$ T cells secreted TNFα, suggesting that they were similar $CD8^+$ T-cell subsets.

3.6. Inflammatory T-cell subsets are predictive biomarkers of HO after posttraumatic elbow surgery

Comparison of the patients who relapsed after combination therapy revealed higher levels of $IFN\gamma^+CD8^+$ T (3.753 % vs 12.90 %, P < 0.0001, Fig. 4A) and $IL17^+CD4^+$T (3.420 % vs 5.560 %, P = 0.0281, Fig. 4B) cells in relapsed patients with HO than in non-relapsed patients. Our findings suggest that the subsets of $IFN\gamma^+CD8^+$ T and $IL17^+CD4^+$T cells may be excellent biomarkers to evaluate the therapeutic efficacy of HO patients. In addition, we investigated whether these cell subsets could be used as peripheral blood monitoring indicators of HO development after posttraumatic elbow surgery. Furthermore, we investigated whether immunological intervention could be carried out to prevent HO in specific patients. To this end, we selected 91 patients who underwent posttraumatic elbow surgery at The Second Affiliated Hospital of Zhejiang University. We quantified the peripheral $IFN\gamma^+CD8^+$ T and $IL17^+CD4^+$T cells of these patients during follow-up. Of the 91 patients, four developed HO of the elbow joint 1–3 months after their initial operation. The proportion of peripheral $IFN\gamma^+CD8^+$ T (3.2 % vs 12.13 %, P < 0.0001; Fig. 4C) and $IL17^+CD4^+$T (2.9 % vs 8.1 %, P = 0.0019; Fig. 4D) cells in patients with HO was also higher than that in non-HO patients. Taken together, our findings suggest that $IFN\gamma^+CD8^+$T and $IL17^+CD4^+$T cells exhibit good predictive yield for HO development. However, the $IFN\gamma^+CD8^+$T cells showed considerably more significance.

3.7. Inflammatory T-cell subsets promoted osteoblast differentiation

It has been reported that IL17 and IFNγ could promote osteogenesis, which participates in the progression of HO. RNA sequencing revealed significantly activated BMP and Wnt signaling pathways in patients with HO (Fig. 4E and G). We further investigated whether

the different clinical outcomes in patients with HO demonstrated distinct transcriptional Fig. 5programs in the tissue and peripheral blood. A bubble plot of the upregulated pathways shows the unique functions of each cluster in Fig. 4F. Similarly, visual dot plots of modularity scores show enriched functional pathways in relapsed clusters compared to those in non-relapsed clusters (Fig. 4G). Th17 cell differentiation and the TNF signaling pathway were consistently upregulated in the relapsed group. The HO-relapsed cluster was enriched in genes associated with PI3K-AKT, MAPK, SMAD, and WNT signaling, which contribute to osteoblast differentiation (Fig. 5C–E). Some genes related to skeletal muscle movement that we previously mentioned, such as *ACTA1*, *MYH2*, *MYH7*, *MYL2*, *TNNI1*, and *MYL1*. MYBPC1 is upregulated in the tissue relative to the peripheral blood of HO patients. We found that these genes are also upregulated in patients with HO recurrence compared with those without recurrence (Fig. 5D). This suggests that the probability of HO recurrence is associated with the upregulated expression of these genes. In addition, there are also some genes such as *CCL9, CXCL14, CTSW* and *GNCY*, which are related to immune cells upregulated in recurrent patients. Among them, *CTSW* and *GNCY* are genes related to CD8+T cell toxicity (Fig. 5D). Furthermore, *CTSW* expression was upregulated in the peripheral blood of patients with recurrent HO. This is consistent with the research results that CD8+T cells that produce effector molecules can predict the recurrence of HO [31,32](Fig. 5E). Interaction analysis also revealed that inflammatory signals mainly activated the MEK, ERK, MAPK, Osterix, and SP7 pathways that promote osteogenesis (Fig. 5F).

To further investigate the effect of $CD8^+$T cells on MSC osteogenesis, the MSCs were cultured in a medium supplemented with BMP-2, with or without $CD8^+$T cell subsets sorted from the peripheral blood of healthy donors or HO patients. The osteogenesis markers, such as alkaline ALP production and OCN expression, were quantified. Compared to coculturing with healthy donor-derived $CD8^+$T cells, MSCs exhibited a higher ALP activity when co-cultured with $CD8^+$T cells derived from patients with HO (0.77 % vs 1.88 %, $P = 0.0004$; Fig. 5A). However, this effect was abrogated by neutralized anti-IFNγ antibody (1.88 % vs 0.87 %, $P = 0.0007$; Fig. 5A). Similarly, *OCN* mRNA expression was elevated in MSCs co-cultured with patient with HO-derived $CD8^+$T cells (1.57 % vs 3.07 %, $P = 0.001$; Fig. 5B). However, it was suppressed by neutralized anti-IFNγ antibody (3.07 % vs 1.82 %, $P = 0.0021$; Fig. 5B). These results demonstrate that activated $CD8^+$T cells promote osteogenesis through IFNγ secretion. Identification of the downstream effects of BMP signaling further revealed elevated expression levels of target genes such as *COL1A1* and *ALPL* in the tissues of patients with HO (Fig. 5C).

4. Discussion

The development of traumatic HO is a complicated process with substantial health effects. The incidence of asymptomatic HO after surgery for elbow fractures or fracture-dislocations is 18.78 % [2]. Herein, we found that among trauma causes, terrible triad injuries accounted for the highest number of HO cases (50 %), similar to a previous study that reported an incidence of 58.3 % [33].

The immune response is essential for host defense but can be prolonged or aberrantly activated under specific conditions. The immune system also plays an essential role in bone remodeling, which involves a balance between osteoblast and osteoclast activity. Bone marrow stromal cells are the main source of osteoblasts, whereas osteoclasts are primarily derived from macrophage fusion. Several types of immune cells have potential roles in HO formation. In previous studies, many diseases, such as osteoporosis, ankylosing spondylitis, and multiple myeloma, which are related to osteoblast and osteoclast activity have also tested immune cell changes in peripheral blood [34–36]. However, there is currently no research to explore the changes in the peripheral blood of patients with HO.

The results of the present study were considered with past literature that explored the roles of B and T cells in physiological bone homeostasis. Current evidence suggests that heterogeneous T-cell populations modulate osteogenesis. In the previous study, the $IFN\gamma^+$ $CD4^+$T cells have been reported to induce osteoclast formation through the expression of RANKL in the presence of M-CSF [27]. However, another study demonstrated that IFNγ inhibits murine osteoclast formation by degrading TRAF6 [33]. Our study highlighted the role of the peripheral immune system in the pathogenesis HO. In the present study, the peripheral lymphoid cell subsets were altered during HO treatment. These findings suggests that the subsets of $IFN\gamma^+CD8^+$ T and $IL17^+CD4^+$T cells may be excellent biomarkers of therapeutic efficacy in patients with HO. In this study, MSCs co-cultured with patient with HO-derived $CD8^+$T cells promoted osteogenesis, as demonstrated by higher $IFN\gamma^+CD8^+$T cell levels in patients with HO, which rapidly declined after treatment. Thus, $IFN\gamma^+$ $CD8^+$T cells have huge prospects for application as predictive biomarkers for HO recurrence.

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Ethics approval

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine on November 25, 2022 (Approval number: 2022–1013).

Consent to participate

Written informed consent was obtained for each participant.

Consent to publish

The authors affirm that human research participants provided informed consent for publication of the images in Fig. 1.

Data availability

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in the National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation/ Beijing Institute of Genomics, Chinese Academy of Sciences (GSA-Human: HRA003596) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa-human.

CRediT authorship contribution statement

Zengfeng Xin: Writing – original draft, Resources, Funding acquisition, Data curation. **Junhua Chen:** Writing – original draft, Supervision, Investigation, Data curation. **Fengbo Huang:** Validation, Methodology, Investigation, Formal analysis. **Siyu Guo:** Supervision, Investigation, Formal analysis. **Yihan Yao:** Visualization, Investigation, Formal analysis. **Yang Tang:** Investigation, Data curation. **Hang Li:** Writing – review & editing, Software, Investigation. **Qinghua Lv:** Writing – review & editing, Supervision, Investigation. **Ting Zhang:** Writing – review & editing, Resources, Funding acquisition, Data curation.

Declaration of competing interest

This work was supported by the grant from the National Natural Science Foundation of China (82173089, T. Z.), Natural Science Foundation of Zhejiang Province (LY21H100004, T. Z. and LGF22H060010, Z. X.), and Medical Science and Technology Project of Zhejiang Province (2021KY697, Z. X. and 2021RC063, T. Z.).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33851.

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