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Original Article

Effect of immune function changes after splenectomy on fracture healing in patients with compound injury

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Abstract: Objective: To investigate the effects immune function changes after splenectomy on fracture healing. Methods: We randomly selected 20 patients with fractures combined with splenic rupture as Group A, and 20 patients only with fractures as Group B. Results: Comparison within groups, the numbers of monocytes and lymphocytes and the levels of TNF-α and IL-6 dynamically changed in peripheral blood. With time extending, bone grey ratio and bone mineral density ratio increased in both groups. Comparison between groups, the numbers of monocytes and lymphocytes and the levels of TNF-α and IL-6 were significantly lower in Group A than in Group B in peripheral blood at each time point (all $P<0.05$). In hematoma tissue, the numbers of TNF-α and IL-6-positive macrophages and lymphocytes were also significantly lower in Group A than in Group B (all $P<0.05$). Bone grey ratio and bone mineral density ratio were significantly lower in Group A than in Group B at each time point (all $P<0.05$). Conclusion: Immune function changes after splenectomy affect fracture healing. We should retain spleen as much as possible to improve fracture healing.

Keywords: Splenectomy, immune, fracture healing

Introduction

Spleen, a largest lymphoid organ in human body, besides these functions including blood storage, hematopoiesis, hemofiltration and destroying blood, also possesses the immune functions against infection and tumor. As a largest immune organ, spleen contains 25% of macrophages, 25% of T-lymphocytes and 10%-15% of B-lymphocytes [1, 2], and can secrete many cytokines. Among numerous cytokines, tumor necrosis factor (TNF-α) and interleukin (IL-6) play an important role in anti-infection, wound repair and fracture healing [3-8]. Inflammation-stimulating macrophages are an important source of TNF-α and IL-6, but T-lymphocytes, B-lymphocytes and monocytes also can produce IL-6, and IL-6 may lead to osteoblastic activation [4]. TNF-α and IL-6 are also involved in repair and reconstruction of bone [5, 6]. It is reported that lack of macrophages and T-lymphocytes can slow wound healing [7]. It has been unclear that whether splenectomy affects fracture healing in the patients with compound injury?

After fractures, the formation of hematoma containing peripheral blood cells, immunocytes and cytokines, indicates the beginning of inflammatory reaction which triggers cascade reaction of fracture healing; so human body's immune function is essential for fracture healing [3-9]. It has still been unclear whether immune function changes after splenectomy affect fracture healing. In this study, we explored the effect of splenectomy on fracture healing through determining the contents of monocytes, lymphocytes, TNF-α and IL-6 in both peripheral blood and hematoma tissue to observe immune function changes, and through analyzing X-ray films using image analysis system to evaluate fracture healing.

Subjects and methods

All study methods were approved by the Ethics Committee of Shengjing Hospital of China Medical University. All patients gave written formal consent to participate.

Subjects

Inclusion criteria were ① age 15-60 years, male or female; ② patients without brain trauma because brain trauma can accelerate fracture healing [10]; ③ open or closed diaphyseal

Table 1. General data in the two groups

	Group A	Group B	*P* values
Case (n)	20	20	
Age (year)	34.83±7.4	33.13±8.2	0.501*
Male [n (%)]	16 (80.0%)	13 (65.0%)	0.288**
Gustillo typing [type (n)]	I (8), II (12)	I (6), II (14)	0.507**
Fracture sites			0.781**
Femur (n)	7	10	
Tibia (n)	10	8	
Ulna (n)	1	1	
Radius (n)	2	1	
Injury severity score (ISS)	27.67±3.9	25.78±4.1	0.143*
From injury to operation (day)	6.44±3.2	6.06±3.8	0.734*
Follow-up duration (month)	18.51±3.2	18.22±4.6	0.819*

Notes: Age, ISS, duration from injury to operation and follow-up duration are expressed as mean±standard deviation. *Indicates one-way ANOVA. **Indicates chi square test.

fractures in four limbs with more than 20 of injury severity score (ISS) [11] except open-fracture Gustilo III; ④ excluding the patients who had a history of surgery for fractures or bone disease, or had a history of long-term usage of steroids, immunosuppressive agents or non-steroidal anti-inflammatory drug; ⑤ surgery: In the patients only with fractures, for open fractures, emergent debridement, would closure and external fixation were first performed; and then internal fixation was conducted after patient's condition was stable. For closed fractures, bone traction was first performed, and then internal fixation was conducted after patient's condition was stable. In the patients with fractures combined with splenic rupture, splenectomy and treatment of open fractures were simultaneously carried out within 8 h after injury, and other procedures were the same as that in Group B. Surgery in all patients of this study was performed by the same surgeon; ⑥ complete follow-up data. Forty patients in line with inclusion criteria were selected and were divided into fractures combined with splenic rupture group (Group A, 20 patients) and only fractures group (Group B, 20 patients).

Determination of contents of monocytes, lymphocytes, TNF-α and IL-6 in peripheral blood

In all patients, peripheral blood were respectively taken on the first day after fracture, the day before operation (mean fifth day after fracture), the day after operation (mean seventh day after fracture) and the fourteenth day after fracture. The 4 time points were all after splenectomy. Blood samples were immediately centrifuged followed by storage at -80°C. The levels of TNF-α and IL-6 were determined by ELISA (Quantikine, R&D system, Minneapolis, MN). The numbers of monocytes and lymphocytes were detected using blood cell analysis counter (Beckman Coulter LU750, US).

Immunohistochemistry

Samples: In both groups, hematoma tissues with a thickness of less than 5 mm around fracture ends were taken during operation, and then were fixed with FAA liquid for 12 h. Samples were washed with alcohol to remove FAA liquid, and then were dehydrated in gradient alcohol (70%→80%→95%→anhydrous alcohol→anhydrous alcohol with each solution for one hour). Transparency was performed respectively using anhydrous alcohol and xylene (1:1)→xylene→xylene with each solution for one hour. Samples were embedded in paraffin, and were sectioned at a thickness of 5 μm followed by coating at 60°C for 30 min.

Immunohistostaining: After deparaffinage and hydration, samples were washed with PBA followed by addition of 3% of H_2O_2 at room temperature for 10 min. Samples were washed with PBS followed by antigen retrieval. Goat serum was added at room temperature for 20 min. After redundant liquid was removed, 50 μl of primary antibody was added in the samples at 4°C overnight, and then the samples were incubated at 37°C for 45 min. Secondary antibody (40-50 μl) was added at 37°C for one hour. Samples were washed with PBS followed by DAB coloration for 5-10 min. After washed with PBS, samples underwent counterstaining with campeachy for 2 min followed by dehydration, transparency and mounting for observing the contents of lymphocytes, macrophages, TNF-α and IL-6 in hematoma tissues.

Radiology

X-ray films were taken in each patient by a same experienced radiologist one day, one month and three months after operation. Bone grey ratio and bone mineral density ratio were determined by analysis of X-ray films using image

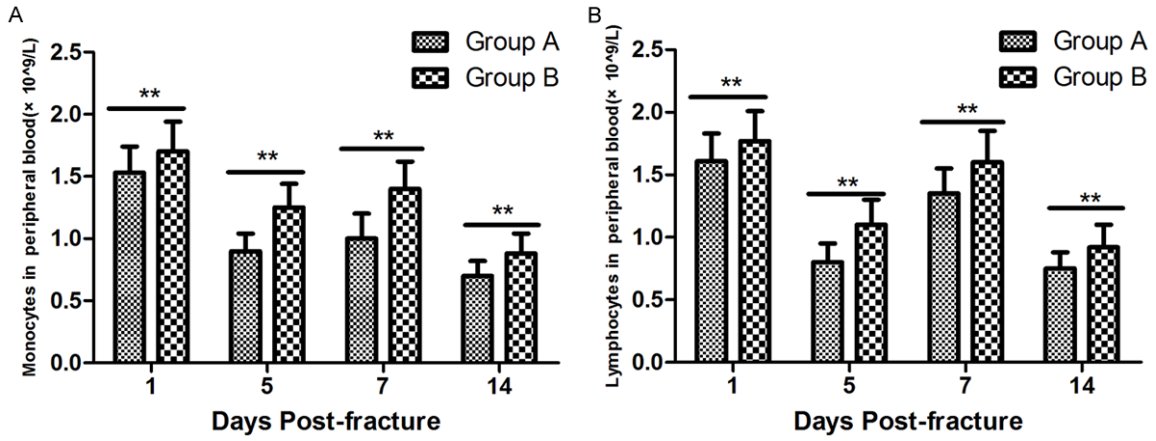

Figure 1. Comparisons of monocyte count and lymphocyte count in peripheral blood between Group A and group B at each time point. A: Comparison of monocyte count between both groups; B: Comparison of lymphocyte count between both groups.

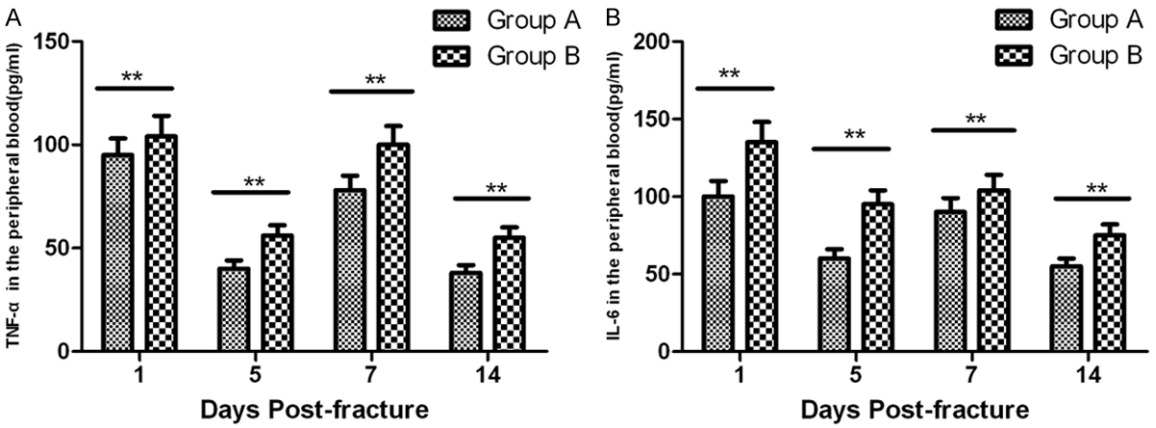

Figure 2. Comparison of tumor necrosis factor (TNF-α) and interleukin (IL-6) in peripheral blood between Group A and group B at each time point. A: Comparison of TNF-α between both groups. B: Comparison of IL-6 between both groups.

analysis system (Leica, Quantiment 570, Germany) to evaluate fracture healing.

Statistical analysis

Statistical treatment was performed using SPSS19 software (SPSS, Chicago, IL, USA). Data were expressed as mean ± standard deviation, and standard errors were calculated. ANOVA and Turkey were used in back testing. Spearman's analysis was used in comparisons between groups. Statistical significance was established at $P<0.05$.

Results

There was no statistical difference in age, sex, fracture site, ISS, duration from injury to operation, and follow-up duration between the two groups (all $P>0.05$, **Table 1**).

After splenectomy, immune function changes were a dynamic process during haematoma formation. Comparison within groups: In peripheral blood of each group, the numbers of monocytes and lymphocytes, and the levels of TNF-α and IL-6 were the highest on the first day after fracture, and then significantly decreased on the fifth day ($P<0.05$ as compared with that on the firs day). On the seventh day after fracture, the numbers of monocytes and lymphocytes, and the levels of TNF-α and IL-6 were lower than that on the first day but higher than that on the fifth day (all $P<0.05$). Two weeks after fracture, the numbers of monocytes and lym-

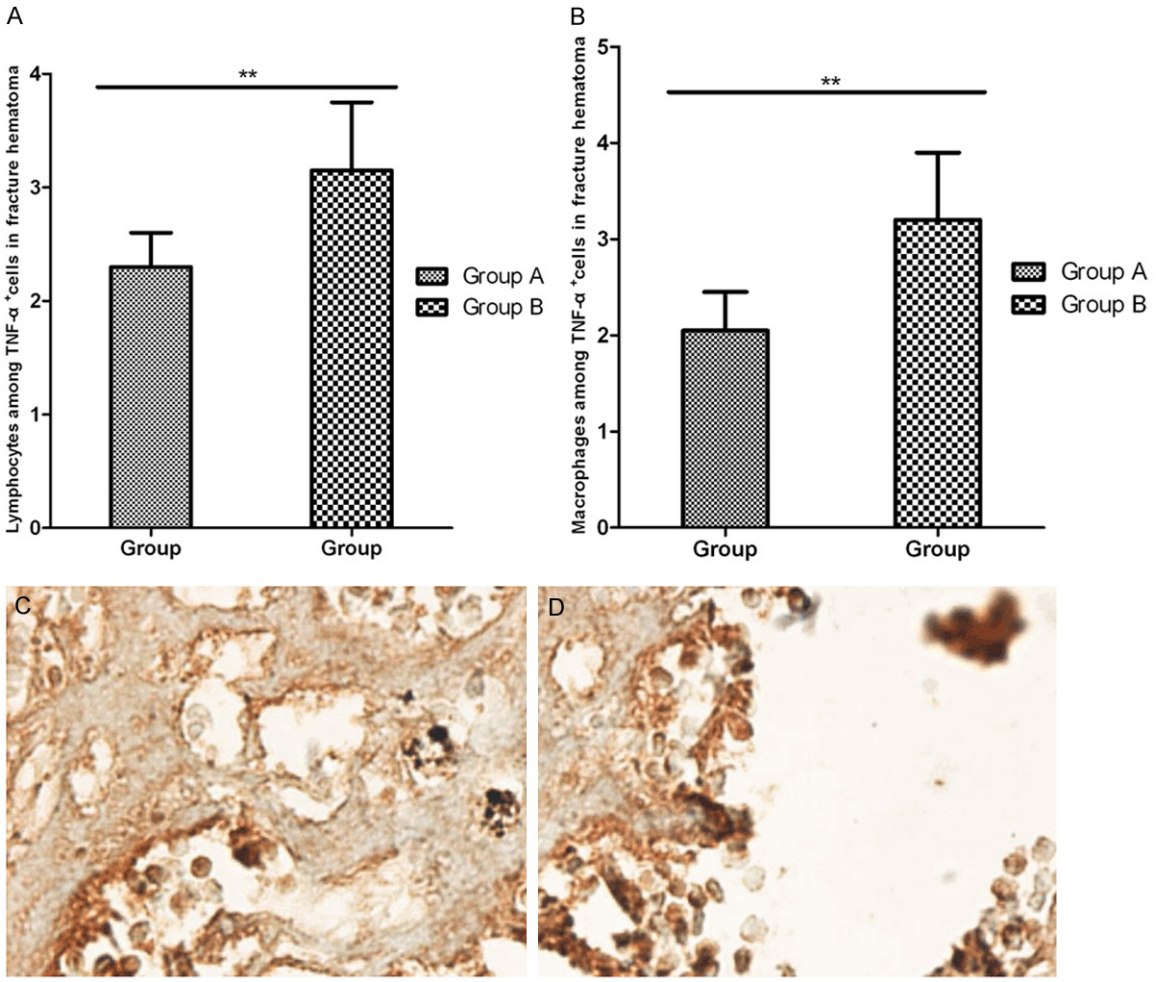

Figure 3. Expressions of tumor necrosis factor (TNF-α)-positive lymphocytes and macrophages in soft tissue. A: Comparison of TNF-α positive lymphocytes in soft tissue between both groups. B: Comparison of TNF-α positive macrophages in soft tissue between both groups. C: Immunohistochemistry indicates TNF-α in lymphocytes and macrophages in group A ×200. Notes: Black arrows indicate TNF-α in lymphocytes and blue arrows indicate TNF-α in macrophages. D: Immunohistochemistry indicates TNF-α in lymphocytes and macrophages in group B ×200. Notes: Black arrows indicate TNF-α in lymphocytes and blue arrows indicate TNF-α in macrophages.

phocytes, and the levels of TNF-α and IL-6 were lower than that on the fifth day ($P<0.05$). With time extending, bone grey ratio and bone mineral density ratio increased in both groups after fracture. Comparison between the two groups: In peripheral blood, the numbers of monocytes and lymphocytes, and the levels of TNF-α and IL-6 were significantly lower in Group A than in Group B at each time point (all $P<0.05$) (**Figures 1**, **2**). In hematoma tissue, the numbers of TNF-α and IL-6-positive macrophages and lymphocytes were also significantly lower in Group A than in Group B (all $P<0.05$) (**Figures 3**, **4**). Bone grey ratio and bone mineral density ratio were significantly lower in Group A than in Group B at each time point (all $P<0.05$) (**Figure 5** and Supplementary Figure 1). Two patients had fracture nonunion in group A and no fracture nonunion occurred in group B 24 months after operation.

Discussion

In previous studies, splenectomy was mostly due to β-thalassemia [12] or cirrhosis-secondary splenomegaly [13]. Little research has been done about immune function change after traumatic splenectomy, especially about the effect of immune function change on bone healing during hematoma. Both osteocytes and immune cells are derived from bone marrow and they have many common regulatory factors,

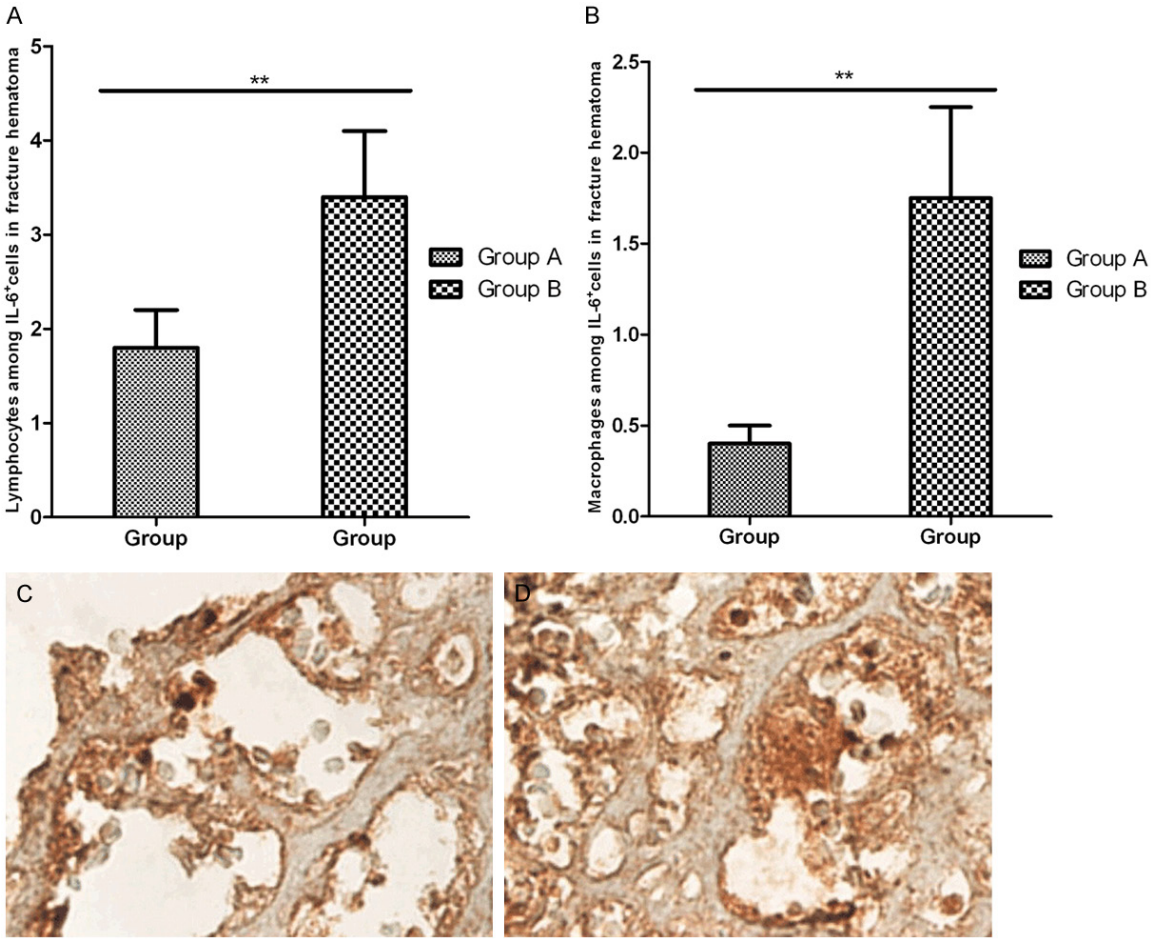

Figure 4. Expressions of interleukin (IL-6)-positive lymphocytes and macrophages in soft tissue. A: Comparison of IL-6 positive lymphocytes in soft tissue between both groups. B: Comparison of IL-6 positive macrophages in soft tissue between both groups. C: Immunohistochemistry indicates IL-6 in lymphocytes and macrophages in group A ×200. Notes: Black arrows indicate IL-6 in lymphocytes and blue arrows indicate IL-6 in macrophages. D: Immunohistochemistry indicates IL-6 in lymphocytes and macrophages in group B ×200. Notes: Black arrows indicate IL-6 in lymphocytes and blue arrows indicate IL-6 in macrophages.

receptors and signal transduction pathways during maturation and activation, and macrophages and osteoclasts are from the same progenitor cells; so people believe that osteocytes are associated with immune cells, and raise the concept of immunoosseous [14]. It has been confirmed that there is a complex regulatory network between osteocytes and immune cells. T cells have bidirectional regulation effects on osteoclasts [15], and T cell-secretory cytokines can regulate differentiation of osteoblasts [16]. Therefore, immune system and immune cells are closely related to fracture and bone metabolism.

Haematoma formation after fracture indicates the beginning of inflammatory reaction and appropriate inflammatory reaction is essential for bone healing [17]. Inflammatory cells first enter haematoma and participate into inflammatory reaction by secreting cytokines and other inflammatory mediators. In early stage of fracture (1-4 h), immune cells begin to increase, and within 24 h after fracture lots of neutrophilic granulocytes accumulate in the injured region to prevent infection with their strong phagocytosis. The number of granulocytes in the initial fracture hematoma was twice as much as that in peripheral blood [15]. Within 48-96 h after haematoma formation, macrophage migration reaches peak value which is very important for fracture healing because in addition to phagocytizing cell debris, macrophages also can secrete a variety of cytokines, including

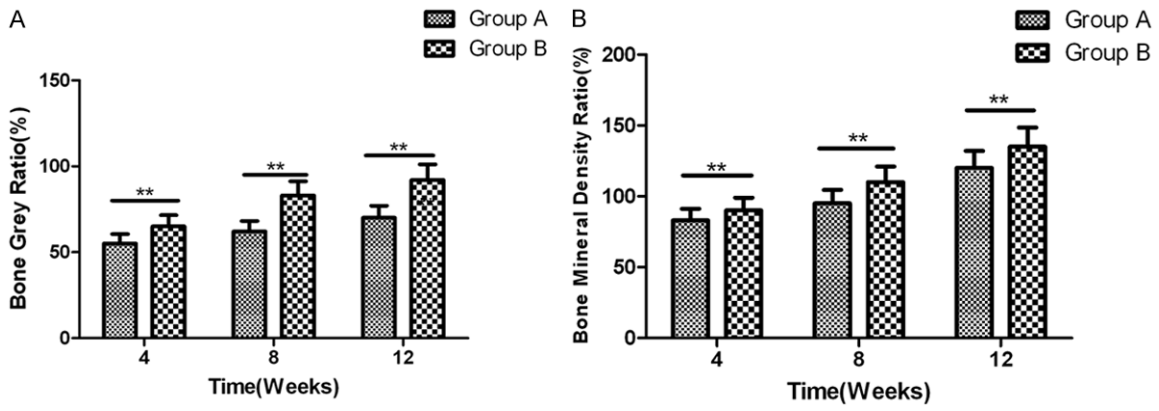

Figure 5. Bone grey ratio and bone mineral density ratio in X-ray films at each time points in both groups. A: Comparison of bone grey ratio between both groups. B: Comparison of bone mineral density ratio between both groups.

TNF-α, IL-1, IL-6, IL-8, IL-12, transforming growth factor-β (TGF-β), platelet-derived growth factor and insulin-like growth factor-1 to promote proliferation and differentiation of fibroblasts, formation of collagen protein and angiogenesis [18]. Removal of macrophages led to delayed union, but replenishment of macrophages promoted collagen synthesis and tissue repair [7]. Similar to macrophages, removal of all lymphocytes inhibited tissue healing [19]. IL-6 and TNF-α, proinflammatory cytokine, increase in initial stage of inflammatory reaction and play an important role in angiogenesis [20]. With time extending, levels of IL-6 and TNF-α increase in fracture hematoma and reach peak value within 1-2 h and 4-24 h after fracture [20]. Over-expression of proinflammatory cytokines, such as IL-6 and TNF-α, or expression imbalance between proinflammatory cytokines and anti-inflammatory cytokines extended inflammatory reaction period and inhibited vascularization, finally affecting bone healing [21].

Early hematoma is very important for bone healing, but the mechanism about the effects of inflammatory reaction, immune cells and related cytokines in hematoma on bone healing has not been completely clear. To observe the relation between bone healing and early immune function changes, we determined the contents of cells (lymphocytes, monocytes, macrophages) and cytokines (IL-6 and TNF-α) in both peripheral blood and fracture hematoma tissue. Our results indicated that the contents of monocytes, lymphocytes, IL-6 and TNF-α in peripheral blood were significantly lower in Group A than in Group B (P<0.05), and the contents of macrophages, lymphocytes, IL-6 and TNF-α in fracture hematoma tissue were also significantly lower in Group A than in Group B (P<0.05), demonstrating that splenectomy led to reductions of immune-related cells and cytokine in both peripheral blood and fracture hematoma tissue. Our results also displayed that bone grey ratio and bone mineral density ratio were all lower in Group A than in Group B (P<0.05), illustrating that splenectomy inhibited bone healing. Inflammatory reaction or inflammatory factors have positive regulation effect on bone healing in certain range, but prolonged inflammatory reaction or inflammatory factor over-expression will produce negative effect on bone healing. Therefore, we will further explore positive and negative effects of these immune cells and inflammatory factors in our future studies.

Cytokines, such as IL-6 and TNF-α, are derived from splenic macrophages. In this study, reduction of cytokines in Group A at different time points may be associated with splenectomy, insufficient compensation of lymphatic organs (thymus gland and lymph nodes), and postoperative immunologic suppression. In this study, splenectomy led to reduction of immune cells, and both immune cells and cytokines decreased in peripheral blood and fracture hematoma tissue at different time points, demonstrating that immune cell is an important source of cytokines. Bone grey ratio and bone mineral density ratio determined by image analysis system can objectively evaluate bone healing [22]. Bone grey ratio and bone mineral density ratio were significantly lower in Group A than in Group B at

each time point (all $P<0.05$), and 2 patients had fracture nonunion in group A. These results confirm that the levels of immune cells and cytokines during hematoma formation affect bone healing.

Much attention has been paid to effect of inflammatory reaction on bone healing. It has been reported that in rat fracture models with severe chest trauma, bone healing is inhibited [23]. This may be related to changes in systemic and local inflammatory reaction. However, the effect of immune function changes after splenectomy on bone healing has not been reported. In this study we revealed that immune function changes after splenectomy affect bone healing by determining the contents of immune cells (lymphocytes and monocytes) and cytokines (IL-6 and TNF-α) in peripheral blood and fracture hematoma tissue. In addition, our results were reliable because age, sex, fracture site, ISS, duration from injury to operation, and follow-up duration were comparable between the two groups.

There were some limitations in this study. Firstly, we did not determine lymphocyte subpopulations. Secondly, the effect of laparotomy on bone healing remains to be further confirmed. Thirdly, fracture hematoma tissues at different time points failed to be obtained from human body, which affected dynamic observation of immune changes in fracture hematoma tissue. We will further explore these problems above in our future studies.

Conclusion

Splenectomy can decrease contents of immune cells and cytokines in peripheral blood and fracture hematoma tissue, and can affect bone healing. Therefore, we should retain spleen as much as possible to promote bone healing in clinical practice.

Disclosure of conflict of interest

None.

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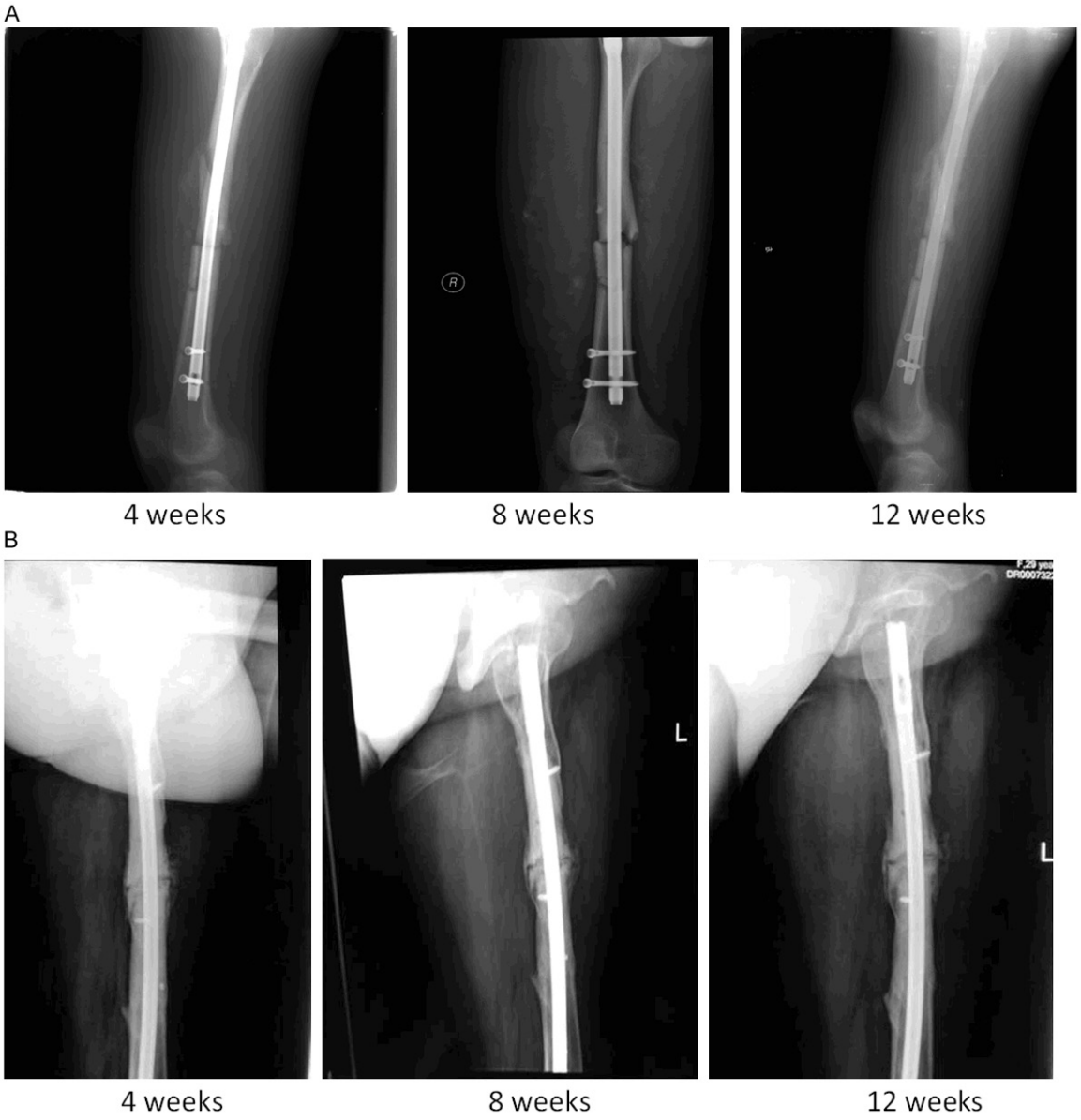

Supplementary Figure 1. X-ray films at each time point. A: X-ray films in group A. B: X-ray films in group B.