Review

The roles of immune cells in bone healing; what we know, do not know and future perspectives

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  \item Key events occurring during the bone healing include well-orchestrated and complex interactions between immune cells, multipotential stromal cells (MSCs), osteoblasts and osteoclasts. Through three overlapping phases of this physiological process, innate and adaptive immune cells, cytokines and chemokines have a significant role to play. The aim of the escalating immune response is to achieve an osseous healing in the shortest time and with the least complications facilitating the restoration of function. The uninterrupted progression of these biological events in conjunction with a favourable mechanical environment (stable fracture fixation) remains the hallmark of successful fracture healing. When failure occurs, either the biological environment or the mechanical one could have been disrupted. Not infrequently both may be compromised. Consequently, regenerative treatments involving the use of bone autograft, allograft or synthetic matrices supplemented with MSCs are increasingly used. A better understanding of the bone biology and osteoimmunology can help to improve these evolving cell-therapy based strategies. Herein, an up to date status of the role of immune cells during the different phases of bone healing is presented. Additionally, the known and yet to know events about immune cell interactions with MSCs and osteoblasts and osteoclasts and the therapeutic implications are being discussed.
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Introduction

The interaction between bone cells, inflammatory mediators and constituents of the immune system involved in bone repair, continue to be of great scientific interest to researchers and clinicians [1–12]. Investigation of the critical role of immune cells during the bone healing is ongoing. Depletion of T- and B-lymphocytes is associated with impairment in bone mineralisation and maturation of osteoblasts with delayed repair and remodelling phases and delayed healing as demonstrated in experimental models [13,14]. Additionally, Cho et al. demonstrated that resident macrophages (osteal) are significantly involved in parathyroid hormone-dependent bone healing [15]. Although there are no experimental models for NK cell depletion in fractures, an important role of NK cells during bone repair has been implied when a high level of interferon-gamma (IFN-γ) was detected in the diaphyseal regions of fractured femur in mice lacking T- and B-lymphocytes [16]. Conversely, as shown in immune-compromised animal model, bone marrow (BM) transplantation greatly enhanced the process of bone healing [17]. In addition to experimental findings, immune-compromised HIV patients can have delayed or non-union of fractures [18]. Thus, both animal and human studies confirmed the critical importance of innate and adaptive immune cells.

While the outer layer of cortical bone carries the weight bearing function, inner cancellous bone contains BM, a niche for different cell types including bone progenitor cells and multipotential stromal cells (MSCs). MSCs are classically identified as cells with the adherence capacity, which also express surface molecules CD90, CD73, CD105, but not hematopoietic lineage markers and are able to differentiate into bone, fat and cartilage cells [19]. Beside inflammatory cells and MSCs, two types of bone resident cells, osteoclasts and osteoblasts also play critical roles during the process of bone healing. Osteoclasts are large multinucleated cells are differentiated from monocyte lineage cells and have a bone degradation activity [20]. In contrast, the function of osteoblasts is the bone formation and they are derived from MSC-differentiated bone progenitor cells. Each of the immune cells has both distinctive and common functions with each other or MSCs during the phases of bone healing (Fig. 1). In this study, we review the vital role of the immune cells and their interactions with bone cells and MSCs (Fig. 2) and how this would affect the outcome of fracture healing.

Inflammatory phase

An early event of the injury of bone is the interruption of blood supply and platelet aggregation with the release of platelet-derived pro-inflammatory cytokines, Interlukin-6 (IL-6), Interlukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-α). These cytokines stimulate the homing of lymphocytes and monocyte/macrophages into the fracture site. As shown in
animal models, T- and B-lymphocytes are recruited at the fracture site after 3 days of injury and then reduced in numbers with the start of cartilaginous callus formation [14,21]. This phase also involves formation of haematoma, which traps inflammatory cells that further produce pro-inflammatory cytokines and growth factors. This haematoma is crucial and its removal causes a defective bone healing [22,23]. The main cellular events taking place during inflammatory phase are presented below.

**Clearing of damaged areas**

Initially, neutrophils arrive to the fracture site as detected in a rat model of fracture [24]. Neutrophils have an anti-septic effect and clear the damaged cells and debris [25,26]. Other cells that help to erode the damaged edges of bones are osteoclasts. Receptor activator of nuclear factor kappa-B ligand (RANKL) that is produced by activated T-lymphocytes and NK cells can induce the differentiation of the osteoclasts from monocytes [27–29]. A role
of B-lymphocytes during inflammatory phase of bone healing has been also linked to osteoclast formation [30]; but this role seems to be tightly controlled as B-lymphocytes can also suppress the osteoclast generation and enhance the apoptosis of osteoclasts [31]. In addition to immune cells and osteoclasts, recruited MSCs into fracture site could be involved in the clearing of dead tissues at the fracture site by phagocytosis of apoptotic cells [32]. This is associated with enhancement of MSC osteogenesis and secretion of interleukin (IL)-8, monocyte chemoattractant protein-1, and RANTES that could stimulate the homing of T cells to the inflamed area. Together, different cells can directly or via crosstalk work early at the bone injury site to clear the debris and pave the way for healing to start.

**Migration of MSCs**

MSCs widely exist within the skeleton in the periosteum, BM and bone. Those MSCs that directly participate in the fracture healing can originate from different sources. Whether localised at the fracture site (periosteum and endosteum) or migrating to the bone injury site, MSCs were shown to have a vital activity in fracture healing [33–35].

It has been demonstrated that MSCs are recruited into the injured bone site under the influence of an inflammatory chemokine, Stromal Derived Factor-1 (SDF-1) [36]. The mechanism of SDF-dependent migration of MSCs involves the activation of alpha serine/threonine-protein kinase (AKT) and extracellular-signal-regulated kinases (ERK) signalling pathways [37,38]. Inflammatory TNF-α also mediates the invasion of MSCs into the bone-healing site [39]. Macrophage-derived chemokines, MCP-1 and monocyte inflammatory protein 1 alpha (MIP-1α) have been also linked to MSC migration to the bone healing site [40]. Recently, it has been found that chemokine; CXCL7 that produced by NK cells enhances MSC migration [41]. Overall, immune cells such as macrophages and NK cells as well as inflammatory cytokines and chemokines can act together to help the homing of MSCs into the fracture-healing site.

**Preparation for the repair phase; licensing of MSCs**

The term licensing is commonly used to describe the activation of MSCs to perform immunosuppressive functions. A group of cytokines that produced within inflammatory milieu are responsible for the licensing of MSCs and can act alone e.g. IFN-γ or in combination [42]. IFN-γ can trigger the proliferation and immunomodulatory function of MSCs via the Kynurenine-depen-
dent mechanism [43]. Likewise, TNF-α induces the proliferation and immunosuppressive function of MSCs using the NF-κB pathway [44]. Thus, NK cells and T-lymphocytes are linked to the licensing of MSCs as major sources of TNF-α that is highly expressed during both inflammatory and repair phases [45]. Together with IFN-γ and TNF-α, MSCs that are activated by IL-1 can perform immunosuppressive functions associated with the production of prostaglandin 2 (PGE2) and IL-8 [46]. Furthermore, it has been shown that IL-17 is another licensing cytokine that can enhance the immunosuppressive functions of MSCs both in vivo and in vitro [47]. In summary, multiple licensing cytokines help to programme MSCs towards immunosuppressive activity in order to control the inflammatory phase of healing.

Other inflammatory mediators and cells follow the wave of MSC ‘licencing’ cytokines to directly enhance the osteogenic potential of MSCs. Toll-like receptors (TLRs) stimulate MSC migration and osteogenic differentiation utilising NFκB-β and PI3 kinase signalling pathways [48]. Additionally, macrophages existing in the fractured bone are a source of bone morphogenetic proteins (BMPs) and Oncostatin M that enhance the proliferation and osteogenic function of MSCs [49,50]. Furthermore, activated monocytes induce the expression level of Cbfα1/Runx2 and alkaline phosphatase (ALP) by MSCs and hence drive the bone formation [51]. In contrast, a conditioned media from CD4 T-lymphocytes and not CD8 has been shown to increase the osteogenesis markers of MSCs [52]. Furthermore, innate lymphocytes cells (ILCs) that produce tissue reparative cytokines such as interleukin-22 (IL-22) [53–55] also seem to induce the osteogenic activity of MSCs. Recently, an in vitro study has shown that IL-22 can induce the osteogenic capacity of licensed BM MSCs [56]. Likewise, once MSCs are licensed, IL-17 induces osteogenesis by increase the expression of osteogenic proteins in MSCs, Cbfα1/Runx2 and collagen [14].

In conclusion, the inflammatory microenvironment delivers important signals that help the preparation of MSCs, proliferation and immunomodulation and then the osteogenesis.

**Reduction of immune cell response and the end of inflammatory phase**

The control of immune cell response is critical to reduce the inflammation and aid the switch into repair phase. Interestingly, the levels of TGF-β2 and TGF-β3 reach the peak at the end of inflammation most likely to control the immune response and finalise the inflammatory phase [57]. As mentioned above, MSCs are licensed to exert their immunosuppressive role. MSCs can induce the generation of anti-inflammatory CD4+CD25+Foxp3+ T reg lymphocytes with the production of immunosuppressive cytokine, IL-10 [58]. Besides its effect on T reg lymphocytes, MSCs directly induce the apoptosis and suppress the proliferation and functions of pro-inflammatory Th1 and Th17 subsets [59,60]. Furthermore, MSCs can decrease the function and the migration of B-lymphocytes via the down-regulation of the chemokine receptors; CXCR4, CXCR5 and CCR7 [61]. In addition to adaptive immune cells, MSCs are able to inhibit the proliferation, secretary and cytotoxicity functions of cytokine-activated NK cells [62,63] as well as inhibition of the differentiation functions of monocyte-derived dendritic cells [64].

MSCs employ these immunosuppressive effects via different soluble molecules including TGF-β, indoleamine 2,3-dioxygenase (IDO), inducible Nitric oxide synthases (iNOS), PGE2, IL-1 receptor antagonist and Tumour necrosis factor-inducible gene 6 (TSG6) [65–68]. Recently, other mechanisms of MSC-dependent immunomodulation have been described. It has been shown that MSC-derived extracellular vesicles have a strong immunosuppressive effect on T- and B-lymphocytes as well as NK cell functions [69]. Furthermore, MSCs can programme macrophages to display anti-inflammatory M2 phenotype that suppresses both innate and adaptive immune responses via IL-10 and TGF-β dependent mechanisms [70]. Collectively, MSCs, which licensed by inflammatory signals act in turn to suppress the inflammatory responses of immune cells as a negative feedback mechanism. This mechanism helps the ignition of repair phase of bone healing. Nevertheless, the effects of some immune cells and cytokines continue to have a role during the repair and remodelling phases.

**Repair phase**

The repair phase involves the differentiation of MSCs into either osteoblasts when the broken bone edges are immaculately aligned (primary healing) [71] or chondroblasts that proliferate forming a cartilaginous structure called soft callus (secondary healing). The soft callus is then mineralised and converted into bone callus with irregularly arranged (woven) bone, which is invaded by new blood vessels in a process called endochondral ossification. In addition to the differentiation function, MSCs support new blood vessel formation via metalloproteinase-dependent mechanisms [72].

Certain immune cells are known to participate in the repair phase. Macrophages participate in the induction of angiogenesis
and a substantial reduction in macrophages is associated with impaired vascularisation and delayed formation of callus as revealed in CCR2−/− mice model [73]. Bone-lining macrophages participate in the intramembranous bone healing as shown in mouse model of tibial fracture [74]. Furthermore, macrophages can regulate MSC differentiation into osteoblasts, as mentioned in previous section [75]. Macrophages have been detected in invading vessels throughout the ossification of mouse long bones [76] and they efficiently produce matrix metalloproteinases (MMPs) to degrade the cartilage matrix [77,78]. These MMPs have a central role in soft-to-hard callus switch [79–81] and any dysregulation of these enzyme activities has been linked to the fracture non-union [82]. The deposition of collagen type I is another function for macrophages and this is associated with up regulation of macrophage macrosialin protein [74]. The multifunction of macrophages highlights their unique importance during the repair phase of either primary healing or endochondral ossification.

Other immune cells also reappear during the mineralisation of cartilaginous callus. This includes T- and B-lymphocytes that were found to be located in a close contact with osteoblasts and osteoclasts [83]. Both types of adaptive lymphocytes produce TNF-α, which trigger the death of mature chondrocytes aiding the transition from cartilage into bone [84,85]. Importantly, the effect of TNF-α on chondrocytes involves up-regulation of MMPs and angiopoietin coordinating both of angiogenesis and ossification of soft callus [86]. IL-17 is another cytokine that can affect the conversion of soft callus into hard callus. IL-17 can inhibit the chondrogenic differentiation of MSCs via the suppression of a key chondrogenesis transcriptional factor, SRY-box 9 (SOX9) and its activator cAMP-dependent protein kinase (PKA) [87]. Additionally, an in vitro work showed that IL-17 also enhances the MSC differentiation into osteoblasts [88]. This all indicates that adaptive lymphocytes can actively participate in the endochondral ossification.

Several growth factors are needed to support bone healing particularly during the repair phase including platelet-derived growth factor (PDGF), TGF-β, Insulin-like Growth Factor (IGF), fibroblast growth factor-1 (FGF-1) and BMPs that promote the proliferation and the chondrogenic differentiation of MSCs as well as deposition of collagen [49,89,90]. Overall, the conversion of soft callus into hard callus is highly controlled by macrophages, T- and B-lymphocytes and various cytokines and growth factors demonstrating the continuation of immune-bone interactions even after the end of the infammation phase.

Remodelling phase

The remodelling of woven bone into normal lamellar bone is related to the balance between osteoblast and osteoclast functions. The osteoblast/osteoclast function is controlled by MSCs, macrophages and cytokines such as TNF-α and IL-17. As mentioned above, the osteoblast formation from MSCs is influenced by various growth factors such as TGF-β family members, BMPs and IGF [91]. However, MSCs have an inhibitory effect on monocyte differentiation into osteoclast via the production of Osteoprotegerin (OPG) [92]. In contrast, RANKL and M-CSF secreted by osteoclasts can improve the survival and the function of osteoclasts [93,94]. Macrophages seem to maintain the bone forming/resorption balance by augmentation of the osteoblast activity and as being the progenitors of osteoclasts [95]. Osteal macrophages are also responsible for coordinating the crosstalk between osteoblasts and osteoclasts [96]. Together, MSCs and macrophages seem to have contrasting effects on osteoclasts to maintain the balance during the remodelling.

The role of IL-17 in the remodelling of hard callus indicates a possible involvement of T-lymphocytes during this phase. The downstream effect of IL-17 on the osteoblasts includes the up-regulation of the osteogenic mediators, bone sialoprotein, collagen and osteocalcin [14]. At the same time, IL-17 enhances the expression of RANKL on MSCs enhancing the osteoclastogenesis when co-cultured with peripheral blood mononuclear cells (PBMCs) [97]. Another cytokine, TNF-α produced by MSCs and osteoblasts during late phase of bone healing [45] can also influence both osteoblasts and osteoclasts functions demonstrating its vital role in the remodelling phase [98,99]. Overall, specific immune cells and mediators keep the bone healing process under control till the end.

The uncontrolled immune cell response and defective bone healing

Systemic inflammatory diseases and local sepsis at the bone injury site are linked to complicated healing including non-union [100]. MSCs extracted from non-union tissues have an impaired proliferative capacity and function compared to healthy controls [101–104]. Nevertheless, these non-union MSCs retain their osteogenic differentiation when activated in vitro [103,105]. This indicates that the healing microenvironment including the effect of immune response could be the main biological player in fracture non-union.

An exaggerated activation of neutrophils using oxygen free radicals is associated with defective healing of bone fracture [106], while an induced neutropenia in animal models of bone defects shows an enhanced osteogenic repair [107]. Likewise, excessive stimulation of macrophages with lipopolysaccharide can decrease their production of BMP-2 causing a delayed bone healing [108]. Additionally, within a chronic inflammatory milieu, monocytes have a higher potential to differentiate into osteoclasts through TNF-α dependent mechanism [109]. Excessively activated NK cells could mediate cytotoxicity against allogeneic or autologous MSCs [62,110–113]. Both Activated T-lymphocytes and B-lymphocytes are well known to release RANKL, which boosts osteoclast differentiation from their progenitors and subsequently provoke the bone lysis [94,114]. Totally, although critical at the early phase of bone healing, excess activation of immune cells has a strong link to defective bone formation.

Several studies have proven that inflammatory cytokines IFN-γ and TNF-α can block the osteogenic differentiation of MSCs [115,116]. TNF-α also works to enhance the expression of Wnt signalling pathway antagonist, Dickkopf-1 (DKK-1) that has an inhibitory effect on the osteoblast formation [117] and inhibits nephronecin, an extracellular matrix protein, which helps the proliferation of osteoblasts [118]. Furthermore, TNF-α stimulates the production of M-CSF by MSCs that in turn, induce the differentiation of osteoclast progenitors [119]. Similarly, IFN-γ has a positive influence on the osteoclastogenesis [120]. Furthermore, IL-1 and IL-17 has been linked to the bone loss within highly inflammatory milieu [121,122]. Altogether, this shows that excess or prolonged inflammation via immune cells or cytokines can be involved in impaired bone healing. Although cytokines such as IFN-γ and TNF-α are key players for MSC licensing, they can exert a negative effect on osteogenic differentiation of MSCs. Therefore, it is vital to determine the exact timings and levels needed of these cytokines to ensure the correct balance between their actions favouring bone healing.

What is unknown?

Despite all the research advances in the osteoimmunology field, more knowledge about cellular interactions during bone healing...
still required. The immune cell-MSC cross talk is essential to complete the inflammatory phase and to initiate the repair phase. However, it is still remaining to reveal if there is a link between iNOS and neutrophils as both cells can be detected early during the inflammation. Furthermore, the in vitro studies indicate complicated interactions between NK cells and MSCs, i.e. NK cells functions can be suppressed by MSCs and NK cells can participate in MSC licensing, but also can kill MSCs [62,63,110–113]. Thus, further research is necessary to understand the biological importance of these interactions NK cells during physiological fracture healing and how this would affect the cell therapy. Also, it will be interesting to locate and identify the functions of innate immune cells, ILCs within healing bone tissues. Similarly, although L-17 are linked to regulation osteoblast and osteoclast activity [123], Th17 cell location in the final stage of the bone healing remains to be investigated. Also, CD4 and CD8 T-lymphocytes seem to have contrasting effects on bone healing. The impact of the variable effects of these subsets on the MSC osteogenic capacity and the exact molecular mechanisms underlying these effects and the time of their participation are not clear yet. All this knowledge will help significantly to improve the therapeutic strategies of complicated fractures.

Therapeutic implications

The use of cell-therapy for non-union of fractures is a promising alternative to conventional bone autograft. According to the diamond concept [12,124–127], the biological elements of these therapies should involve MSCs and growth mediators including those promoting the new vasculature formation. Whether MSCs are delivered within concentrated or not-concentrated mononuclear bone marrow cells [128,129] or as culture-expanded pure population and loaded on matrices [130] or injected subcutaneously [131], their therapeutic use still needs further optimisation. The inflammation status could affect considerably the effectiveness of the regenerative treatments at least in part via their effect on MSCs [132]. Also, the revascularisation of bone graft or matrices are vital to maintain the survival and function within the healing milieu via supplying the nutrition, oxygen supply and regulatory mediators.

Several studies, in which MSCs were used for bone and cartilage repair, have indicated that the failure of therapeutic effect of allogeneic MSCs was associated with signs of activated immune responses [115,133–135]. However, in other studies where the allogeneic MSCs were loaded into scaffolds, an inflammatory response was similar to that induced by autologous MSCs with better outcomes [136–141]. This could be related to limited accessibility of allogeneic MSCs to host immune cells. For autologous MSCs, in vitro studies have shown that NK cells can lyse autologous MSCs similar to allogeneic MSCs [142]. The NK-cell mediated killing of autologous MSCs is related to the low expression of HLA-I molecules and high expression of NK cell activating ligands on the MSC surface [62,110–113]. Thus, it is remaining to investigate if under certain conditions, this mechanism could threaten the fate of the transplanted MSCs for fracture healing. Overall, this clearly indicates how immune response and inflammatory milieu can greatly affect the activities of both allogeneic and autologous MSCs. Also, the choice of the intervention therapy time in relation to inflammation status is an essential challenge to be addressed.

The in vitro polarisation of macrophages into either pro-inflammatory M1 or anti-inflammatory M2 that are also involved in tissue repair and angiogenesis, becomes possible via differentiation of peripheral blood monocytes utilising specific cytokines [143,144]. Importantly, it has been shown that both subsets together highly support the angiogenesis. This role is mediated via M1 cells that produce the angiogenesis prompting factors, VEGF, IL-8, bFGF and RANTES. Additionally, M2 cells have been proposed to enhance the blood vessel fusion, vascular remodelling and regulation of M1 activity [145]. These findings had a great potential to be applied in the regenerative bone therapies to fabricate scaffolds that help to polarise macrophages and consequently supporting blood vessel formation during bone healing [145] together with promoting the proliferation and polarisation of bone progenitors cells to achieve a completed healing process.

Conclusions

Bone healing constitutes a successive process with three phases starting with critical inflammation; in which both innate and adaptive immune cells as well as cells of macrophage-osteoclast lineage help the removal of bone debris, antisepsis and preparation of MSCs for next repair phase. In turn, licensed MSCs work to control the inflammatory phase and differentiate directly into osteoblasts or most commonly into chondroblasts forming soft callus. During this repair phase, certain immune cells and mediators play an important role to convert soft callus into hard callus and formation of new blood vessels. Finally, bone remodelling is mediated via interplay between osteoblasts and osteoclasts under influence of MSCs, macrophages and probably Th17 lymphocytes. The excess activation of the immune mediators can inhibit the osteogenic differentiation of MSCs. Thus a delicate balance between the functions of immune cells, MSCs and bone cells are critical for healthy bone healing. The therapeutic use of MSCs for bone loss and fractures should consider enhancing the bone forming capacity of MSCs as well as microenvironment particularly the inflammation status. Additionally, a new generation of biomaterials is needed to help the delivery of the appropriate type and concentration of growth factors/cytokines enhancing both osteogenesis and angiogenesis. The developed knowledge about details of cellular interactions during the bone healing will help to improve the outcomes of MSC-based therapy used for complicated bone healing.

References


