Role of skeletal macrophages in fracture repair: A systematic review

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Abstract. In the field of bone research, the importance of the function of skeletal macrophages (sMΦ) and their crucial role in immune homeostasis and bone regeneration has been extensively studied. The aim of the present systematic review was to summarize the role of sMΦ in bone fracture healing and to evaluate their potential for immunoregulatory therapy in bone regeneration. A systematic literature search of PubMed and Embase® was performed to retrieve studies on the role of sMΦ in bone injury repair. The Systematic Review Centre for Laboratory animal Experimentation tool was used to assess the risk of bias of the studies included. A total of four articles were included in the present review. A relatively high risk of bias was identified in the included articles as none of the assessors in these studies were blinded. sMΦ were defined by the surface markers F4/80^{+}, Mac-2$^{-/low}$, TRAP^{-}, CD169^{+}, Ly6G^{-} and CD115^{low}. All of the studies provided support for the essential role of sMΦ in intramembranous ossification or endochondral ossification during fracture healing. F4/80^{+}Mac-2^{-}CD169^{+} sMΦ are a promising therapeutic target for immunoregulatory therapy of bone repair due to their essential role in bone formation and homeostasis. Future studies aimed at profiling and modulating sMΦ to promote bone regeneration are required.

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Introduction

Trauma is the fifth leading cause of death, resulting in more fatalities than diabetes and infectious diseases in China, and thus places a substantial burden on healthcare systems across the world (1). A recent retrospective study which included >500,000 Chinese subjects reported that the population-weighted incidence rate of traumatic fractures in the general population was ~3.2 per 1,000 individuals (1).

Despite the considerable developments in terms of internal and external fixation systems, bone fractures may still fail to heal under certain circumstances, including bone non-union or pseudarthrosis, causing painful and delayed bone healing (2). Clinical studies focusing on facilitating bone healing and restoration of normal biomechanical properties following bone fracture have shown that such methods may allow patients to recover and return to normal life relatively quicker than conventional methods (1-3).

Healing of fractures is initiated by the inflammatory cascade, followed by the recruitment of various immune and mesenchymal cells, as well as the formation of hematomas that further develop into vascularized and innervated granulation tissue (4). Following this initial stage of repair, callus tissue, characterized by the formation of woven bone, which may bridge the injury sites, is formed, followed by the bone remodelling phase (5). Although the inflammatory response is essential and beneficial to initiate bone repair, dysregulated or chronic inflammation may severely impair bone healing (6). Previous studies have shown that macrophages and other interleukin (IL)-17-producing γδ T cells promote bone healing (7,8), and that cytotoxic T cells may impair bone repair (9). IL-10-producing B cells, which suppress excessive and/or prolonged inflammation, may also contribute to bone healing (4). However, the underlying mechanisms of the effects of immune reaction on bone homeostasis during fracture healing remains to be determined.

In recent years, tissue-resident macrophages have been garnered increasing attention, not only because of their

Table I. Search strategy for studies published from inception of the database to December 23rd 2019.

A, PubMed[a]	Number of results
('Osteal tissue macrophages' OR 'osteal tissue macrophage' OR 'osteal macrophage' OR 'osteal macrophages' OR 'bone resident macrophages' OR 'bone resident macrophage' OR 'tissue resident macrophages' OR 'tissue resident macrophage' OR 'skeletal macrophages' OR 'skeletal macrophage' OR 'osteomacs' OR 'osteomac' OR 'resident tissue macrophages' OR 'resident tissue macrophage) AND (Broken Bones' OR 'Bone, Broken' OR 'Bones, Broken' OR 'Broken Bone' OR 'Bone Fractures' OR 'Bone Fracture' OR 'Fracture, Bone' OR 'Spiral Fractures' OR 'Fracture, Spiral' OR 'Fractures, Spiral' OR 'Spiral Fracture' OR 'Torsion Fractures' OR 'Fracture, Torsion' OR 'Fractures, Torsion' OR 'Torsion Fracture' OR 'Fracture' OR 'Fractures' OR 'Fractures, Bone')	76
B, Embase®	
i) 'osteal tissue macrophages' OR 'osteal tissue macrophage' OR 'osteal macrophage' OR 'osteal macrophages' OR 'bone resident macrophages' OR 'bone resident macrophage' OR 'tissue resident macrophages' OR 'tissue resident macrophage' OR 'skeletal macrophages' OR 'skeletal macrophage' OR 'osteomacs' OR 'osteomac' OR 'resident tissue macrophages' OR 'resident tissue macrophage'.mp.	933
ii) exp fracture/	275,697
iii) 'bone fracture' OR 'bone fractures' OR 'fracture' OR 'fractures'.mp.	366,616
iv) ii and iii	373,991
v) i and iv	17

[a]MeSH terms searched. mp. multi-purpose; /, terms searched.

important roles in innate immunity, but also in homeostasis and regeneration (6,10-12). Multiple subsets of tissue-resident macrophages have been identified in different organs or tissues, including microglial cells in the brain, Kupffer cells in the liver and Langerhans cells in the skin (13). Bone-resident macrophages are divided into erythroblastic island macrophages, haematopoietic stem cell niche macrophages and skeletal macrophages (sMΦ) (4,6,14,15). sMΦ, also called osteal macrophages or osteomacs, have been reported to significantly contribute to bone homeostasis and regeneration (16,17).

The aim of the present review was to systematically summarize the contribution of sMΦ in bone repair, and evaluate their potential as a therapeutic target for promoting bone regeneration and other bone diseases.

Materials and methods

Search strategy. A systematic search of the PubMed and Embase® databases (from inception to December 23rd, 2019) for studies investigating the function of sMΦ in bone injury repair was performed. This review was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (18), with search key words including 'osteal tissue macrophages', 'bone resident macrophages', 'skeletal macrophages', 'bone resident macrophage' and 'bone fractures'. The detailed search strategy is presented in Table I including a list of all search items used, names of the database searched and the publication period included.

Inclusion and exclusion criteria. Studies were included if they met the following criteria: i) Relevant to evaluating the effect of sMΦ in bone repair or regeneration; ii) full-length research articles were available; and iii) studies were published in English. Reviews, correspondences, case reports, expert opinions and editorials were all excluded.

Quality assessment and statistical analysis. The Systematic Review Centre for Laboratory animal Experimentation tool (19) was used to assess the risk of bias of included studies, with the types of bias including: Selection bias, performance bias, attrition bias, detection bias, reporting bias and other biases. The response was defined as 'Low risk of bias' or 'High risk of bias' for each item in the checklist. For ideal methodological quality, the percentage of 'Low risk of bias' was required to be ≥80% (20). If it was not possible to make a judgment based on the present information, a rating of 'Unclear risk of bias' was assigned. Finally, a sum of the percentage of bias for each study was calculated.

Data extraction. Data extraction was performed by two reviewers independently. Disagreements were resolved by consensus or discussion amongst co-investigators. The extracted data were characteristics of the study samples, general and detailed methodology characteristics, and study results.

Statistical analysis. All statistical analysis was performed using SPSS version 25 (IBM Corp.). A one-way ANOVA and Brown-Forsythe test were used to compare groups. $P<0.05$ was considered to indicate a statistically significant difference.

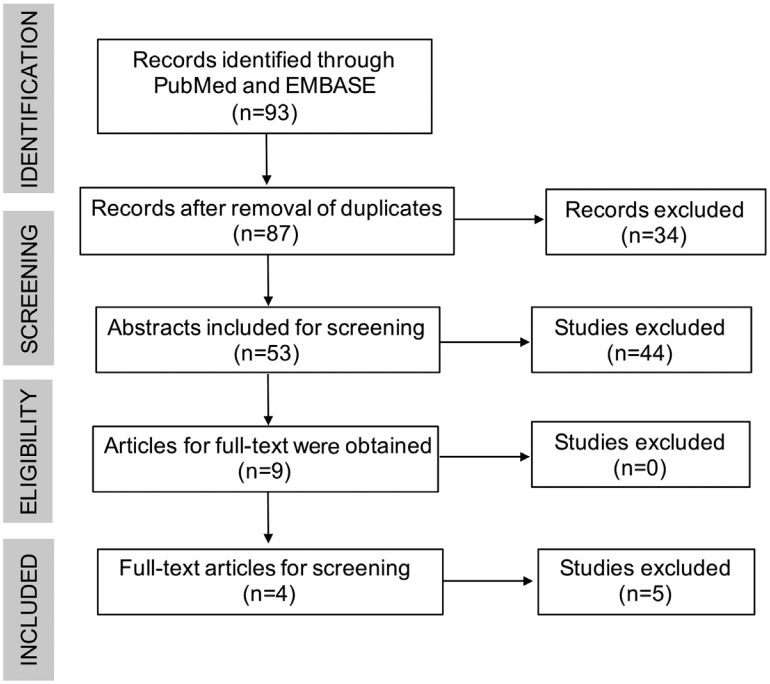

Figure 1. Flowchart of the study selection process.

Results

Details of the study selection process are presented in Fig. 1. The systematic search resulted in retrieval of 93 articles. After removing duplicates, 87 articles remained for first-stage screening. By reviewing the titles and abstracts, 3 articles were deemed irrelevant. No additional articles were included by checking the references. A total of 9 relevant articles were identified, the full text of which were assessed for eligibility. Finally, 4 articles that met all of the inclusion criteria were identified and included in the present systematic review (10,16,17,21).

The results of the risk of bias assessment are presented in Fig. 2. The mean percentage of low risk bias was 45% [95% confidence interval (CI), 12.6-77.4%], the mean percentage of high risk bias was 7.5%, (95% CI, 0.0-24.5%) and the mean percentage of unclear bias was 45.0%, (95% CI, 12.6-77.4%). $P<0.05$ in the Brown-Forsythe test indicated there was a significant difference between these 3 bias groups. There was a relatively high risk of bias associated with the blinding of the investigators and animals, since none of the assessors in these studies were blinded, and reports on allocation, random outcome assessment and incomplete outcome data were not well documented. There was a low risk of bias for baseline characteristics, random housing, selective information and other potential biases in the studies evaluated.

Table II presents the major characteristics of the studies included in the present systematic review. In all of the studies, mice were used as the experimental animals, with an age of 11-13 weeks. Of the four studies, three utilized the tibial fracture model and the remaining study used a femoral fracture model. Furthermore, three studies used immunohistochemistry combined with flow cytometry for identification and characterization of sMΦ. Specific surface markers used to define sMΦ were $F4/80^{+}$, $Mac\text{-}2^{-/low}$, $TRAP^{-}$, $CD169^{+}$, $Ly6G^{-}$ and $CD115^{low}$. In addition, all of the studies concluded that sMΦ have an essential role in fracture healing, and the mechanisms are summarized in Fig. 3.

Discussion

The aim of the present study was to summarize the results of previous studies assessing the role of sMΦ in bone healing. Previous studies supported the involvement of sMΦ in fracture healing, and identified the underlying cellular and molecular mechanisms and their utility in novel immunoregulatory therapy in bone regeneration.

Alexander *et al* (17) assessed the effects of sMΦ and inflammatory macrophages in bone healing and showed that $F4/80^{+}Mac^{-/low}$ sMΦ formed a distinctive canopy-like structure over cuboidal osteoblasts located on the surface of new bone. The number of $F4/80^{+}Mac^{high}$ inflammatory macrophages was considerably lower than that of sMΦ during the early and late anabolic phases of tibial fracture repair, which heals primarily via intramembranous ossification (16). $F4/80^{+}$ macrophages were present in all phases of fracture healing and were required for matrix deposition and bone mineralization. Systematic depletion of $F4/80^{+}$ macrophages notably suppressed bone deposition and mineralization (21). Furthermore, due to the relationship in the lineage of macrophages and osteoclasts, osteoclasts were specifically ablated using osteoporotegerin treatment to study the effect of an absence of osteoclasts on bone healing (21). It was shown that osteoporotegerin treatment resulted in significantly impaired bone resorption, but did not compromise $CT1^{+}$ woven bone deposition, which further confirmed the importance of $F4/80^{+}$ macrophages that were prominently sMΦ in bone healing (22). The systematic depletion approach of macrophages using lysozyme M-driven Cre recombinase, Csf1r promoter, clodronate liposome or antibody is also able to reduce inflammatory macrophages

Table II. Summary of studies included in the systematic review.

First author, year (Refs.)	Species investigated	Sample characteristics	Bone injury model	Identification methods of macrophages	Markers of sMΦ used	Role of sMΦ in bone repair
Alexander *et al*, 2011 (17)	Mouse	11-12 weeks old	Tibial fracture	FC, IHC	F4/80+, Mac-2-/low, TRAP-	i) Participated in intramenbranous ossification. ii) Required for CT1+ matrix deposition and bone mineralization.
Raggatt *et al*, 2014 (21)	Mouse	11-12 weeks old	Femoral fracture	FC, IHC	F4/80+, Mac-2-	Promoted anabolism during endochondral callus formation.
Vi *et al*, 2015 (10)	Mouse	12 weeks old	Tibial fracture	IHC	F4/80+, TRAP-	Maintained bone homeostasis and promoted fracture repair by enhancing the differentiation of mesenchymal progenitors.
Batoon *et al*, 2019 (16)	Mouse	11-13 weeks old	Tibial fracture	FC, IHC	CD169+, F4/80+, Ly6G-, CD115 low	Supported osteoblasts during both bone homeostasis and repair.

sMΦ, skeletal macrophages; TRAP, tartrate-resistant acid phosphatase; CT1, collagen type 1; FC, flow cytometry; IHC, immunohistochemistry; Ly6G, lymphocyte antigen 6 complex locus G6D.

Figure 2. Risk of bias assessment. The Systematic Review Centre for Laboratory animal Experimentation was used to assess the risk of bias in the included studies. Green, low risk of bias; red, high risk of bias; yellow, unclear risk of bias.

and osteoclasts (23). Therefore, specific ablation of sMΦ by targeting a specific surface marker in fracture models is necessary (23).

In addition, macrophages were shown to promote endochondral callus formation following bone fracture (21). In a mouse femoral fracture model, which primarily heals via endochondral ossification, Batoon *et al* (15) found that F4/80$^+$ Mac-2$^+$ inflammatory macrophages were abundant in the granulation tissue, which was fully established 7 days after fracture surgery. However, the presence of sMΦ, defined as F4/80$^+$Mac-2 cells-, were relatively rare at this reparative stage. Furthermore, during soft-to-hard callus transition, both sMΦ and inflammatory macrophages were abundantly present in the maturing callus. F4/80+ macrophage depletion at the start of the early anabolic phase significantly impeded soft callus formation and the progression of anabolism in endochondral ossification (15). Furthermore, Alexander *et al* (14) suggested that macrophages have a significant influence on both cartilage and bone deposition during endochondral ossification. The presence of F4/80$^+$ macrophages throughout the entire process of fracture repair and macrophage deficiency may result in smaller fracture calluses, but increased fibrotic calluses, which results in delayed bone repair (10).

The crosstalk between sMΦ and osteoblasts/osteoclasts is currently being investigated. Batoon *et al* (16) demonstrated that

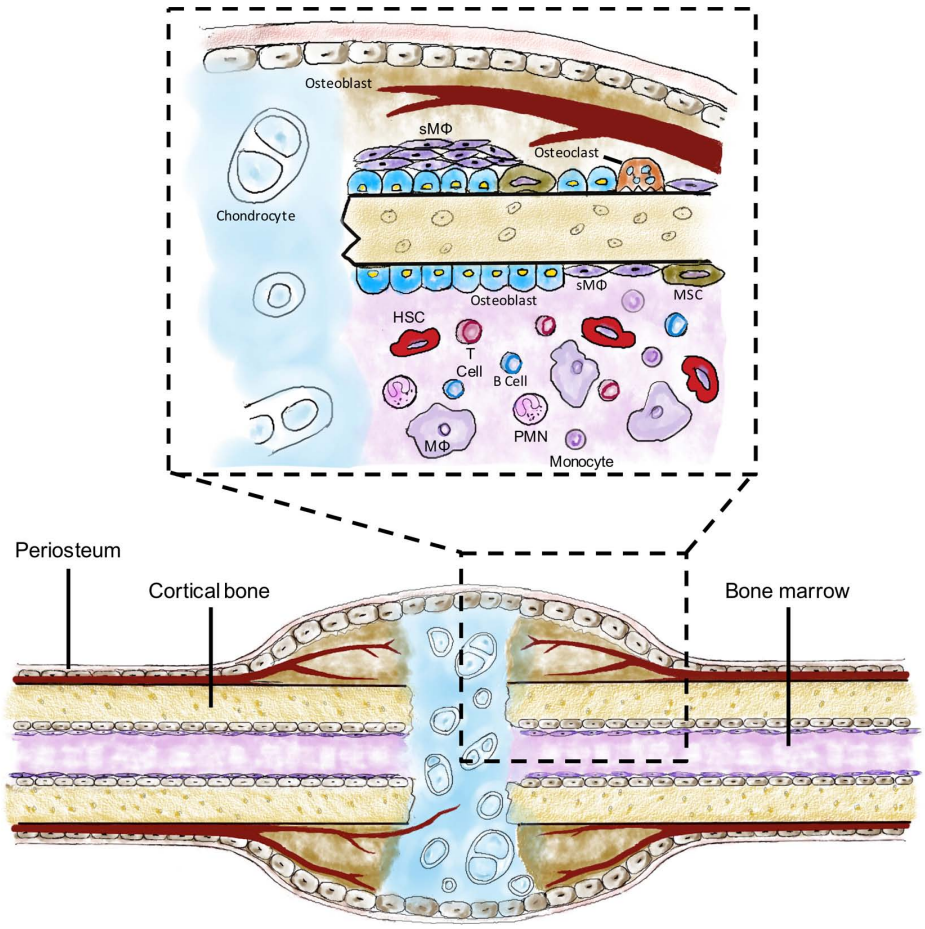

Figure 3. Participation of sMΦs in fracture repair. Lower panel: sMΦs located at the border of cortical bone form a sMΦ lining. During the anabolic phase, woven bone bridges the injury sites, and the bone fracture heals via endochondral ossification and intramembranous ossification. Inset: Immune cells and MSCs are recruited to the fracture site. sMΦs form a canopy-like structure over the cuboidal-shaped osteoblasts. MΦ, macrophage; sMΦ, skeletal MΦ; MSC, mesenchymal stem cell; HSC, hematopoietic stem cell; PMN, polymorphonuclear leucocyte.

CD169, a cell surface antigen expressed by mature tissue-resident macrophages, may be used to discriminate osteoclasts and sMΦ. $CD169^+$ sMΦ depletion may significantly compromise osteoblastogenesis and bone repair in bone injury, primarily via promoting both endochondral ossification or intramembranous ossification (16). Furthermore, increasing the proliferation of sMΦ in callus tissue by administering colony-stimulating factor-1, which may target sMΦ and promote its proliferation, was reported to promote bone repair (17,21). Although the mechanisms by which sMΦ promotes fracture healing remain elusive, the NF-κB signalling pathway, bone morphogenetic proteins and oncostatin M are thought to be essential in sMΦ-mediated osteogenesis (24,25). Ablation of sMΦ was indicated to significantly impair osteocalcin expression and osteoblast mineralization *in vivo* and *in vitro* (11). Furthermore, the interaction between sMΦ and osteoclasts may also be a point of interest. Macrophage-deficient mice exhibited functionally active osteoclast activities, but were characterized by decreased sMΦ at the bone surface and impaired bone formation (10,26). These results emphasize the importance of sMΦ in bone healing, and highlight the potential role of sMΦ as a therapeutic target for bone regeneration. Thus, a more in-depth understanding from a global perspective of molecular profiles and phenotypes adopted by sMΦ in the bone environment is required.

An increasing number of studies have shown that tissue-resident macrophages are able to adopt tissue-specific phenotypes and functions and may acquire self-renewal capacity (10,16,17,21). Multiple studies have confirmed the essential roles of macrophages in skeletal homeostasis and bone repair (10,16,17,21); however, direct evidence of the function of sMΦ in bone biology remains insufficient, due to the heterogeneity of macrophage clusters and the lack of sMΦ-specific biomarkers (27). With the development of cutting-edge techniques, including optimized next-generation sequencing technologies (28), for use in life science investigations, a single-cell sequencing approach may be a suitable means of profile the involved macrophages, thus assisting in the identification of the heterogeneity of sMΦ during fracture repair.

The present systematic review provided an overview of the roles of sMΦ in bone healing. Several biomarkers defining sMΦ were identified based on the available literature. The present study is limited by the high risk of bias with regard to blinding and sequence generation in the reviewed studies. Another limitation is that due to the shortage of sufficient studies on this topic, the importance of sMΦ in fracture healing may be under- or overestimated.

In conclusion, a growing body of evidence strongly supports the notion that $F4/80^+Mac\text{-}2^-CD169^+$ sMΦ may serve as a promising therapeutic target for immunoregulatory therapy in

bone repair, due to their essential role in bone formation and homeostasis. Further investigation aiming to modulate sMΦ, with the aim of promoting bone regeneration, are required.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZW and OKSL conceived and designed the study. ZW acquired the data. ZW, LYS, YFW, ZH, YD, CWL and SMK analyzed and interpreted the data. ZW wrote the manuscript. ZW, SMK, and OKSL revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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