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REVIEW

Unraveling macrophage contributions to bone repair

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Macrophages have reemerged to prominence with widened understanding of their pleiotropic contributions to many biologies and pathologies. This includes clear advances in revealing their importance in wound healing. Here we have focused on the current state of knowledge with respect to bone repair, which has received relatively little scientific attention compared with its soft-tissue counterparts. Our detailed characterization of resident tissue macrophages residing in bone-lining tissues (osteomacs), including their pro-anabolic function, exposed a more prominent role for these cells in bone biology than previously anticipated. Recent studies have confirmed the importance of macrophages in early inflammatory processes that establish the healing cascade after bone fracture. Emerging data support that macrophage influence extends into both anabolic and catabolic phases of repair, suggesting that these cells have prolonged and diverse functions during fracture healing. More research is needed to clarify macrophage phase-specific contributions, temporospatial subpopulation variance and macrophage specific-molecular mediators. There is also clear motivation for determining whether macrophage alterations underlie compromised fracture healing. Overall, there is strong justification to pursue strategies targeting macrophages and/or their products for improving normal bone healing and overcoming failed repair.

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Introduction

Bone fracture is a common and increasing medical affliction that results from both traumatic injury and disease-related bone fragility. In 1998, in the USA alone over 13 million fractures were seen by physicians.[1] Even when adequate bone repair is achieved within the expected time frames, treating fractures is costly[2] and this is protracted in 10–20% of cases in which healing is delayed or failed.[3,4] The most common cause of bone fragility is osteoporosis, and the socioeconomic burden of osteoporotic fractures alone is an increasing problem for health-care systems internationally.[5] In the USA in 2007, inpatient costs due to low-energy fractures exceeded $25 billion.[1] Fractures are predominantly treated via orthopedic management. Current biological approaches (for example, teriparatide and strontium ranelate) are modestly effective or not broadly applicable, creating a treatment gap in the management of fracture and osteoporosis. Reducing the overall burden of fracture will require a multipronged approach, including the following: reduction of fracture healing time frames in healthy individuals; overcoming compromised healing associated with disease/risk factors; reigniting healing in delayed nonunion settings; and reducing the osteoporosis-associated fracture rate. A prerequisite for all of these approaches is availability of an affordable, effective and broadly applicable pro-anabolic therapy. Clearly, benefit can be gained from improved understanding of fracture repair. In physiology, and particularly in disease, cells of the immune and osseous systems have a dynamic interplay such that each system has a multilevel influence on the fate and functionality of the other. There are still substantial knowledge gaps in understanding these interactions, subsequently limiting our ability to harness or temper these outcomes clinically. Evidence is accumulating in support of immune macrophages having an important functional input during multiple stages of fracture healing. This review will overview known, as well as speculate on, potential macrophage contributions to bone repair.

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Resident Tissue Macrophages and Osteomacs

Macrophages are heterogeneous myeloid lineage cells, the majority of which differentiate from bone marrow (BM) hematopoietic stem/progenitor cells via intermediary monocytes circulating in the blood (**Figure 1**).[6,7] They are 'the chameleon cell' of higher organisms and have one of the most diverse and adaptive transcriptomes, expressing a large range of cell surface receptors, growth factors, pro- and anti-inflammatory cytokines, chemokines, proteolytic enzymes and many other cellular products.[7,8] Their highly attuned responsiveness and diverse expression capacity translate into macrophages having a dynamic phenotype, capable of rapidly responding to minor changes within their environment. As a result, macrophage classification is challenging. Many subpopulations exist, and their phenotype upon *ex vivo* and *in vitro* manipulation is unstable.[9] The first branching of the macrophage 'phylogenetic tree' divides these cells into resident tissue or immune macrophages (**Figure 1**). Resident macrophages infiltrate both lymphoid and non-lymphoid tissues during early development and have ongoing presence in nearly all tissues throughout adult life, with varying turnover rates, density and complexity.[6,10,11] In the absence of inflammation or damage, resident macrophages have important ubiquitous as well as tissue-specific contributions during development, homeostasis and repair (for a comprehensive review, see Stefater *et al.*[6]).

Of particular relevance here, we have identified that both periosteal and endosteal bone-lining tissues contain a resident tissue macrophage population that we termed osteomacs. They were present in resting osteal tissues and were increased at sites undergoing active bone anabolism.[12–14] Osteomacs have pivotal roles in bone and BM homeostasis through the support of osteoblast maintenance and functional activity.[13,15] In mice, osteomacs expressed numerous myeloid lineage markers including but not limited to F4/80, CD115, Mac-3 and CD68 (**Figure 2** and refs 12,13 and unpublished data). Osteomacs express minimal if any tartrate-resistant acid phosphatase (osteoclast marker) or Mac-2/galectin-3 (inflammatory macrophage marker), confirming their classification as resident macrophages (**Figure 2**). The identification and characterization of osteomacs have invigorated interest in macrophage contributions to bone biology, pathology and repair.

Inflammatory Macrophages

Recruited inflammatory macrophages are derived from a distinct population of blood monocytes that rapidly infiltrate tissues compromised by injury, inappropriate functioning and/or infection (**Figure 1**).[16] Depending on the environmental queues encountered within an infected/injured tissue, inflammatory macrophages are polarized toward an appropriate activation pathway that spans a broad spectrum. The extreme ends of this spectrum are described as classically activated macrophages (commonly referred to as M1, **Figure 1**) or as alternatively activated macrophages (commonly referred to as M2, **Figure 1**). M1 macrophages are critical in destroying foreign organisms and in fighting infection.[17] In contrast, M2 macrophages have been documented in wound healing, tissue repair, debris scavenging and angiogenesis.[18] Overall, the polarization phenotype adopted by a macrophage can have a major influence over healing progression and outcome.[19] We

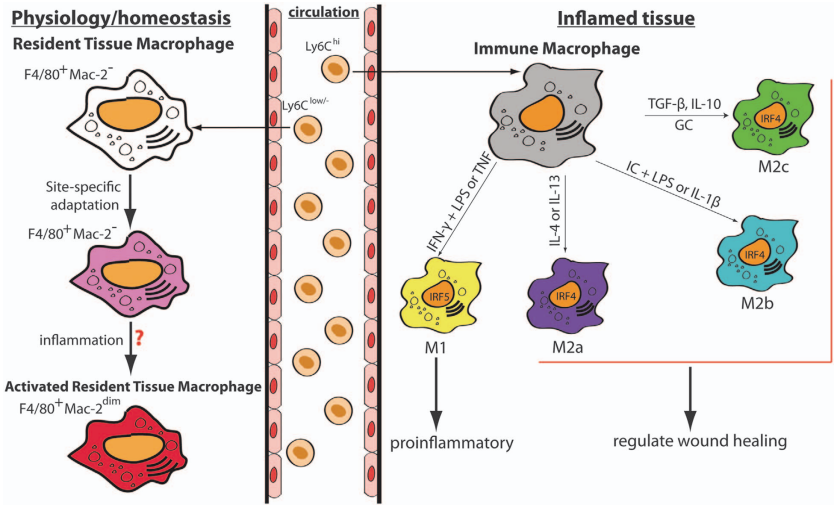

Figure 1 Resident tissue and immune macrophages. A key attribute of macrophages is their ability to polarize to distinct phenotypes that express unique biomarkers and discrete molecules. Under physiological conditions (left panel), resident tissue macrophages are derived from circulating Ly6C$^{low/-}$ monocytes that undergo site-specific adaptation once in their resident tissue.[11] In mice, resident tissue macrophages express the pan-macrophage marker F4/80 but lack Mac-2/galectin-3 expression.[11,12] In response to inflammation, it is possible that these resident tissue macrophages become activated. In the setting of bone injury, a population of F4/80$^+$ Mac-2^{dim} cells can be identified that are distinct from their F4/80$^+$ Mac-2$^+$ inflammatory relatives.[12] In a setting of inflammation induced by injury, damage or infection (right panel), inflammatory macrophages are recruited from circulating Ly6C^{hi} monocytes.[11] These inflammatory monocytes acquire either a classically (M1) or alternatively (M2) activated phenotype depending on the stimuli encounter. The M1 phenotype is typically induced by interferon (IFN)-γ, microbial stimuli such as lipopolysaccharides (LPS) and/or cytokines including TNF.[40] The M2 phenotype can be further divided into M2a, b and c subsets that have distinct characteristics.[40,64] Example stimuli for the M2 subsets are as follows: M2a macrophages, interleukin (IL)-4 and IL-13; M2b macrophages, immune complexes (IC) plus IL-1β or LPS; M2c macrophages, transforming growth factor-β (TGF-β), IL-10 or glucocorticoids.[40] M1 macrophages express the nuclear transcription factor interferon regulatory factor (IRF)-5 and M2 cells express IRF-4.[65] M1 cells drive cytotoxic, proinflammatory responses promoting a Th1 helper immune response. In contrast, M2 cells are associated with anti-inflammatory processes, Th2-type immune responses and/or with aiding wound regeneration and angiogenesis.[40]

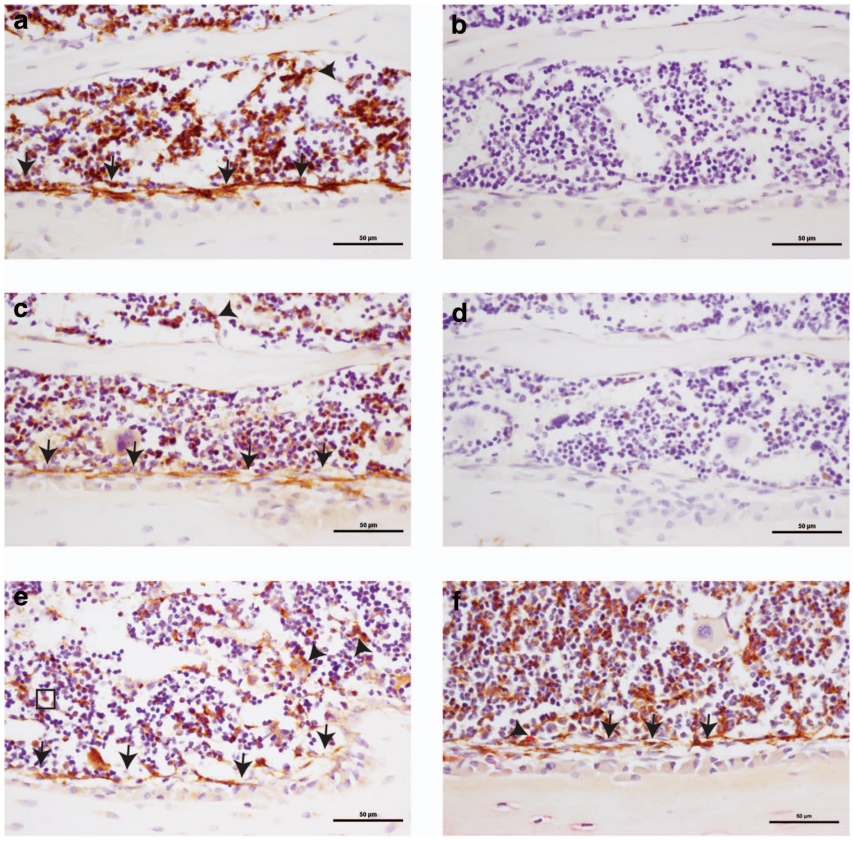

Figure 2 Osteomacs are resident tissue macrophages. (**a**–**f**): Immunohistochemical staining performed as previously described[12] in sagittal cross-sections of cortical long bone from 4-week-old C57Bl/6 mice within the diaphyseal region. Sections were stained with a panel of myeloid, monocyte and macrophage markers. All sections were counterstained with hematoxylin; **a** through **d** are serial sections. (**a**) Staining with anti-F4/80 antibody (brown) demonstrating F4/80$^+$ osteomacs (arrows) forming a canopy over cuboidal osteoblasts that stained for osteocalcin and collagen type I expression in near serial sections (data not shown). (**b**) Specificity of staining was confirmed using a relevant isotype control antibody. These canopy osteomacs also expressed the mature macrophage marker CD68 (**c**, arrows), but were negative for the inflammatory macrophage marker Mac-2/galectin-3 (**d**). Canopy osteomacs were also positive for the pan-myeloid marker Mac-3 (**e**, arrows). (**f**) F4/80$^-$ tartrate-resistant acid phosphatase (TRAP) double staining illustrated that F4/80$^+$ osteomacs (arrows) did not express the osteoclast marker TRAP and were distinct from TRAP$^+$ mononuclear osteoclast precursors (arrowhead) within this endosteal bone environment. This expression profile confirms osteomacs as resident tissue macrophages. Images are representative of four mice/group. Original magnification: (**a**–**f**) $\times$ 40.

have demonstrated that, during bone repair, inflammatory macrophages and resident macrophages coexist within the injury site,[12] a phenomenon that has not been well described in the literature and requires ongoing investigation.

The beneficial contributions of inflammatory macrophages to soft-tissue repair have been widely studied.[20–22] In skin wounds, these macrophages participate at multiple stages of healing and influence granulation formation, stromal cell differentiation, vascular integrity, modeling/remolding of extracellular matrix and inflammation.[19] However, it cannot be assumed that macrophage contributions in soft-tissue healing will directly translate into bone repair, particularly when these processes fundamentally differ in their outcomes.

Bone Repair During Fracture Healing

Fracture healing is a highly coordinated and complex process that involves the interplay of many cells, growth factors and extracellular matrix components. It has many commonalities with soft-tissue repair associated with generic wound healing events including inflammation, neo-angiogenesis and replacement of damaged mesenchyme. However, bone repair differs from the repair of most other tissues in that successful healing does not result in scar tissue formation. The repair process needs to regenerate complex three-dimensional structures that have to meet mechanical load specifications. Therefore, bone repair is likely to involve bone exclusive events that require a greater level of prolonged complexity compared with soft-tissue repair and incorporate ongoing communication with surrounding, and potentially distant, undamaged tissues.

At a cellular level, the key participants during fracture healing are endothelial cells, inflammatory cells, osteoblasts, chondrocytes and osteoclasts.[23] The mechanism of bone healing is dependent on mechanical influences, with highly stabilized fractures healing predominantly via direct intramembranous ossification with greatly reduced magnitude of periosteal callus formation and healing progressing predominantly via intramedullary and intra-cortical bridging.[24] In this circumstance, healing initiates with inflammation, transitions to anabolic repair and concludes with prolonged remodeling. The latter phase is designed to reinstate the original bone architecture as closely as possible. Healing of mechanically non-ridged fractures occurs via generation of a periosteal callus that is formed predominantly via endochondral ossification. This repair process can be simplified into four phases: inflammatory, soft callus formation (early anabolic), hard callus formation (late

anabolic) and remodeling, with significant overlap between each of these phases (reviewed extensively in Schindeler *et al.*[25]).

Macrophage Contributions to Inflammation During Fracture Repair

Fracture causes disruption of local tissue vasculature, soft- and hard-tissue integrity and BM architecture and induces hematoma formation. This results in activation of the complement pathway and release of injury-associated signals from the damaged mesenchymal cells. These signals are subsequently detected by resident innate immune cells, which in the case of bones include osteomacs, resident BM macrophages and BM granulocytes. These cells then initiate a cascade of growth factors and inflammatory cytokine and chemokine production that facilitate the recruitment of inflammatory immune cells (granulocytes and inflammatory monocyte/macrophages) that combat infection and phagocytose debris and dead cell remnants.[25] During this inflammatory event, expansion/recruitment of mesenchymal stem/progenitor/precursor cells (be they from local or more distant locations[26]) occurs and this is followed by replacement of the hematoma with a vascularized fibrous connective tissue, known as granulation tissue.[25]

Macrophages are present during the inflammatory phase of fracture healing in both humans[27] and animals.[12,28–31] A suite of known inflammatory macrophage cytokines that are generally associated with M1 responses can be detected at this early stage of fracture healing. In patients with hip fractures, high levels of macrophage-related cytokines including interleukin-1, interleukin-6 and tumor necrosis factor (TNF) have been detected within the first few days after injury.[32] In a mouse model of fracture, interleukin-1 and TNF were localized predominately to macrophages and inflammatory cells in the marrow and periosteum adjacent to fracture sites.[31] In mouse models of fracture in which recruitment of inflammatory macrophages[28] or inflammatory cytokine signaling[33–35] was compromised through germline genetic alterations, there were prolonged negative impacts on fracture healing. These data support the theory that either reduction of inflammatory macrophages (without specific assessment of resident macrophages) or diminution of their inflammatory cytokine output from the time of injury compromises fracture healing. The experimental designs of these studies do not permit definitive differentiation between inflammatory phase-specific contributions of macrophages, potential knock-on effects due to perturbed early inflammatory processes and ongoing inflammatory macrophage contributions to later stages of fracture healing.

Interestingly, amplification of inflammatory macrophage activation either through semisoluble aminated glucan given at the time of fracture[36] or through prolonged delivery of supraphysiological TNF[37] or bacterial products administered for 7 days starting at the time of fracture[38,39] compromised healing. However, macrophage polarization was not directly examined in these studies, but these stimuli are known M1-polarizing agents.[40] These data imply that persistent polarization of macrophages to an M1 phenotype during the inflammatory and possibly early anabolic phase(s) may be detrimental to fracture healing, although this remains to be proven experimentally. In an appropriately regulated immune response, inflammatory macrophages are generally short-lived, remaining within tissues only while there is an immediate threat. Compromised healing and chronic inflammatory disease are associated with inappropriate and prolonged tissue infiltration of inflammatory macrophages.[41] It is possible that one of the mechanisms underlying increased rates of delayed or failed fracture healing in compound fractures is the increased pathogen load in these open wounds resulting in inappropriate or extreme polarization of macrophages. Experimentally, elevated systemic inflammation due to additional soft-tissue trauma injuries impairs fracture healing,[42,43] confirming that persistence of a proinflammatory environment is one mechanism responsible for detrimental fracture healing.[44] Interestingly, low-dose TNF administered to the fracture site at the time of surgery and 1 day post surgery improved fracture healing in mice,[45] suggesting that TNF, and potentially macrophage activation, has complex dose- and time-dependent outcomes on bone healing.

The process of angiogenesis is an indispensable event during skeletal and soft-tissue regeneration, providing essential supply of oxygen and nutrients and enabling the recruitment of key cellular participants. Inadequate revascularization of the fracture site significantly increases the chances of nonunion.[46] Macrophages facilitate neovascularization during embryogenesis, postnatal tissue remodeling, wound repair and tumor progression (reviewed in Nucera *et al.*[17]). During skin repair, angiogenesis of granulation tissue was directed by a non-redundant subset of recruited inflammatory macrophages expressing vascular endothelial growth factor (VEGF)-A. These pro-angiogenic monocyte/macrophages had a mixed M1 and M2 profile.[48] Macrophages pervading the granulation tissue at a fracture site[12,29] are a confirmed cellular source of VEGFs in soft-tissue injury,[48] and VEGFs have been established as important pro-angiogenic molecules in fracture repair.[49] It remains to be definitively established whether macrophages direct vascularization within the granulation tissue during bone repair.

Macrophage Contributions to Early Anabolism During Fracture Repair

Appropriate inflammation and granulation tissue formation are assumed to be prerequisites for transition to the early anabolic phase of fracture healing. At this point, healing mechanisms diverge either toward direct bone deposition by intramembranous ossification (rigidly stabilized fractures) or toward periosteal callus formation via endochondral ossification (nonrigid fixation). In both cases, vital events are mesenchymal stem/progenitor/precursor cell recruitment/expansion (from distant or local pools[26]) and subsequent induction of osteoblastogenesis or chondrogenesis, respectively. Data from animal models support the fact that macrophages are present within repair-associated tissues during this early anabolic phase with potentially sustained presence of robust numbers of macrophages during rigidly stabilized fixation compared with nonrigid repair scenarios.[12,29,30,50] During intramembranous ossification, macrophages were intercalated throughout areas of developing bone matrix,[12] whereas during endochondral repair they were excluded from the developing cartilage but were present in adjacent tissues.[29] Similarly, human fracture tissue studies support that, although macrophage numbers are higher in early fracture samples, they do persist in fracture-associated tissues and have been observed in association with

areas of bone formation.[27] Overall, macrophages are appropriately positioned to directly influence events during the early anabolic phase of healing, suggesting an extension of their contributions beyond early inflammatory events.

Although it has not yet been definitively demonstrated that macrophage-derived signals are directly responsible for the expansion/recruitment of mesenchymal stem/progenitor/precursor at/into fracture sites and for the subsequent induction of their differentiation, indirect evidence is mounting. Macrophages can produce pro-anabolic factors and support *in vitro* osteogenic differentiation and osteoblast full-functional maturation *in vitro*.[13,51,52] Monocyte/macrophage production of oncostatin M has been implicated as a pro-anabolic molecule,[51,52] and *in vitro* evidence has suggested that M1 but not M2 macrophages express oncostatin M.[52] Conversely, emerging data suggest that mesenchymal stem/stromal cells regulate the activation profile of macrophages, driving them more toward an M2 profile,[53] suggesting that this phenotype may also facilitate mesenchymal stem/stromal cells biology.

We have published a study that directly examined macrophage functional contributions during the early anabolic phase of repair. Using a tibial injury model that heals via stabilized fracture repair mechanisms with two inducible macrophage depletion strategies, staggered treatment regimens and comparison with osteoprotegerin treatment, we demonstrated that intramembranous ossification was significantly impaired when macrophages were depleted during the anabolic phase.[12] Additional, but indirect, support for macrophages influencing anabolic outcomes during stabilized fracture repair was provided in a recent study investigating healing in matrix metalloproteinases (MMP)-9 deficient mice.[29] In this model, the absence of MMP9 resulted in a switch from intramembranous to endochondral callus repair in this rigidly stabilized fracture setting[54] and was unexpectedly associated with increased infiltration of macrophages compared with that seen in control mice.[29] These observations are somewhat paradoxical when compared with the wild-type setting, in which fewer infiltrating macrophages were associated with endochondral callus formation in nonrigid fracture repair models.[12,29,30,50] Hankemeier *et al.*[30] similarly suggested that the magnitude of macrophage infiltration into the callus might influence the anabolic mechanism induced, but argued that fewer macrophages were indicative of nonrigid fracture repair via endochondral callus formation. We speculate that the magnitude of macrophage infiltration is secondary in importance to the type of macrophage being recruited/induced. We have shown that both inflammatory macrophages and osteomacs were present in tissues associated with a bone injury but that the latter predominated within the expanding woven bone bridging the injury site. Importantly, increasing osteomacs, but not inflammatory macrophages, during the inflammatory and early anabolic phases of bone healing via colony stimulating factor-1 treatment accelerated the deposition of bone matrix.[12] More detailed investigation is required to characterize the participating macrophage populations, identify their origin and clarify their contributions to the decision of what repair mechanism is initiated during fracture healing.

Few, if any, published studies have provided clear evidence for direct macrophage contributions to anabolic processes during endochondal callus formation. Altered endochondral callus formation has been reported as a result of manipulation of macrophages and or macrophage-expressed molecules, but the experimental designs did not specifically target these molecules during the anabolic phase.[28,29,34,45] Therefore, altered inflammatory progression/resolution cannot be ruled out as a contributing mechanism to the observed compromised fracture repair. Carefully timed manipulation of macrophages and/or their products is required to elucidate the pro-anabolic contributions of macrophages to bone repair via endochondral ossification. Overall, evidence is mounting in support of the fact that macrophages are apex regulatory cells in driving early anabolic processes in fracture repair irrespective of the ossification mechanism.

Macrophage Participation in Hard Callus Formation and Remodeling

In nonrigid fractures, soft callus needs to be converted to hard boney callus in order to provide a temporary support structure to the bare mechanical load. The boney callus is then remodeled to reinstate the original bone structure. Although these are distinct events occurring in site-specific locations, they generally occur simultaneously within the fracture zone, are interdependent and even overlap with the early anabolic events. This makes it challenging to dissect out cellular and molecular contributions to the individual processes, and very little, if any, published literature definitively deconstructs these events. When attempting to dissect macrophage contributions in these later phases, additional care is needed to distinguish their role from that of osteoclasts/chondroclasts, which, before this phase, had only minor roles in the repair process.[55] This is challenging because of the close lineage and functional relationship of these cells. Osteoprotegerin and other receptor activators of nuclear factor-kappa B ligand targeting methods can be used to specifically block osteoclasts/chondroclasts without having a direct impact on macrophages. We do not know of any method by which targeting of macrophages can be achieved without potential consequences on osteoclasts/chondroclasts.[12]

Nuclear factor-kappa B ligand inhibition in animal fracture models has established the importance of osteoclasts/chondroclasts in cartilaginous-to-boney callus transition.[55–57] Profound inhibition of osteoclast formation was confirmed in most models. Interestingly, the remaining callus consisted of mineralized and vascularized cartilage[55] that was mechanically sound.[57] MMPs,[55] particularly MMP-9[54] and MMP-13,[58] have been implicated as important molecular mediators of soft-to-hard callus transition. *In vitro* data support the fact that macrophages can efficiently produce MMPs and degrade the cartilage matrix,[59,60] with MMP production being a forte of M1 macrophages.[61] Interestingly, F4/80$^+$ macrophages were shown to be present in invading vascular canals during the formation of the primary and secondary ossification centers in mouse long bone development.[62] Overall, macrophage contributions to the early stages of cartilage remodeling should not be ruled out.

We have implicated osteomacs in the ongoing support of osteoblast maintenance and function[13] and have predicted that osteomacs and/or a similar macrophage population participate in bone formation during hard callus evolution. We have demonstrated that osteomacs persist during the remodeling phase of bone healing after tibial injury,[12] supporting this

hypothesis. Circumstantial support for this hypothesis includes mRNA expression of macrophage macrosialin protein correlating with the expression of collagen IX in fractures.[30] In addition, colony stimulating factor-1 treatment over an extended time course (14 days) in rabbits resulted in a significantly increased mineralized callus area at 4 weeks post fracture, with no coincident increase in osteoclast or osteoblast number.[63] Macrophages were not assessed in this study, but given that they are a major colony stimulating factor-1 target it raises the possibility that either increased macrophage numbers or enhancement of a particular macrophage phenotype contributed to the observed increase in callus mineralization.

Conclusions and Future Directions

The accumulating direct and indirect literature supports the fact that macrophages are appropriately located within fracture-associated tissues, with the required detection and translation molecule repertoire to initiate, influence and coordinate both generic wound healing mechanisms as well as bone exclusive catabolic and anabolic outcomes. However, more definite investigations are required to achieve a comprehensive understanding of these contributions. This includes better temporospatial mapping of macrophages throughout fracture repair, during both rigidly stabilized and nonrigid healing scenarios, with *in situ* confirmation of specific macrophage subpopulations and polarization status. To gain greater understanding of the full gambit of macrophage functional contributions and the molecular mediators involved, more precise and phase-specific manipulation of macrophages and/or their molecular repertoire is required. We now feel that there is sufficient evidence to motivate the investigation of macrophage alterations, particularly inappropriate polarization status, being an underlying cause of compromised fracture repair. This includes compromised repair in the setting of infection, systemic inflammation, osteoporosis and diabetes. Finally, cautious optimism is reasonable in proposing macrophages as a viable target for therapeutically enhancing fracture healing and overcoming failed repair. We hypothesize that, within fractures, macrophages acting as an apex regulatory cell, with specific subpopulations sensing instructions from multiple inputs throughout the healing time frame and translating these into biological responses. We predict that manipulation of an apex regulator will become a more successful approach in anabolic bone therapy compared with current approaches that target downstream effector cells and/or molecular mediators. However, this approach will have its challenges, as a delicate balance likely exists between enhancing beneficial and detrimental macrophage functionality, and this may vary depending on the stage of healing or the underlying cause of compromised repair.

Conflict of Interest

The authors declare no conflict of interest.

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