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Macrophage Polarization and Bone Formation: A review

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Abstract The contribution of inflammation to bone loss is well documented in arthritis and other diseases with an emphasis on how inflammatory cytokines promote osteoclastogenesis. Macrophages are the major producers of cytokines in inflammation, and the factors they produce depend upon their activation state or polarization. In recent years, it has become apparent that macrophages are also capable of interacting with osteoblasts and their mesenchymal precursors. This interaction provides growth and differentiation factors from one cell that act on the other and visa versa—a concept akin to the requirement for a feeder layer to grow hemopoietic cells or the coupling that occurs between osteoblasts and osteoclasts to maintain bone homeostasis. Alternatively, activated macrophages are the most likely candidates to promote bone formation and have also been implicated in the tissue repair process in other tissues. In bone, a number of factors, including oncostatin M, have been shown to promote osteoblast formation both in vitro and in vivo. This review discusses the different cell types involved, cellular mediators, and how this can be used to direct new bone anabolic approaches.

Keywords Osteoblast · Mesenchymal stem cells · Macrophage · Polarization · Oncostatin M · STAT3

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Introduction

Monocytes and macrophages are heterogeneous population of cells that can switch their phenotypic and functional properties in response to signals from their microenvironment during normal homeostasis and in disease. The activation state, or polarization, of the macrophage is determined by numerous factors including tissue location and the cells, cytokines, and other mediators it encounters. In terms of bone, the pro-inflammatory properties of macrophages are often reported in the context of arthritic joint destruction; however, there is an emerging body of literature implicating macrophages in the accrual of bone mass and as vital modulators of the tissue repair process in bone. This review will focus on how macrophage polarization affects osteoblast fate during normal homeostasis and in disease.

Macrophage Polarisation

Over 100 years, since Elie Metchnikoff described these cells of phagocytic ability, the world of macrophage biology has seen an ever-growing diversity in their types and functions [1]. In the 1960s, macrophages were being given more diverse properties based on classical activation; antigen-dependent, but non-specific enhanced, microbicidal activity, by encountering bacillus Calmette-Guerin (BCG), and Listeria [2]. By the early 1990s, the concept that there could be both classically and alternatively activated macrophages was supported by the findings of Stein and colleagues showing that IL-4 treatment induced inflammatory macrophages to adopt an alternative activation phenotype, distinct from that induced by IFN-γ, characterized by a high capacity for endocytic clearance of mannosylated ligands, enhanced MHC class II antigen expression, and reduced pro-inflammatory cytokine secretion

[3]. Ultimately leading to the adoption of the M1/M2 terminology following the observation of differential macrophage function in cells obtained from T helper (Th)1 or Th2 dominant mouse strains [4]. Following this nomenclature, M1 refers to 'classical' activation of macrophages by IFN-γ whereas M2 refers to 'alternative' activation of IL-4 and IL-13. There have been further additions to this scheme including the addition of toll-like receptor ligands such as lipopolysaccharide (LPS) or the use of GM-CSF and M-CSF as M1 and M2 differentiation factors, respectively.

In recent times, the notion of classifying macrophages as classically activated/inflammatory (M1) and alternatively activated/regenerative (M2), regardless of how many additional subtypes you might like to add, has fallen out of favor to be replaced with concept of a continuum of different activation states according to the environment that the macrophages are exposed to, and the transcription factors, cytokines, and cellular functions they exhibit. It has been proposed that three principles—the source of macrophages, definition of the activators, and a consensus collection of markers—should be used to describe macrophage activation [5]. This is supported by an increasing body of literature into the genomics of macrophage polarization [6–8]. Although the current thinking encourages a full description of the source and stimulation of the macrophage, it is important to note that M1/M2 terminology is still in wide spread use and much of the work quoted in this review will be using this wording as it was generated prior to the newly suggested guidelines.

Evidence from a number of different studies in vivo and in vitro has generally indicated that identifying the activated states of macrophages and targeting the macrophage polarization from M1 to M2 or vice versa might be served as novel diagnostic or therapeutic strategies for multiple diseases. Macrophage activation is involved in the outcome of many diseases, including metabolic diseases, allergic disorders (such as airway hyperreactivity), autoimmune diseases, cancer, and bacterial, parasitic, fungal, and viral infections. Hence, macrophage polarization plasticity has important therapeutic implications and will be discussed in the context of bone disorders.

Bone Destruction and Macrophages

In 1972, the mononuclear phagocyte system was proposed to classify macrophages, monocytes, and their precursor cells based on similarities in the morphology, function, origin, and kinetics of the phagocytes [9]. Cells of the mononuclear phagocyte series, including hematopoietic marrow cells, blood monocytes, and peritoneal macrophages, have the capacity to differentiate into bone resorbing osteoclasts placing osteoclast ontology firmly within this lineage of cells [10–13]. Myeloid-derived suppressor cells have also been described to contribute to the osteoclast precursor pool in inflammatory arthritis [14, 15]. Thus, for many years, the perceived contribution of monocyte/macrophages to bone destruction was as a source of precursors and pro-inflammatory cytokines. With the discovery of RANKL in 1998 and the advent of 'osteoimmunology', the ever-expanding links to other cell types modulating bone turnover are at an all time high [16]. With this in mind, it is important not to just perceive the monocyte/macrophage lineage as a forerunner to the osteoclast but additionally as vital modulators of bone homeostasis in their own right.

A great deal of what is known about osteoclastogenesis and the immune system has arisen from the study of disease, particularly rheumatoid arthritis (RA) [17]. In RA, synovial fibroblast proliferation is accompanied by extensive neovascularization and perivascular and interstitial infiltration of the synovium with lymphocytes, plasma cells, and activated macrophages [18]. The success of anti-TNF therapy for RA was underpinned by research showing that monocyte/macrophages are producing TNF in response to the cells and cytokines in the arthritic joint [19, 20]. TNF and other proinflammatory cytokines have been shown to promote osteoclastogenesis directly by increasing precursor numbers and/or differentiation, as well as indirectly via osteoblasts and other stromal cells to increase RANKL production [21–23]. Classical macrophage activation is associated with high levels of these cytokines so it is tempting to ascribe pro-inflammatory macrophages as cells promoting osteoclastogenesis whilst the alternatively activated macrophages would inhibit this process. Furthermore, TNF has been described as capable of switching CD11b+F4/80+ cells (M-CSF treated murine bone marrow) from Ly6C-Gr1-M2 to Ly6C+Gr1-CD11c+ and Ly6C-Gr1-CD11c+M1 cells. Pretreatment of the M-CSF-treated murine bone marrow with TNF to increase the number of osteoclast precursors led to increased the numbers of osteoclasts from both the Ly6C+Gr1- and Ly6C-Gr1- groups suggesting that the role of TNF is to expand osteoclast precursors by switching the differentiation of M-CSF-induced M2 to M1 macrophages with enhanced osteoclast forming potential [24].

Orthodontic tooth movement (OTM) is associated with inflammatory bone remodeling. Forced tooth movement increased M1-like macrophage polarization as determined by increased expression of TNFa and iNOS. The distance of OTM, the number of TRAP-positive osteoclasts and CD68+ macrophages, and the expression of TNF-α and iNOS were increased by exogenous TNF addition whilst anti-TNF reduced these features suggesting that M1-like macrophage polarization promotes alveolar bone resorption to allow tooth movement [25]. Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a complication observed following high dose of zoledronate for the prevention of osteolytic bone lesions in breast and prostate cancer patients and multiple myeloma.

Zhang et al. demonstrated that elevated IL-17 expression correlated with an increased M1/M2 macrophage ratio at the local mucosal tissue of non-healing extraction socket of BRONJ patients and in a murine model of the disease. In the mice, blocking of IL-17 activity reversed the alteration in M1/M2 macrophages ratio and incidence of BRONJ. Adoptive transfer of M2 macrophages (bone marrow in M-CSF for 6 days followed by 48 h of IL-4) decreased serum IL-17 and incidence of BRONJ [26]. Both of these studies support the notion of the M1 macrophage being associated with bone destruction and the M2 with tissue repair.

Bone Formation and Macrophages

A characteristic feature of arthritic disorders is focal erosions in articular bone. This is broadly attributed to an inflammatory cytokine driven increase in osteoclast formation with a concomitant decrease in osteoblast activity. In the K/BxN model of serum transfer arthritis, osteoblasts present at site of inflammation lacked markers of maturation and less mineralized bone was formed at bone surfaces adjacent to inflammation compared to surfaces adjacent to normal bone marrow [27]. In this system, the resolution of inflammation restored osteoblast differentiation and function [28]. In the clinical setting, the RA joint is associated with extensive osteoclastic bone destruction in the absence of bone repair as well as generalized osteoporosis [18, 29]. As such, it could be thought that inflammation inhibits bone formation yet other forms of arthritis such as ankylosing spondylitis (AS) show both bone erosions in the joints and excessive bone formation at the enthesis, where tendons and ligaments insert into the bone.

Enthesitis, inflammation of the enthuses, occurs in spondyloarthritides (SpA), including AS, but enthesitis can also be associated with endocrinological, metabolic, traumatic, and degenerative conditions [30]. In this situation, new bone formation occurs at the site where inflammation has been. Animal models of AS have shown that inhibition of TNF did not affect the severity and incidence of joint ankylosis suggesting that the process of entheseal ankylosis may be independent of TNF [31]. Evidence from genetic studies, in vitro models, human expression studies, and animal models supports a central role of the IL-23/IL-17 axis in the pathogenesis of SpA [32, 33]. Sherlock and colleagues recently described the essential role of IL-23 in enthesitis by acting on IL-23 receptor (IL-23R)(+), RAR-related orphan receptor gammat (ROR-gammat)(+)CD3(+)CD4(-)CD8(-), stem cell antigen 1 (Sca1)(+) entheseal resident T cells to produce IL-22, and activate signal transducer and activator of transcription 3 (STAT3)-dependent osteoblast-mediated bone remodeling thus leading to entheseal bone formation [34].

Could there also be a role for monocyte/macrophages in promoting bone formation? Conceptually, this would have parallels to the coupling of osteoblasts and osteoclasts whereby factors and cell surface markers on one cell promote the formation and/or activation of the other [35]. Early studies gave some initial clues reporting enhanced osteogenic differentiation and growth of marrow stromal cells or calvaria osteoblasts co-cultured with monocyte/macrophage lineage cells as evidenced by increased alkaline phosphatase activity and collagen I synthesis [36, 37]. These reports also highlighted the proximity of macrophage lineage cells to bone cells in vivo and a role for monocyte/macrophage-derived osteoinductive soluble factors such BMP2 in osteoblast survival and differentiation [38].

In arthritic disorders, studies into any potential contribution of the macrophages to bone formation are overshadowed by the predominant role that inflammatory macrophages play in the clinical features of the disease. In 2008, Chang et al. described a discrete population of resident macrophages, OsteoMacs, intercalated throughout murine, and human osteal tissues. The removal of OsteoMacs from calvarial osteoblast preparations led to decreased in bone nodule formation in vitro. In vivo, macrophage depletion using the macrophage-Fas-induced apoptosis (MAFIA) mouse caused complete loss of the osteoblast bone-forming surface indicating a vital role of macrophages in osteoblast survival and function [39]. Efficient fracture repair relies on early inflammation with the recruitment of monocyte macrophages to the fracture site [40]. Osteomacs have been described as critical mediators of endochondral and intramembranous bone healing in murine models of bone injury [41, 42]. Osteomacs were in direct contact with matrix-producing and mineralising osteoblasts and were distinct from infiltrating inflammatory macrophages as characterised by high expression of Mac2. Depletion of osteomacs significantly suppressed new bone formation whereas specifically expanding osteomacs, but not their $Mac2^{high}$ inflammatory counterparts, resulted in a significant increase in new mineralised matrix [41]. In a murine femoral fracture model, IHC demonstrated that inflammatory macrophages (F4/80(+)Mac-2(+)) were localized with initiating chondrification centers and persisted within granulation tissue at the expanding soft callus front. Resident macrophages (F4/80(+)Mac-2(neg)), including osteal macrophages, were predominated in the maturing hard callus. Ablation of macrophages using the MAFIA mice abolished or reduced callus formation supporting the conclusion that inflammatory macrophages were required for initiation of fracture repair. The exact contribution of both inflammatory and resident macrophages to anabolic bone repair and the factors they produce remain to be elucidated [42].

Osteonecrosis (ON) is another example of inflammatory bone loss. Experimentally, injection of methylprednisolone in mice led to the infiltration of M1 macrophages and expression of TNF in the necrotic zone during the early stages of disease progression. TNF levels gradually decreased and a

larger M2 cell population presented in the necrotic zone in the late stage of ON. At this late stage, histologic findings of appositional new bone formation around the necrotic bone suggested that M2 macrophages could be beneficial for resolving inflammation and promoting tissue repair [43]. It is evident from these studies that cell–cell interactions between macrophages and osteoblasts and their progenitors are critical for bone formation; however, the osteogenic factors derived from either cell type that are involved these interactions remained less clear.

Macrophage-derived Factors in Osteoblast Differentiation

Using an in vitro human system, we showed that monocytes and macrophages could promote the osteogenic differentiation of bone marrow derived mesenchymal stem cells (MSC), the precursor of the osteoblast. This process required direct cell–cell contact leading to the production of a soluble factor and was dependent on prostaglandin E2 (PGE2) and cyclooxygenase 2 (COX2). This soluble factor was shown to induce STAT3 phosphorylation and was identified as oncostatin M (OSM) [44] (Fig. 1). Another study by Guihard et al. showed that monocyte/macrophages activated via LPS or endogenous ligands similarly induced osteoblast formation from MSC due to the production of OSM. The authors found that classically activated inflammatory M1 and not M2 macrophages were responsible for OSM production via a COX2 and PGE2 regulatory loop. Two other gp130 cytokines, IL-6 and leukemia inhibitory factor, were induced in this system and showed

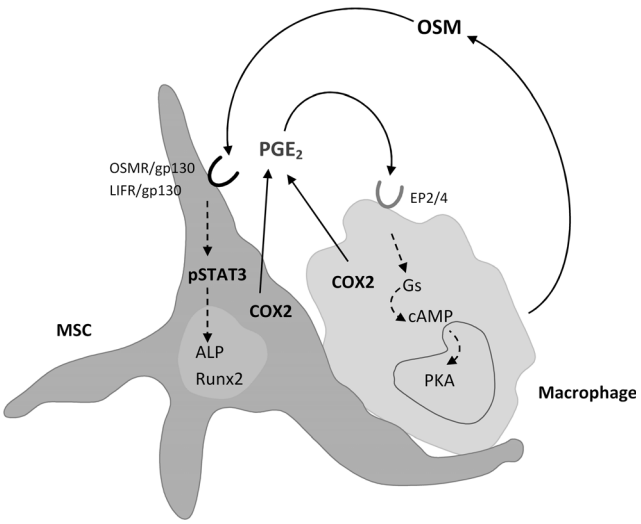

Fig. 1 Cell–cell contact between MSCs and macrophages results in the production of PGE_2 and acting via the EP2/4 receptors on the macrophages to induce OSM production. OSM acts via the OSM and LIF receptors on the MSC to activate STAT3 phosphorylation and switch on a program of osteoblast differentiation genes. STAT3 signaling also leads to the upregulation of the receptors for OSM to amplify its effects

similar effect on MSC differentiation [45]. A third study also showed OSM-driven osteoblast formation from MSC using conditioned media of macrophages derived from cord blood. These macrophage populations were then treated with M-CSF plus IL-4 (to induce alternative activation) or with GM-CSF, IFN-γ, and LPS to represent classical activation. In this case, conditioned media from IL-4-treated macrophages stimulated osteoblastic maturation in MSC, whilst the classically-activated macrophages did not [46]. This leads to a conflict between these two studies although work from our own laboratory is in favor of supporting a role for alternatively activated macrophages as the most potent inducers of osteoblast formation.

Monocytes and macrophages are known producers of OSM [47] which has been shown to increase osteogenic differentiation and mineralisation both in vitro and in vivo. Transgenic mice overexpressing OSM develop osteopetrotic bones and enlarged hind limbs [48], whilst directed OSM expression in mouse knee joints stimulated periosteal bone formation [49]. We, and others, have also shown that injection of OSM over calvarie of 5-week-old male C57BL/6 mice leads to an increase in calvarial thickness, mineral apposition rate, mineralizing surface/bone surface, and bone formation rate/bone surface [44, 50]. OSM regulates osteoblast differentiation through rapidly inducing the transcription factors C/EBPδ and C/EBPβ, and subsequent activation of transcription factor Runx2 but also by strongly inhibiting expression of sclerostin, an osteocyte-derived mineralisation inhibitor [50]. In addition, OSM can activate STAT3 signaling in osteoblasts [44, 51] leading to increased ALP activity which can be abrogated by both tyrosine and threonine/serine kinase inhibitors [52] and overexpression of a STAT3 dominant negative in MSC [44]. OSM signaling through STAT3 has also been shown to directly target Wnt5a [53, 54] that promotes osteogenic differentiation of MSCs. Furthermore, the activation of STAT3 by OSM can induce expression of c-Fos [55]. All these studies provide evidence of the possible mechanisms by which monocytes induce MSC osteogenic differentiation through OSM.

As previously discussed, an early phase of inflammation is associated with fracture repair. In a murine tibial injury model of intramembranous bone formation, OSM was expressed during this inflammatory phase and the depletion of macrophages repressed OSM expression. OSM deficient mice showed reduced STAT3 activation during the hematoma stage of repair leading to a significant reduction in the amount of new intramedullar woven bone at the injured site [56]. The exact contribution of inflammation, as opposed to mechanical destabilization, to the pathogenesis of osteoarthritis and the formation of osteophytes are undecided. OSM, in combination with TNF, has formerly been shown to stimulate cartilage degradation via matrix metalloproteinase-13 [57]. In a recent study, OSM was higher in fluid and tissue from 32 patients

with knee OA compared with the controls. In vitro, OSM increased osteoblast proliferation and differentiation via a downregulation of Notch signaling molecules [58]. Whilst not providing direct evidence, it is tempting to speculate a role for OSM in osteophyte formation however this remains to be proven.

Much of the work investigating MSC interactions with monocytes and the factors involved has been due to the immunomodulatory properties of MSC. Macrophages co-cultured with MSCs showed an increased expression of CD206, increased production of IL-10 and IL-12p40, and reduced production of TNFα, IL-6, and IL-12p70 [59–61]. MSC inhibited the upregulation of CD86 and MHC class II in LPS-stimulated macrophages impairing their ability to activate antigen-specific CD4+ T cells whilst increasing their phagocytic capacity [60]. These studies show that MSCs can polarise macrophages into a phenotype resembling alternatively activated macrophages, an environment that on balance appears to be in accord with these macrophages promoting bone formation and tissue repair (Fig. 2). PGE_2 has been identified as a major factor involved in the immunomodulatory properties of MSCs [59–61]. Recent in vivo studies using scaffolds impregnated with MSC to promote bone formation have shown that MSCs induce mobilization of macrophages and induce their functional switch from pro-inflammatory to an alternatively activated phenotype via PGE_2 production. Subsequently, there is the formation of a bone regenerative niche through the recruitment of endothelial and osteogenic precursors from the bone marrow [62]. PGE_2 has been reported to have many important roles in bone including osteoclast and osteoblast formation and function, bone mechanotransduction, and repair [63, 64]. It has been demonstrated in bone fracture sites that infiltrating macrophages have elevated expression of COX2 and that this is required for bone repair [65]. PGE_2 has been shown to directly promote osteoblast differentiation [66–68] but can also induce OSM production in monocytes and macrophages [69], which could suggest that COX2 expressing macrophages at fracture sites are critical for bone repair at least in part due to their production of OSM.

However, OSM is not the only STAT3 activating factor, and activation of STAT3 is not the only signaling pathway associated with osteoblast differentiation [70, 71]. We have shown that constitutive activation of STAT3 enhances osteogenesis of MSCs accompanied by upregulation of ALP and RUNX2 as well as downregulation of Dickkopf homolog 1 (DDK1). In addition, constitutively active STAT3 induced the expression of the OSM receptor (OSMR) and leukemia inhibitory factor receptor (LIFR) making osteoblast progenitors more responsive to OSM [44, 50]. Bone formation by entheseal resident T cells depends on IL-22 production [34], and in other systems, MSC/macrophage interaction is mediated by IL-10 production [61]—both of these cytokines lead to signaling via STAT3. The interleukin-6 (IL-6) family cytokines, of which OSM is a member, act via gp130 and stimulate STAT3 phosphorylation. There was a profound reduction in trabecular bone mass when gp130 was deleted in the entire osteoblast lineage (Osx1Cre gp130 f/f) and also when this deletion is restricted to osteocytes (DMP1Cre gp130 f/f) [72].

Cyclic AMP-signaling via PGE_2, as well as SMAD signaling via BMP-2, results in osteoblast differentiation [73]. Whilst there is a documented role for PGE_2 in both macrophage polarization and in osteoblastogenesis, there is scant direct evidence for BMP2 in conjunction with macrophages

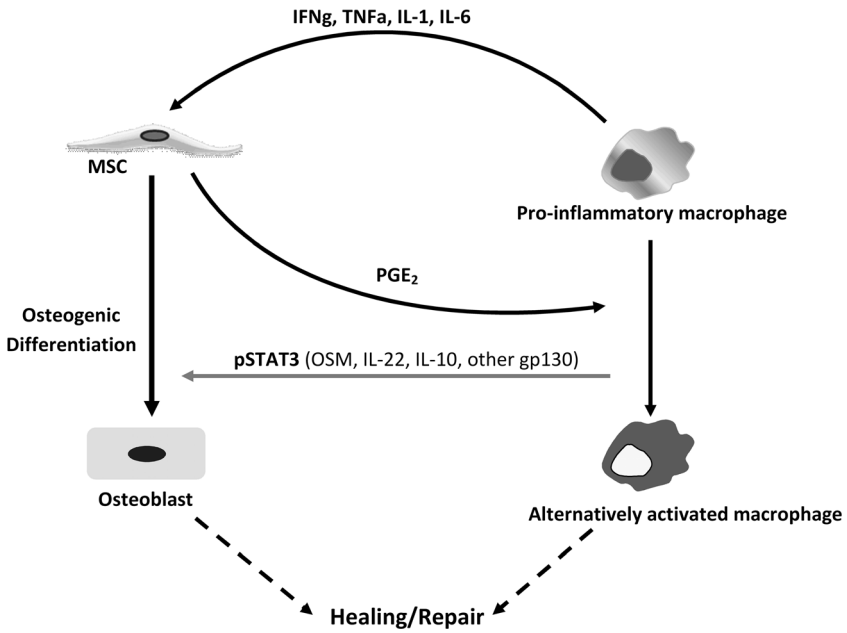

Fig. 2 MSCs are activated by pro-inflammatory mediators such as IFNγ to exert their immunoregulatory abilities including macrophage polarisation towards an alternatively activated phenotype. PGE_2 has been shown to be involved in this process. In turn, OSM from the macrophage induces STAT3 phosphorylation and promotes osteoblast differentiation from MSC, and thus, inflammation is dampened and the tissue repair process is initiated

beyond the ability of PGE_2, acting via the EP2 and EP4 receptors on osteoblast precursors, to induce BMP production. To date, most of the Wnt signaling interactions in bone have focused on osteoblast–osteoclast crosstalk, and there is little literature on the role of polarized macrophages in this signaling pathway.

Conclusions

The world of macrophage biology has entered into an exciting phase, and their contribution to both bone formation and destruction is a growing field of research. The advent of the genomic era has provided us with more insight than ever as to the diversity of macrophage activation states in both normal homeostasis and in disease [5, 8]. Macrophages are an integral part of bone tissue that regulate normal osteoblast differentiation from mesenchymal progenitors and bone formation [39, 74]. During inflammation, osteoblast precursors encounter pro-inflammatory macrophages that one might predict would inhibit bone formation. However, the resolution of inflammation and subsequent tissue repair process is a tightly regulated. MSC, as osteoblast precursors, has been reported to induce a switch for a pro-inflammatory phenotype to an alternatively activated macrophage phenotype, and the weight of evidence to date supports a role for these cells in inducing osteoblast formation to promote bone tissue repair. OSM is the most documented of the macrophage-derived factors that promote this process but there are certainly more to be discovered in the coming years. It is the clues from these investigations that will direct the next generation of bone anabolic therapies.

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