Role of bone marrow macrophages in controlling homeostasis and repair in bone and bone marrow niches

Title: Role of bone marrow macrophages in controlling homeostasis and repair in bone and bone marrow niches

Author: Simranpreet Kaur Liza Jane Raggatt Lena Batoon David Arthur Hume Jean-Pierre Levesque Allison Robyn Pettit

PII: S1084-9521(16)30247-6
DOI: http://dx.doi.org/doi:10.1016/j.semcdb.2016.08.009
Reference: YSCDB 2110

To appear in: *Seminars in Cell & Developmental Biology*

Received date: 24-6-2016
Revised date: 9-8-2016
Accepted date: 9-8-2016

Please cite this article as: Kaur Simranpreet, Raggatt Liza Jane, Batoon Lena, Hume David Arthur, Levesque Jean-Pierre, Pettit Allison Robyn.Role of bone marrow macrophages in controlling homeostasis and repair in bone and bone marrow niches.*Seminars in Cell and Developmental Biology* http://dx.doi.org/10.1016/j.semcdb.2016.08.009

Role of bone marrow macrophages in controlling homeostasis and repair in bone and bone marrow niches

AUTHORS: Simranpreet Kaur[a], Liza Jane Raggatt[a], Lena Batoon[a], David Arthur Hume[b], Jean-Pierre Levesque[a] and Allison Robyn Pettit[a].

AFFILIATIONS:

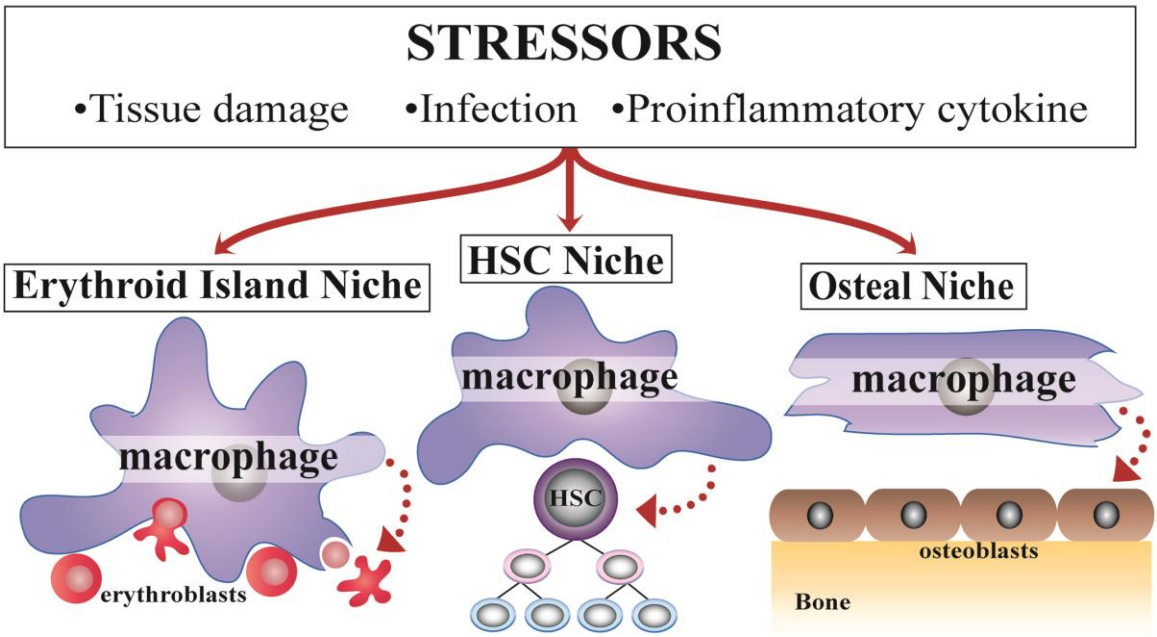
[a] Blood and Bone Diseases Program, Mater Research Institute - The University of Queensland, Faculty of Medicine and Biomedical Sciences, Translational Research Institute, 37 Kent Street, Woolloongabba, 4102, Australia.

[b] The Roslin Institute and Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Roslin, Midlothian EH25 9PS, Scotland, United Kingdom.

Author Email addresses: SK, simranpreet.Kaur@mater.uq.edu.au; LJR, liza-jane.raggatt@mater.uq.edu.au; LB, lena.batoon@mater.uq.edu.au; DAH, david.hume@roslin.ed.ac.uk; JPL, jp.levesque@mater.uq.edu.au; and ARP, allison.pettit@mater.uq.edu.au

CORRESPONDING AUTHOR: Allison R Pettit. Mater Research Institute-The University of Queensland, Translational Research Institute, 37 Kent St, Woolloongabba, QLD, 4102, Australia. Phone: +6173443 7575; Fax: +6173163 2550; and Email: allison.pettit@mater.uq.edu.au

Graphical Abstract



Abstract

Macrophages, named for their phagocytic ability, participate in homeostasis, tissue regeneration and inflammatory responses. Bone and adjacent marrow contain multiple functionally unique resident tissue macrophage subsets which maintain and regulate anatomically distinct niche environments within these interconnected Tissues. Three bone-bone marrow resident tissue macrophages have been characterised; erythroblastic island macrophages, haematopoietic stem cell niche macrophages and osteal macrophages. The role of these macrophages in controlling homeostasis and repair in bone and bone marrow niches is reviewed in detail.

Keywords: macrophages, stem cell niches, haematopoiesis, bone and bone marrow

Table of contents

1. Introduction

1.1 Interdependence of Bone and Bone Marrow

Bone marrow (BM), the principal haematopoietic organ in adults, is encased within bone, the primary function of which is to provide mechanical support and contribute to endocrine homeostasis. The bone is not just an inert site of residence for BM; they are interdependent organs that have reciprocal regulatory mechanisms throughout life [1]. Parallel functional decline in both systems is a major contributor to loss of productivity and well-being during aging [2] and disease [3, 4]. Clear understanding of the cellular and molecular mechanisms responsible for the reciprocity is lacking. Herein we will discuss accumulating evidence suggesting that macrophages are a cellular link between these organs, focusing on macrophage contributions to haematopoietic systems within the BM as well as homeostasis and repair of bone.

1.2 Resident Tissue Macrophages

Macrophages, first described by Élie Metchnikoff, form a heterogeneous population of cells with diverse and adaptive transcriptomes [5, 6]. All tissues of the body contain resident tissue macrophages, with hyaline cartilage being the possible exception. Resident tissue macrophages play significant roles in tissue/niche homeostasis, phagocytosis of cellular debris, tissue damage/injury repair, immune surveillance and inflammation resolution [7, 8]. The mononuclear phagocyte system (MPS) was proposed to encompass this collection of functionally disparate but related cells. The MPS conceptualized that in postnatal tissues macrophages are continually replenished from blood monocytes derived from BM haematopoiesis [9, 10]. Recent ontogeny studies have indicated that self-renewal of tissue macrophages also contributes to homeostasis [11-13]. Only one of these ontogeny studies has

attempted to map BM macrophage origin [11] and showed that a subset of BM macrophages can self-repopulate while ontogeny of bone resident macrophages (osteal macrophages, osteomacs) has not been investigated.

1.3 Macrophages within the Bone and Marrow Environment

BM topography is subtle and complex, involving an intricate patchwork of functionally designated microenvironments/niches [14]. The BM and bone contain multiple distinct resident macrophage populations that contribute to these specific niches and their specialised functions. The first recognised resident macrophages within BM stroma were the central macrophages within erythroblastic islands (erythroblastic island macrophages, EIM) [15]. A more recent discovery is the BM resident macrophage population involved in maintenance of hematopoietic stem cell (HSC) [16] that will be referred to here as HSC niche macrophages. Lastly, osteomacs, reside within the specialised tissues lining bone, including the endosteum which is continuous with adjacent BM [17]. See Figure 1 for schematic representation of the confirmed macrophage subsets within bone and BM. Given the large number of functional niches within BM and bone, it is likely that new resident macrophage subsets will emerge.

1.4 *In Vivo* Models of Macrophage Depletion

In vivo mouse models of macrophage depletion have been used to study functional contributions of macrophages to erythropoiesis, HSC niches as well as bone homeostasis and repair. The Mafia (**ma**crophage **F**as **i**nduced **a**poptosis) transgenic mice contain a drug-inducible *Fas* suicide gene regulated by the *c-fms* promoter [16, 18, 19]. Inducible and efficient broad spectrum macrophage depletion is achieved in many tissues (significant reductions in F4/80$^+$ cells) [20] including BM macrophages [16, 20, 21] and osteomacs [18, 21]. However depletion efficacy within specific BM macrophage subsets has not been

explicitly reported and warrants more rigorous attention given recent improvements in subset phenotyping, as detailed below. Caution is needed when using the Mafia model as the transgene is also expressed by $CD11b^+$ myeloid cells including monocytes and myeloid precursors [16, 20], osteoclasts [19], granulocytes [16, 20]and dendritic cells [16, 20, 22]. Macrophage recovery occurs rapidly if depleting agent delivery is ceased [19, 20].

Clodronate liposomes have been used extensively to deplete macrophages *in vivo* [23] including those in bone [18, 19, 21] and BM [16, 24, 25]. Internalisation of the clodronate-loaded liposome is required for apoptosis induction and consequently professional phagocytes are preferentially depleted in this model [23]. Consequently phagocytic potential of different macrophage subsets dictates depletion kinetics and sensitivity. For example, in BM EIM are particularly susceptible to clodronate liposomes [16], but granulocytes [16] and myeloid precursors are preserved. Both osteomacs and osteoclasts are efficiently depleted by clodronate liposomes [26]. An additional nuance of this model is that delivery route and delivery dose/regimen impact on the macrophage depletion specificity and sensitivity including target organ variation [21, 23]. As discussed in detail below, both the Mafia model and clodronate liposome delivery induce HSC mobilisation, implying that both approaches target HSC niche macrophages.

The CD169-diphtheria toxin (DT) receptor (DTR) mouse is a more refined *in vivo* macrophage depletion tool [27]. It is a knock in model where human DTR cDNA has been recombined into the mouse CD169 gene. Thus DTR expression is regulated by the endogenous CD169 promoter. CD169 expression is restricted to a subset of tissue macrophages and is expressed by approximately 30% of BM $F4/80^+$ cells which includes HSC niche macrophages [28], EIM [24] and osteomacs [26]. This model avoids many

undesired off-macrophage targets, including osteoclasts [26]. Other models of *in vivo* macrophage depletion have also been employed, but have yet to be rigorously characterised or broadly reproduced [29].

2. Macrophages and Erythropoiesis

2.1 Role of EIM in Regulating Erythroblastic Island Niches

Erythroblastic islands comprise a central macrophage clustering numerous erythroblasts spanning the multistep erythroid maturation process (Figure 1) [15]. EIM are essential for erythroblasts survival during their maturation to generate functional enucleated reticulocytes. The function of EIM, recently reviewed in detail [30], falls into three broad categories: a) secretion of trophic cytokines, b) iron transport [31], and c) phagocytosis and degradation of extruded nuclei [32, 33]. BM macrophages can express erythropoietin (EPO) [34, 35] the principal growth factor regulating erythropoiesis. Macrophages are also EPO responsive suggesting complex feedback loops may exist in response to EPO regulation of erythroid islands [36]. Macrophages can also express other factors that promote erythropoiesis including insulin-like growth factor-1[37, 38] and bone morphogenetic protein (BMP) 4 [39], but direct confirmation of EIM expression and necessity for erythroid island integrity is lacking. Interestingly, all of the molecules have also been implicated in bone biology with dominant pro-anabolic effects [40-42] with the latter two also implicated in HSC homeostasis [43-45]. EIM also contribute to heme synthesis and iron recycling by incorporating iron into ferritin. The ferritin is then transported to erythroblasts for the synthesis of large amounts of hemoglobin [46]. Finally, through complex adhesion interactions the EIM aid in the enucleation process and ultimately phagocytosis and degradation of the approximate 200 billion daily extruded pyrenocytes [32, 33]. Failure in this function derails erythropoiesis and leads to severe anaemia [33, 47].

The importance of these EIM niche roles was recently validated through robust and sustained *in vivo* depletion of EIM results in collapse of BM erythropoiesis with loss of all maturation stages of erythroblasts and reticulocytes while pro-erythroblasts were unaffected [24, 48]. The loss of erythrocyte production within BM did not produce peripheral anaemia, presumably through a combination of extramedullary erythropoiesis and increased erythrocyte lifespan [48]. Repeat transient macrophage depletion via intermittent treatment with clodronate liposome (engulfment causes apoptosis of macrophages) did not reduce the number of BM erythrocytes but did result in decreased hemoglobin concentration and mean hemoglobin content, supporting the important role of EIM in erythrocyte hemoglobin synthesis [49].

Phenotypic profiling of EIM has recently advanced to reveal a unique combination of typical myeloid-macrophage markers including CD11b, F4/80 (Figure 2A, circle) and CD169 [24, 48] plus the EIM-associated antigen ER-HR3 (Figure 2B, circle) [50] in combination with the traditional granulocyte antigen Ly6G [24]. They also express adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 [24, 51] which mediates erythroblast adhesion to EIM [51, 52]. The improved knowledge of EIM identity will inform more elegant approaches to elucidating EIM biology and disrupted mechanisms in blood diseases.

2.2 Role of EIM in Stress-Induced Erythropoiesis and Blood Diseases

Erythropoiesis is increased in response to circumstances that place elevated pressure and/or attrition on erythrocyte supply, including blood loss, anaemia, disease/infection, or after exposure to exogenous factors (*e.g.* recombinant EPO). These stressors often drive extramedullary blood production to rapidly expand erythropoietic capacity [49]. Recent

studies have confirmed the critical role of EIM in stress-induced erythropoiesis. Macrophage depletion either prior to or after phlebotomy-induced anaemia [48, 49], phenylhydrazine-induce haemolytic anaemia [48] or exogenous EPO driven erythropoiesis [49] significantly impaired erythropoietic recovery.

Relative expression levels of specific transcription factors (*e.g.* Gata1 and PU.1) regulate the counterbalance between erythropoiesis and myelopoiesis [45, 53], presumably to protect the HSC pool from exhaustion (discussed in section 3.1). As EIM express pattern recognition receptors and granulocyte-colony stimulating factor (G-CSF) receptors, they are well placed to adapt erythropoiesis to inflammation and infections that generally cause a high demand on myelopoiesis. For instance, G-CSF is abundantly released in the circulation following acute inflammation and infections and drives granulocyte production, maturation and survival. G-CSF also causes a profound loss of EIM in the BM resulting in a complete shut-down of medullar erythropoiesis [24]. Bacterial lipopolysaccharides (LPS) cause a similar loss of medullar EIM and erythropoiesis, an effect mediated by toll like receptor 4 (TLR4) [54].

The essential role of EIM in regulating and supporting erythropoiesis makes them a viable therapeutic target in diseases of either hyper- or hypo-erythrocyte production/deficiency. Liposome encapsulated EPO was targeted to erythroid islands via EIM phagocytosis and was more effective than systemic recombinant EPO delivery in a model of renal anaemia [55]. Conversely, depletion of EIM in mouse models of polycythaemia vera [48, 49] and β-thalassemia [49] reduced the elevated erythropoietic activity associated with these pathological conditions [49]. Therapeutic feasibility and efficacy of targeting EIM in blood diseases warrants further investigation.

3. Macrophages and Haematopoiesis

3.1 HSC Niche Regulation by Resident BM Macrophages During Homeostasis

HSC reside within the mammalian BM and by definition have both life-long self-renewal potential and the capacity to produce all cells required to replenish the blood and immune systems throughout life. Thus HSC must balance conflicting demands of self-renewal and extensive proliferation to replenish exhausted leukocytes and erythrocytes. Prolonged extensive HSC proliferation can produce progressive exhaustion of the HSC pool leading to BM failure. To meet these conflicting demands, a pool of HSC remains dormant/quiescent, acting as a back-up against progressive exhaustion [56, 57]. This store of dormant HSC can be rapidly recruited to proliferate in response to stress if required, and return to dormancy once the challenge is resolved [56]. Both intrinsic and extrinsic factors determine HSC fate decisions and it is likely that different types of niches make substantial contributions toward imposing and supporting these different fates [58, 59].

Separate niches have been defined within the BM that harbor either active HSC or dormant HSC (Figure 1). The precise location, support cell constitution and extrinsic molecular mediators controlling HSC within these two niches remain controversial [60, 61]. Enrichment of HSC has been reported within the metaphyseal endosteal arteriolar regions [60-64] or within peri-sinusoidal niches [60, 61, 65, 66]. The endosteal hypoxic areas of BM may be associated with low blood perfusion and dormant HSC [56, 58, 63, 67, 68]. Whole mount imaging of bone and BM provided additional evidence that quiescent HSC are enriched in the endosteal region adjacent to arterioles [62], while cycling/active HSC located near peri-sinusoidal niches where their greater metabolic demand is better served by higher oxygen content [59, 60, 62]. Within this hierarchy of niches (Figure 1), HSC are supported by specific stromal cells including mesenchymal stem/progenitor cells and pericytes (nestin$^+$ or

leptin receptor$^+$), early committed osteolineage progenitors (osterix$^+$ cells), non-myelinating Schwann cells, sympathetic nerves and endothelial cells [69]. Macrophages also contribute to HSC niche homeostasis [16, 28, 70, 71] and dissection of their specific niche functional contributions and molecular mediators is in its infancy [60]. All these elements coalesce to create a complex regulatory network influencing HSC quiescence, proliferation, and self-renewal [60, 61].

Macrophages are localised in HSC niche microenvironments (Figure 2) [18] and support other niche cellular components *in vitro* [16, 18, 72]. Their likely function has been assessed using *in vivo* models of macrophage depletion, including Mafia transgenic mice (contains drug-inducible *Fas* suicide gene regulated by *c-fms* promoter) [16], clodronate liposome in wild type mice [16, 28], or CD11b- and CD169-based inducible depletion mouse models [28]. Macrophage depletion disrupted endosteal HSC niche components including suppression of osteoblast lineage cells and down regulation of HSC retaining factors [16, 18]. This subsequently induced HSC niche collapse leading to HSC mobilisation from BM to blood and extramedullary lymphoid organs [16, 28]. Additionally, as discussed in Section 3.2 below, these observations were further validated in models of haematopoietic stress [16, 70].

The unique HSC niche macrophages phenotype is still uncertain. Winkler *et. al.* first demonstrated that collapse of HSC niches was instigated by depletion of a mixed population of CD11b$^+$F4/80$^+$ cells that had variable Ly6G expression [16]. While they also express CD115, major histocompatibility complex class II and CD68, expression of CD169 provided clear distinction of HSC niche macrophages from other BM myeloid populations including monocytes [28] but not necessarily from other BM resident macrophages. Subsequent studies achieved further honing of HSC niche macrophage phenotype by a process of elimination as

$CD169^{+}Ly6G^{+}$ BM macrophages were confirmed to be EIM. Dutta *et. al.* demonstrated the macrophage expression of VCAM-1 was important for retention of HSC in splenic myelopoiesis niches [73], which likely extrapolates to BM niches but expression overlaps with EIM [24]. Finally, Hur *et. al.* showed that CD234 (also known as duffy antigen receptor for chemokines, DARC) is expressed by macrophages that are in direct contact with quiescent HSC [74], although caution is needed as its expression is not myeloid restricted [75]. In summary, HSC niche macrophages of the mouse are currently defined as $CD11b^{+}F4/80^{+}Ly6G^{neg}CD169^{+}VCAM\text{-}1^{+}CD234^{+}$ BM macrophages. It is yet to be determined whether this subpopulation encompasses a phenotypically and/or functionally homogenous subset and functional confirmation of this subset as the only BM macrophage subset required for HSC niche maintenance is lacking. Given HSC mobilisation efficiency reduced concomitantly with the narrowing of the specificity of macrophage *in vivo* depletion in models [28], there is likely to be at least some redundancy, if not diversity, in HSC niche macrophages, creating resilience in the HSC niche system.

Evidence suggests that HSC niche macrophages regulate HSC via both direct [74] and indirect (i.e via other niche support cells) [16, 28, 72] actions. Hur *et. al.* have demonstrated that binding of CD234 on HSC niche macrophages stabilises surface CD82 expressed by long term (LT)-HSC residing in endosteal arteriolar regions of the BM [74] and promotes HSC quiescence. Depletion of $CD169^{+}$ macrophages, which included depletion of $CD234^{+}$ BM macrophages, decreased HSC cell surface CD82 and increased their proliferation and differentiation [74]. Independent validation of these observations is needed, especially given other niche components have also been reported to express CD234 [75].

HSC niche macrophage support of osteoblast lineage cells has also be implicated in HSC niche regulation [16] and the specific cellular and molecular mechanisms will be discussed in more detail in Section 4. Of specific relevance here, loss of p62 (also known as sequestosome-1; major role in ubiquitinated protein turnover through autophagy pathways) in osteoblasts upregulated nuclear factor (NF)-κB signalling which subsequently inhibited osteoblast differentiation and C-C motif chemokine (CCL-4) production [72]. This in turn modulated BM hematopoietic stem and progenitor cell (HSPC) chemotactic response to CXCL12 [76] and resulted in increased traffic of HSPC out of the BM [72]. Importantly, direct cell contact with macrophages sustained the required low level NF-κB activity in osteoblasts, via p62, needed to support osteoblast maintenance and homeostasis [72]. Similarly, *Chow et al*, indicated that HSC niche supportive BM macrophages provided indirect support through $Nestin^{+}$ mesenchymal stem cells with macrophage depletion significantly reducing expression of HSC retention molecule genes such as *Cxcl12, Angpt1, Kitl and Vcam1* in $Nestin^{+}$ mesenchymal stem cells coupled with reduced HSC-attracting C-X-C motif chemokine ligand (CXCL)12 expression in BM stromal cells [28]. HSC niche macrophages appear to be the primary sensors of homeostatic mechanisms that influence the size and activity of the HSC niche pool. Specifically, circadian rhythms that influence HSPC egress into the circulation are triggered by macrophage elimination of aged neutrophils in the BM [77]. This mechanism could also provide feedback to BM HSC niches to meet increased haematopoietic demand created by distant tissue injury or infection [77].

3.2 HSC Niche Maintenance by Macrophages during BM Stress

Evidence supports that HSC niche macrophages respond directly to stimuli in times of BM injury, infection or inflammation and translate these stress signals to modulate HSC niche dynamics to achieve ‘emergency hematopoiesis’. An equally important outcome is whether

HSC niche macrophages orchestrate protection of the pool of long-term repopulating HSC (LT-HSC) required for longevity of the haematopoietic system. Exposure to the pro-inflammatory factor G-CSF, which is commonly used to mobilized HSC for collection and subsequent clinical transplantation [78], substantially reduced BM [16] but not splenic [24] macrophages. This suggests that G-CSF may selectively target BM HSC niche macrophages resulting in a site-specific adaptation to this stressor. The loss of BM macrophages triggered loss of osteoblast lineage cells and concomitantly reduced HSC niche regulating factors in the BM [16] and induced HSC to proliferate [79]. This response was dependent on G-CSF receptor expression by $CD68^+$ macrophages [70]. Human monocytes and macrophages also express high levels of G-CSF receptor mRNA [80] indicating this response is unlikely to be species specific.

Chemotherapy treatment, using either cyclophosphamide [71] or 5-fluorouracil [74], appears to trigger a similar cascade of HSC niche collapse. In both cases BM macrophages and HSC transiently resist the myeloablative treatment but once BM macrophages succumb, HSC egress from BM rapidly follows [71, 74]. These observations suggest that there is a threshold of BM stress, and once reached the BM is no longer a viable environment for HSC residence and that macrophages may be a key element of the rheostat monitoring this stress threshold. Once the threshold is reached, HSC mobilisation occurs so that other suitable non-BM niches can be temporarily engrafted and extramedullary haematopoiesis initiated to sustain blood and immune cell supply. Given evolutionary pressure to sustain haematopoiesis, it is likely that multiple different mechanisms exist to protect HSC integrity. In a murine bacterial infection model, interferon-γ increased the number of BM macrophages but this was associated with a decrease in the BM HSC pool [25, 81]. It was postulated that the expanded macrophage subset, which was driven by interferon-γ, promoted HSC quiescence/dormancy

[25], similar to recent functional characterisation of CD234$^+$ BM HSC niche macrophages [75]. This may be a mechanisms to protect the LT-HSC pool in the context of systemic infection that has compromised both BM and/or other suitable extramedullary niches [81].

Following a period of increased demand for haematopoiesis, the HSC niche must recover. This process is clinically relevant in BM transplantation and during recovery from chemotherapy. F4/80$^+$ macrophages were one of the first cells to repopulate the endosteal environment following cessation of a mobilising G-CSF regimen [16], followed by expansion of osteoblast-lineage cells and recovered HSC niche factor expression [16, 71]. HSC niche reassembly is presumably a pre-requisite for recreating a permissive environment for HSC to repopulate BM post mobilization, a phenomenon that may extend to HSC transplantation settings. This possibility somewhat mimics the primordial wave where resident tissue macrophages exist in developing embryos prior to definitive haematopoiesis [12, 82]. Indeed, the fact that F4/80$^+$ macrophages are the first mature cell output of haematopoiesis and form the centre of embryonic haematopoietic islands in the foetal liver [83] is also suggestive of macrophages being a focal point for HSC niche formation. Embryonic macrophages also degrade extracellular matrix with matrix metalloproteinase (MMP)-9, aiding HSC migration from the aorta-gonad-mesonephros to haematopoietic organs [84] where they establish definitive haematopoiesis. Even though the post-transplant BM environment is vastly different to the developing embryo, BM macrophages have been reported to survive lethal doses of irradiation [11, 85] and potentially chemotherapeutic drugs [71, 74] suggesting they are at least available to contribute to reformation of HSC niches after these stressors. In fact, radiation induced injury or 16,16-dimethyl prostaglandin (PG) E2 treatment upregulated cyclooxygenase-2 (COX-2) expression in α-SMA$^+$ BM macrophages which further enhanced PGE2 production [86] and improved the homing, survival and proliferation of PGE2 receptor

expressing HSC post-transplant [87]. This pathway is unlikely to be restricted to α-SMA$^+$ macrophages as many other macrophage populations express COX-2 in response to inflammation or tissue injury [88, 89]. Clear understanding of resilience or recovery of HSC niche macrophages post-transplantation may reveal therapeutic strategies for improving transplantation outcomes or reducing the toxicity of chemotherapy.

4. Macrophages in Bone Homeostasis and Bone Repair

4.1 Osteal Macrophages

Bone is a dynamic tissue that undergoes continual adaptation during vertebrate life to maintain structural integrity, regenerate in response to damage and regulate mineral homeostasis. Homeostatic control of bone integrity involves the balanced interaction of matrix forming (mesenchymal) and matrix degrading (haematopoietic) cells. Osteoblast lineage cells develop through a multi-stage maturation progressing from relatively inactive bone-lining cells to bone forming osteoblasts and subsequently, a portion of these, undergo terminal differentiation into osteocytes. The latter form a cellular network within the mineralized matrix from where they detect mechanical strain and damage. Both osteoblasts and osteocytes contribute to incorporation of mineral into the bone organic matrix [90, 91]. Chondrocytes are responsible for production of cartilage matrix which is used as an intermediary structure during bone formation via endochondral ossification. Osteoclasts are terminally differentiated multinucleated myeloid lineage cells responsible for removal of bone matrix. In the early 1980s, a candidate third participant in bone homeostasis was identified in the form of a periosteal F4/80$^+$ macrophage population [92]. The distribution and function of these cells, termed osteomacs, in bone homeostasis and repair has become clear from more recent studies [17, 93, 94].

Dynamic events in bone often occur in a coordinated site-specific manner with bone surfaces referred to as either resting or active (Figure 1). On resting bone surfaces, the bone lining tissues consists of osterix$^+$ bone lining cells interspersed by osteomacs. The endosteum (bone surface in contact with the BM) at resting sites is a single cell layer and therefore osteomacs are in direct contact with the bone surface (Figure 1 and [18]). The periosteum (peripheral bone surface in contact with skeletal muscle) is more complex including an inner cambium and outer capsule layers (Figure 2D). The cambium contains osteomacs (Figure 2D, arrows) while the capsule contains a potentially distinct F4/80$^+$ resident tissue macrophage population (Figure 2D, arrowheads). Osteomacs express a suite of common pan-macrophage markers including F4/80, CD115, CD68 and Mac-3 but do not express the osteoclast marker tartrate-resistant acid phosphatase [17, 18, 24, 94] or osteoblast lineage markers [18]. An equivalent CD68$^+$ osteomac population was also detected in human endosteum in normal bone [18] and pathological bone formation associated with prostate cancer [26]. Thus far, no unique marker distinguishes osteomacs from other BM macrophage subsets, but unlike EIM, endosteal osteomacs do not express the ER-HR3 antigen (Figure 2A and B, arrows). Therefore definitive designation of osteomacs currently requires confirmation of their anatomical location (within 3 cell diameters of a bone surface). This limitation continues to hinder characterization of the osteomac-specific molecular signature and consequently the molecules involved in mediating their bone specific functions.

4.2 The Role of Osteomacs in Bone Formation

Despite limited molecular characterisation of osteomacs, their striking distribution at sites of bone formation provided insight into at least one of their functional roles in bone [18]. At active bone forming sites on endosteal (Figure 2A and C) and periosteal (Figure 2D-F) surfaces osteomacs are intimately associated with bone forming osteoblasts, including

formation of a canopy-like structure over endosteal modelling surfaces (Figure 2A and C, arrows). Given the endosteum is continuous with adjacent BM and that many molecular mediators have activity in both bone and BM homeostasis, it is possible that the site specific formation of a continuous layer of endosteal osteomacs achieves microenvironmental partitioning by forming a phagocytic barrier (Figure 1). This barrier may be needed to prevent inappropriate disturbances within the adjacent BM as a bystander impact of bone dynamics.

In vitro evidence using both human and mouse macrophages [18, 29, 72, 95-99], including primary macrophages isolated from bone tissues [18], demonstrated that these cells promote mesenchymal maturation along the osteoblast lineage and/or osteoblast functional maturation. Osteomacs are also integral for parathyroid hormone anabolic actions [21]. It is unclear whether direct cell contact is necessary [72] or if secreted factors [18, 29, 72, 95-99] or even monocyte/macrophage exosomes [100] can at least partially mediate these macrophage actions. However, it is clear that macrophages can provide pro-anabolic support to osteoblasts and that osteomacs are appropriately located to achieve this function *in vivo*. Only two macrophage-derived molecules have been directly implicated in driving osteoblast maturation: oncostatin M [96] and bone morphogenetic protein-2 [98]. It is unlikely that a single molecular pathway is responsible for pro-anabolic effects of macrophages on osteoblasts, as macrophages under various conditions can express a wide array of pro-anabolic molecules.

The extrinsic stimuli that promote pro-bone anabolic function in macrophages are not well defined. We originally showed that elevated extracellular calcium, as would be expected at sites of bone remodelling [93], greatly enhanced macrophage pro-anabolic function [18]. The

pro-inflammatory stimulus LPS also promoted human monocyte/macrophage support of osteoblast differentiation and function [96]. This outcome was somewhat unexpected given that chronic inflammation generally causes bone loss and LPS delivery is used as an *in vivo* bone loss model [101]. Recently, Michalski *et. al.* observed that $CD206^+$ anti-inflammatory/reparative macrophages secrete the pro-anabolic molecule transforming growth factor (TGF)-β1 after efferocytosis of apoptotic osteoblasts [102]. This potential feedback loop could extend bone formation beyond the functional lifespan of the first wave of osteoblasts at any given anabolic site, consistent with the more extended time required to complete bone formation in comparison to bone resorption [93]. Macrophages play a vital role in monitoring the microbiota, and this has been shown to impact on homeostatic mechanisms within the gut [103]. Macrophages could also contribute to impacts of microbiota on bone [104] but direct evidence is lacking. More refined *in vivo* studies research are needed to better characterise extrinsic signal impacts on osteomac/macrophage functional polarization and how this influences bone homeostasis.

In vivo approaches using inducible or targeted macrophage depletion strategies in mouse models have provided confirmation of osteomac/macrophage pro-anabolic function. This was first shown using the Mafia mouse model in which induced conditional depletion of macrophages resulted in rapid loss of osteoblast bone-forming surface [16, 18, 21] and blunted the anabolic actions of parathyroid hormone [21]. An alternative approach used a transgenic mouse in which lysozyme M drives expression of diphtheria toxin causing cell death. Bone-BM macrophages were at best reduced by approximately 50% but a significant bone growth and formation phenotype was observed [29]. Studies using clodronate liposomes have produced mixed outcomes [16, 21]. Short-term, aggressive treatment with clodronate liposomes recapitulated the Mafia model [16]. However, a long-term reduced treatment

schedule was unexpectedly bone anabolic due to a compensatory expansion of $CD68^+$ phagocytic macrophages that was accompanied by an increase in the osteogenic factors Wnt-3a, Wnt-10b and TGF-β1 within the bone-BM environment [21]. As discussed in section 3.2, HSC mobilising regimens of G-CSF resulted in robust depletion of bone-BM macrophages. This was associated with loss of osteoblast bone surface and a reduced bone formation rate [58, 71]. Interestingly, G-CSF treatment does not deplete macrophages in spleen [24] and has limited impact on osteoclasts [16], suggesting its actions on macrophages may be restricted to bone-BM. G-CSF was reported to induce changes in osteocyte biology [105] which may provide some explanation as to why its effects are more site restricted that other *in vivo* macrophage targeted models. The same study demonstrated that targeted depletion of osteocytes resulted in loss of osteomacs [105] implicating cross talk between not only macrophages and osteoblasts on the bone surface, but direct communication between osteocytes and osteomacs.

Expansion of macrophages also provides tentative evidence supporting macrophage pro-anabolic function. Macrophage proliferation and differentiation is controlled by CSF-1 [106]. CSF-1 is also required to generate progenitors for osteoclasts, and CSF-1-deficient mice are severely osteoclast deficient [107]. However, CSF-1 alone cannot increase osteoclast formation *in vivo*, even in a pro-inflammatory environment [108]. Exogenous CSF-1 increases systemic and/or local bone volume in a number of models [109-112]. The anabolic actions of CSF-1 have been assumed to be due to increased osteoclast-osteoblast coupling and consequently enhanced remodelling, without consideration of the potential role of osteomacs or other macrophage subsets.

4.3 Role of Osteomacs and Inflammatory Macrophages in Bone Repair

Macrophages are central contributors to reparative inflammation, coordinating both the injury response and tissue regeneration [113]. Bone fracture causes disruption of local tissue vasculature, soft and hard tissue integrity, BM architecture and induces hematoma formation. Resident macrophages initiate a cascade of growth factor, inflammatory cytokine and chemokine production that facilitate recruitment of inflammatory immune cells that combat infection and phagocytose debris and dead cell remnants [114]. The inflammatory event drives expansion/recruitment of endothelial, neuronal and mesenchymal stem/progenitor/precursor cells leading to replacement of the hematoma with a vascularized and innervated fibrous granulation tissue [114]. Anabolic healing mechanisms are initiated at the fracture site and are influenced by fracture biomechanics, with high rigidity fractures favouring direct bridging by intramembranous ossification and low rigidity fractures healing via periosteal callus formation through endochondral ossification. A structurally viable callus is formed which is gradually remodelled to reinstate normal bone architecture. Thus fractures heal via sequential progression through phases of inflammation, early anabolic, late anabolic and remodelling.

Macrophages, including both osteomacs and inflammatory macrophages, are present during all phases of fracture repair and associate with key repair events in animal models [19, 109] and human tissues [115]. Inflammatory macrophages are anatomically positioned to support key events in the inflammation and early anabolic phases [19]. Osteomacs predominate in the late anabolic [19, 109] and remodelling phases [19, 109] and are specifically associated with maturing bone within the callus [19]. In bone repair/fracture models various broad spectrum myeloid depletion approaches indicate macrophage requirement for initiation and osteomac requirement for optimal progression of fracture healing [19, 29, 109, 116] with repair outcomes proportionate to the number of local participating osteomacs/macrophages [19].

Local administration of CSF-1 in both a tibial injury [109] and a femoral fracture model [19] increased the number of fracture-associated F4/80^{+} macrophages, without a concomitant increase in osteoclasts, and significantly increased boney bridging and soft-callus formation, respectively. Furthermore, systemic treatment with CSF-1 in the first 2 weeks post fracture in a rabbit bone injury model resulted in increased mineralized callus at 8 weeks post-fracture [117]. CSF-1 treatment has already been shown to be safe and efficacious in humans [118]. Based on this evidence CSF-1 treatment is a candidate therapy for fracture repair. Using a tibial injury model, Guihard *et. al.* recently showed, that macrophage production of the anabolic molecule oncostatin M and subsequent STAT3 activation promoted bone healing [119] and therefore more specific therapies based on macrophage pro-anabolic stimuli may be an alternative approach.

5. Conclusions and Conundrums

In the last 10 years significant leaps have been made in the appreciation of macrophage contributions to bone and BM homeostasis and health. Improved characterisation of BM and bone resident macrophage subsets is needed at both the functional and molecular level to reveal the entirety and complexity of the resident macrophage subsets in these physically linked organs. Distinct niches in endosteum and BM may differentially support the various hematopoietic stem and committed progenitor populations [68, 120]. The specific stromal support cell involved within a given niche may define which stem/progenitor cell is preferentially supported [121]. A similarly hierarchy of BM niche supportive macrophage subsets may also exist. Macrophage contributions to the homeostasis of both bone and BM clearly intersect at the level of their support of osteoblast lineage cells but this is unlikely to be the extent of the overlap as both vascular and nerve contributions are integral to both

systems and macrophages dynamically interact with both these compartments. It is clear that we are only scratching the surface of the complex mechanisms regulating the various niches within bone and BM but macrophage involvement in these niches is a common mechanism. Ultimately improved understanding of osteoimmunological mechanisms underlying the peak function and decline of bone and BM will inform approaches to achieve preservation/reinstatement of peak function in disease and aging.

AUTHOR CONTRIBUTION

SK, LJR, JPL and ARP drafted and edited the manuscript. LB and ARP generated the figures. DAH performed critical editing.

ACKNOWLEDGMENTS

SK, LJR, LB, JPL and ARP are supported by the Mater Foundation. ARP is supported by and Australian and New Zealand Bone and Mineral Society Gap Fellowship. JPL is supported by a Research Fellowship from the National Health and Medical Research Council (NHMRC) of Australia (#1044091). The authors' work described in this review was supported by NHMRC project grants 1046590 (JPL), 434515 (JPL) and (1102964) and a Cancer Council Queensland grant (1066051).

REFERENCES

[1] D'Amelio P, Sassi F. Osteoimmunology: from mice to humans. BoneKEy reports 2016;5:802.

[2] Baht GS, Silkstone D, Vi L, Nadesan P, Amani Y, Whetstone H, et al. Exposure to a youthful circulaton rejuvenates bone repair through modulation of beta-catenin. Nature communications 2015;6:7131.

[3] Kim HL, Cho HY, Park IY, Choi JM, Kim M, Jang HJ, et al. The positive association between peripheral blood cell counts and bone mineral density in postmenopausal women. Yonsei medical journal 2011;52:739-45.

[4] Lei Z, Xiaoying Z, Xingguo L. Ovariectomy-associated changes in bone mineral density and bone marrow haematopoiesis in rats. International journal of experimental pathology 2009;90:512-9.

[5] Gautier EL, Shay T, Miller J, Greter M, Jakubzick C, Ivanov S, et al. Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. Nat Immunol 2012;13:1118-28.

[6] Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature 2013;496:445-55.

[7] Davies LC, Jenkins SJ, Allen JE, Taylor PR. Tissue-resident macrophages. Nat Immunol 2013;14:986-95.

[8] Wynn TA, Vannella KM. Macrophages in Tissue Repair, Regeneration, and Fibrosis. Immunity 2016;44:450-62.

[9] van Furth R, Cohn ZA. The origin and kinetics of mononuclear phagocytes. J Exp Med 1968;128:415-35.

[10] van Furth R, Cohn ZA, Hirsch JG, Humphrey JH, Spector WG, Langevoort HL. The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. Bull World Health Organ 1972;46:845-52.

[11] Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, et al. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. Immunity 2013;38:792-804.

[12] Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. Immunity 2013;38:79-91.

[13] Sheng J, Ruedl C, Karjalainen K. Most Tissue-Resident Macrophages Except Microglia Are Derived from Fetal Hematopoietic Stem Cells. Immunity 2015;43:382-93.

[14] Agas D, Marchetti L, Douni E, Sabbieti MG. The unbearable lightness of bone marrow homeostasis. Cytokine & growth factor reviews 2015;26:347-59.

[15] Bessis M. [Erythroblastic island, functional unity of bone marrow]. Revue d'hematologie 1958;13:8-11.

[16] Winkler IG, Sims NA, Pettit AR, Barbier V, Nowlan B, Helwani F, et al. Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs2010.

[17] Sinder BP, Pettit AR, McCauley LK. Macrophages: Their Emerging Roles in Bone. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 2015;30:2140-9.

[18] Chang MK, Raggatt L-J, Alexander KA, Kuliwaba JS, Fazzalari NL, Schroder K, et al. Osteal Tissue Macrophages Are Intercalated throughout Human and Mouse Bone Lining Tissues and Regulate Osteoblast Function In Vitro and In Vivo. The Journal of Immunology 2008;181:1232-44.

[19] Raggatt LJ, Wullschleger ME, Alexander KA, Wu AC, Millard SM, Kaur S, et al. Fracture healing via periosteal callus formation requires macrophages for both initiation and progression of early endochondral ossification. The American journal of pathology 2014;184:3192-204.

[20] Burnett SH, Kershen EJ, Zhang J, Zeng L, Straley SC, Kaplan AM, et al. Conditional macrophage ablation in transgenic mice expressing a Fas-based suicide gene. J Leukoc Biol 2004;75:612-23.

[21] Cho SW, Soki FN, Koh AJ, Eber MR, Entezami P, Park SI, et al. Osteal macrophages support physiologic skeletal remodeling and anabolic actions of parathyroid hormone in bone. Proceedings of the National Academy of Sciences of the United States of America 2014;111:1545-50.

[22] MacDonald KP, Rowe V, Bofinger HM, Thomas R, Sasmono T, Hume DA, et al. The colony-stimulating factor 1 receptor is expressed on dendritic cells during differentiation and regulates their expansion. Journal of immunology 2005;175:1399-405.

[23] van Rooijen N, Hendrikx E. Liposomes for specific depletion of macrophages from organs and tissues. Methods in molecular biology (Clifton, NJ) 2010;605:189-203.

[24] Jacobsen RN, Forristal CE, Raggatt LJ, Nowlan B, Barbier V, Kaur S, et al. Mobilization with granulocyte colony-stimulating factor blocks medullar erythropoiesis by depleting F4/80(+)VCAM1(+)CD169(+)ER-HR3(+)Ly6G(+) erythroid island macrophages in the mouse. Exp Hematol 2014;42:547-61.e4.

[25] McCabe A, Zhang Y, Thai V, Jones M, Jordan MB, MacNamara KC. Macrophage-Lineage Cells Negatively Regulate the Hematopoietic Stem Cell Pool in Response to Interferon Gamma at Steady State and During Infection. Stem cells (Dayton, Ohio) 2015;33:2294-305.

[26] Wu AC, He Y, Broomfield A, Paatan NJ, Harrington BS, Tseng HW, et al. CD169(+) macrophages mediate pathological formation of woven bone in skeletal lesions of prostate cancer. The Journal of pathology 2016;239:218-30.

[27] Miyake Y, Asano K, Kaise H, Uemura M, Nakayama M, Tanaka M. Critical role of macrophages in the marginal zone in the suppression of immune responses to apoptotic cell–associated antigens. The Journal of Clinical Investigation 2007;117:2268-78.

[28] Chow A, Lucas D, Hidalgo A, Méndez-Ferrer S, Hashimoto D, Scheiermann C, et al. Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. The Journal of Experimental Medicine 2011;208:261-71.

[29] Vi L, Baht GS, Whetstone H, Ng A, Wei Q, Poon R, et al. Macrophages promote osteoblastic differentiation in-vivo: implications in fracture repair and bone homeostasis. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 2015;30:1090-102.

[30] Jacobsen RN, Perkins AC, Levesque JP. Macrophages and regulation of erythropoiesis. Current opinion in hematology 2015;22:212-9.

[31] Bessis MC, Breton-Gorius J. Iron metabolism in the bone marrow as seen by electron microscopy: a critical review. Blood 1962;19:635-63.

[32] Kawane K, Fukuyama H, Kondoh G, Takeda J, Ohsawa Y, Uchiyama Y, et al. Requirement of DNase II for definitive erythropoiesis in the mouse fetal liver. Science 2001;292:1546-9.

[33] Toda S, Segawa K, Nagata S. MerTK-mediated engulfment of pyrenocytes by central macrophages in erythroblastic islands. Blood 2014;123:3963-71.

[34] Rich IN, Vogt C, Pentz S. Erythropoietin gene expression in vitro and in vivo detected by in situ hybridization. Blood cells 1988;14:505-20.

[35] Vogt C, Pentz S, Rich IN. A role for the macrophage in normal hemopoiesis: III. In vitro and in vivo erythropoietin gene expression in macrophages detected by in situ hybridization. Exp Hematol 1989;17:391-7.

[36] Lifshitz L, Tabak G, Gassmann M, Mittelman M, Neumann D. Macrophages as novel target cells for erythropoietin. Haematologica 2010;95:1823-31.

[37] Tonkin J, Temmerman L, Sampson RD, Gallego-Colon E, Barberi L, Bilbao D, et al. Monocyte/Macrophage-derived IGF-1 Orchestrates Murine Skeletal Muscle Regeneration and Modulates Autocrine Polarization. Molecular therapy : the journal of the American Society of Gene Therapy 2015;23:1189-200.

[38] Sawada K, Krantz SB, Dessypris EN, Koury ST, Sawyer ST. Human colony-forming units-erythroid do not require accessory cells, but do require direct interaction with insulin-like growth factor I and/or insulin for erythroid development. J Clin Invest 1989;83:1701-9.

[39] Liu M, Jin X, He X, Pan L, Zhang X, Zhao Y. Macrophages support splenic erythropoiesis in 4T1 tumor-bearing mice. PloS one 2015;10:e0121921.

[40] Li C, Shi C, Kim J, Chen Y, Ni S, Jiang L, et al. Erythropoietin promotes bone formation through EphrinB2/EphB4 signaling. Journal of dental research 2015;94:455-63.

[41] Chen Y, Cheung KM, Kung HF, Leong JC, Lu WW, Luk KD. In vivo new bone formation by direct transfer of adenoviral-mediated bone morphogenetic protein-4 gene. Biochemical and biophysical research communications 2002;298:121-7.

[42] Giustina A, Mazziotti G, Canalis E. Growth hormone, insulin-like growth factors, and the skeleton. Endocrine reviews 2008;29:535-59.

[43] Caselli A, Olson TS, Otsuru S, Chen X, Hofmann TJ, Nah HD, et al. IGF-1-mediated osteoblastic niche expansion enhances long-term hematopoietic stem cell engraftment after murine bone marrow transplantation. Stem cells (Dayton, Ohio) 2013;31:2193-204.

[44] Goldman DC, Bailey AS, Pfaffle DL, Al Masri A, Christian JL, Fleming WH. BMP4 regulates the hematopoietic stem cell niche. Blood 2009;114:4393-401.

[45] Lau CI, Outram SV, Saldana JI, Furmanski AL, Dessens JT, Crompton T. Regulation of murine normal and stress-induced erythropoiesis by Desert Hedgehog. Blood 2012;119:4741-51.

[46] Leimberg MJ, Prus E, Konijn AM, Fibach E. Macrophages function as a ferritin iron source for cultured human erythroid precursors. Journal of cellular biochemistry 2008;103:1211-8.

[47] Toda S, Nishi C, Yanagihashi Y, Segawa K, Nagata S. Clearance of Apoptotic Cells and Pyrenocytes. Current topics in developmental biology 2015;114:267-95.

[48] Chow A, Huggins M, Ahmed J, Hashimoto D, Lucas D, Kunisaki Y, et al. CD169(+) macrophages provide a niche promoting erythropoiesis under homeostasis and stress. Nat Med 2013;19:429-36.

[49] Ramos P, Casu C, Gardenghi S, Breda L, Crielaard BJ, Guy E, et al. Macrophages support pathological erythropoiesis in polycythemia vera and beta-thalassemia. Nat Med 2013;19:437-45.

[50] Sonoda Y, Sasaki K. Surface morphology of the central macrophages of erythroblastic islets in the spleen of aged and pregnant mice: an immunohistochemical light microscopic study. Archives of histology and cytology 2008;71:155-61.

[51] Sadahira Y, Yoshino T, Monobe Y. Very late activation antigen 4-vascular cell adhesion molecule 1 interaction is involved in the formation of erythroblastic islands. J Exp Med 1995;181:411-5.

[52] Ulyanova T, Jiang Y, Padilla S, Nakamoto B, Papayannopoulou T. Combinatorial and distinct roles of alpha(5) and alpha(4) integrins in stress erythropoiesis in mice. Blood 2011;117:975-85.

[53] Galloway JL, Wingert RA, Thisse C, Thisse B, Zon LI. Loss of gata1 but not gata2 converts erythropoiesis to myelopoiesis in zebrafish embryos. Dev Cell 2005;8:109-16.

[54] Bisht K, Jacobsen RN, Brunck M, Keech T, McGuirr C, Nowlan B, et al. Hematopoietic stem cell mobilization and erythropoiesis suppression in response to lipopolysaccharides involve two distinct TLR4-depedent mechanisms with different requirement for G-CSF receptor. Exp Hematol. San Diego, USA2016.

[55] Miyazaki Y, Taguchi K, Sou K, Watanabe H, Ishima Y, Miyakawa T, et al. Therapeutic impact of erythropoietin-encapsulated liposomes targeted to bone marrow on renal anemia. Molecular pharmaceutics 2014;11:4238-48.

[56] Wilson A, Laurenti E, Oser G, van der Wath RC, Blanco-Bose W, Jaworski M, et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell 2008;135:1118-29.

[57] Catlin SN, Busque L, Gale RE, Guttorp P, Abkowitz JL. The replication rate of human hematopoietic stem cells in vivo. Blood 2011;117:4460-6.

[58] Winkler IG, Barbier V, Wadley R, Zannettino AC, Williams S, Levesque JP. Positioning of bone marrow hematopoietic and stromal cells relative to blood flow in vivo: serially reconstituting hematopoietic stem cells reside in distinct nonperfused niches. Blood 2010;116:375-85.

[59] Winkler IG, Barbier V, Nowlan B, Jacobsen RN, Forristal CE, Patton JT, et al. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. Nat Med 2012;18:1651-7.

[60] Mendelson A, Frenette PS. Hematopoietic stem cell niche maintenance during homeostasis and regeneration. Nat Med 2014;20:833-46.

[61] Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. Nature 2014;505:327-34.

[62] Kunisaki Y, Bruns I, Scheiermann C, Ahmed J, Pinho S, Zhang D, et al. Arteriolar niches maintain haematopoietic stem cell quiescence. Nature 2013;502:637-43.

[63] Grassinger J, Haylock DN, Williams B, Olsen GH, Nilsson SK. Phenotypically identical hemopoietic stem cells isolated from different regions of bone marrow have different biologic potential. Blood 2010;116:3185-96.

[64] Itkin T, Gur-Cohen S, Spencer JA, Schajnovitz A, Ramasamy SK, Kusumbe AP, et al. Distinct bone marrow blood vessels differentially regulate haematopoiesis. Nature 2016;532:323-8.

[65] Acar M, Kocherlakota KS, Murphy MM, Peyer JG, Oguro H, Inra CN, et al. Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. Nature 2015;526:126-30.

[66] Chen JY, Miyanishi M, Wang SK, Yamazaki S, Sinha R, Kao KS, et al. Hoxb5 marks long-term haematopoietic stem cells and reveals a homogenous perivascular niche. Nature 2016;530:223-7.

[67] Xie Y, Yin T, Wiegraebe W, He XC, Miller D, Stark D, et al. Detection of functional haematopoietic stem cell niche using real-time imaging. Nature 2009;457:97-101.

[68] Levesque JP, Winkler IG. Hierarchy of immature hematopoietic cells related to blood flow and niche. Current opinion in hematology 2011;18:220-5.

[69] Levesque JP, Jacobsen RN, Winkler IG. The role of mesenchymal stem cells in hematopoiesis. In: Atkinson K, editor. “The Biology amd Therapeutic Application of Mesenchymal Cells": Willey-Blackwell; 2016.

[70] Christopher MJ, Rao M, Liu F, Woloszynek JR, Link DC. Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice. The Journal of Experimental Medicine 2011;208:251-60.

[71] Winkler IG, Pettit AR, Raggatt LJ, Jacobsen RN, Forristal CE, Barbier V, et al. Hematopoietic stem cell mobilizing agents G-CSF, cyclophosphamide or AMD3100 have distinct mechanisms of action on bone marrow HSC niches and bone formation. Leukemia 2012;26:1594-601.

[72] Chang KH, Sengupta A, Nayak RC, Duran A, Lee SJ, Pratt RG, et al. p62 is required for stem cell/progenitor retention through inhibition of IKK/NF-kappaB/Ccl4 signaling at the bone marrow macrophage-osteoblast niche. Cell reports 2014;9:2084-97.

[73] Dutta P, Hoyer FF, Grigoryeva LS, Sager HB, Leuschner F, Courties G, et al. Macrophages retain hematopoietic stem cells in the spleen via VCAM-1. The Journal of Experimental Medicine 2015;212:497-512.

[74] Hur J, Choi JI, Lee H, Nham P, Kim TW, Chae CW, et al. CD82/KAI1 Maintains the Dormancy of Long-Term Hematopoietic Stem Cells through Interaction with DARC-Expressing Macrophages. Cell stem cell 2016;18:508-21.

[75] Xu L, Ashkenazi A, Chaudhuri A. Duffy antigen/receptor for chemokines (DARC) attenuates angiogenesis by causing senescence in endothelial cells. Angiogenesis 2007;10:307-18.

[76] Basu S, Broxmeyer HE. CCR5 ligands modulate CXCL12-induced chemotaxis, adhesion, and Akt phosphorylation of human cord blood CD34+ cells. Journal of immunology 2009;183:7478-88.

[77] Casanova-Acebes M, Pitaval C, Weiss LA, Nombela-Arrieta C, Chevre R, N AG, et al. Rhythmic modulation of the hematopoietic niche through neutrophil clearance. Cell 2013;153:1025-35.

[78] To LB, Levesque JP, Herbert KE. How I treat patients who mobilize hematopoietic stem cells poorly. Blood 2011;118:4530-40.

[79] Winkler IG, Wiercinska E, Barbier V, Nowlan B, Bonig H, Levesque JP. Mobilization of hematopoietic stem cells with highest self-renewal by G-CSF precedes clonogenic cell mobilization peak. Exp Hematol 2016;44:303-14.e1.

[80] Joshi A, Pooley C, Freeman TC, Lennartsson A, Babina M, Schmidl C, et al. Transcription factor, promoter, and enhancer utilization in human myeloid cells. J Leukoc Biol 2015.

[81] McCabe A, MacNamara KC. Macrophages: Key regulators of steady-state and demand-adapted hematopoiesis. Exp Hematol 2016;44:213-22.

[82] Takahashi K, Yamamura F, Naito M. Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: a light-microscopic, enzyme-cytochemical, immunohistochemical, and ultrastructural study. J Leukoc Biol 1989;45:87-96.

[83] Lichanska AM, Hume DA. Origins and functions of phagocytes in the embryo. Exp Hematol 2000;28:601-11.

[84] Travnickova J, Tran Chau V, Julien E, Mateos-Langerak J, Gonzalez C, Lelievre E, et al. Primitive macrophages control HSPC mobilization and definitive haematopoiesis. Nature communications 2015;6:6227.

[85] Heylmann D, Rodel F, Kindler T, Kaina B. Radiation sensitivity of human and murine peripheral blood lymphocytes, stem and progenitor cells. Biochimica et biophysica acta 2014;1846:121-9.

[86] Porter RL, Georger MA, Bromberg O, McGrath KE, Frisch BJ, Becker MW, et al. Prostaglandin E2 increases hematopoietic stem cell survival and accelerates hematopoietic recovery after radiation injury. Stem cells (Dayton, Ohio) 2013;31:372-83.

[87] Hoggatt J, Singh P, Sampath J, Pelus LM. Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation. Blood 2009;113:5444-55.

[88] Fournier T, Fadok V, Henson PM. Tumor necrosis factor-alpha inversely regulates prostaglandin D2 and prostaglandin E2 production in murine macrophages. Synergistic action of cyclic AMP on cyclooxygenase-2 expression and prostaglandin E2 synthesis. The Journal of biological chemistry 1997;272:31065-72.

[89] Hinz B, Brune K, Pahl A. Prostaglandin E(2) upregulates cyclooxygenase-2 expression in lipopolysaccharide-stimulated RAW 264.7 macrophages. Biochemical and biophysical research communications 2000;272:744-8.

[90] Tanaka Y, Nakayamada S, Okada Y. Osteoblasts and osteoclasts in bone remodeling and inflammation. Current drug targets Inflammation and allergy 2005;4:325-8.

[91] Xiao W, Wang Y, Pacios S, Li S, Graves DT. Cellular and Molecular Aspects of Bone Remodeling. Frontiers of oral biology 2016;18:9-16.

[92] Hume DA, Loutit JF, Gordon S. The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80: macrophages of bone and associated connective tissue. Journal of cell science 1984;66:189-94.

[93] Pettit AR, Chang MK, Hume DA, Raggatt LJ. Osteal macrophages: a new twist on coupling during bone dynamics. Bone 2008;43:976-82.

[94] Wu AC, Raggatt LJ, Alexander KA, Pettit AR. Unraveling macrophage contributions to bone repair. BoneKEy reports 2013;2:373.

[95] Rifas L, Shen V, Mitchell K, Peck WA. Macrophage-derived growth factor for osteoblast-like cells and chondrocytes. Proceedings of the National Academy of Sciences of the United States of America 1984;81:4558-62.

[96] Guihard P, Danger Y, Brounais B, David E, Brion R, Delecrin J, et al. Induction of osteogenesis in mesenchymal stem cells by activated monocytes/macrophages depends on oncostatin M signaling. Stem cells (Dayton, Ohio) 2012;30:762-72.

[97] Nicolaidou V, Wong MM, Redpath AN, Ersek A, Baban DF, Williams LM, et al. Monocytes induce STAT3 activation in human mesenchymal stem cells to promote osteoblast formation. PloS one 2012;7:e39871.

[98] Pirraco RP, Reis RL, Marques AP. Effect of monocytes/macrophages on the early osteogenic differentiation of hBMSCs. Journal of tissue engineering and regenerative medicine 2013;7:392-400.

[99] Fernandes TJ, Hodge JM, Singh PP, Eeles DG, Collier FM, Holten I, et al. Cord blood-derived macrophage-lineage cells rapidly stimulate osteoblastic maturation in mesenchymal stem cells in a glycoprotein-130 dependent manner. PloS one 2013;8:e73266.

[100] Ekstrom K, Omar O, Graneli C, Wang X, Vazirisani F, Thomsen P. Monocyte exosomes stimulate the osteogenic gene expression of mesenchymal stem cells. PloS one 2013;8:e75227.

[101] Gao Y, Grassi F, Ryan MR, Terauchi M, Page K, Yang X, et al. IFN-gamma stimulates osteoclast formation and bone loss in vivo via antigen-driven T cell activation. J Clin Invest 2007;117:122-32.

[102] Michalski MN, Koh AJ, Weidner S, Roca H, McCauley LK. Modulation of Osteoblastic Cell Efferocytosis by Bone Marrow Macrophages. Journal of cellular biochemistry 2016.

[103] Muller PA, Koscso B, Rajani GM, Stevanovic K, Berres ML, Hashimoto D, et al. Crosstalk between muscularis macrophages and enteric neurons regulates gastrointestinal motility. Cell 2014;158:300-13.

[104] Li JY, Chassaing B, Tyagi AM, Vaccaro C, Luo T, Adams J, et al. Sex steroid deficiency-associated bone loss is microbiota dependent and prevented by probiotics. J Clin Invest 2016;126:2049-63.

[105] Asada N, Katayama Y, Sato M, Minagawa K, Wakahashi K, Kawano H, et al. Matrix-embedded osteocytes regulate mobilization of hematopoietic stem/progenitor cells. Cell stem cell 2013;12:737-47.

[106] Hume DA, MacDonald KP. Therapeutic applications of macrophage colony-stimulating factor-1 (CSF-1) and antagonists of CSF-1 receptor (CSF-1R) signaling. Blood 2012;119:1810-20.

[107] Felix R, Cecchini MG, Fleisch H. Macrophage colony stimulating factor restores in vivo bone resorption in the op/op osteopetrotic mouse. Endocrinology 1990;127:2592-4.

[108] Pettit AR, Ji H, von Stechow D, Muller R, Goldring SR, Choi Y, et al. TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. The American journal of pathology 2001;159:1689-99.

[109] Alexander KA, Chang MK, Maylin ER, Kohler T, Muller R, Wu AC, et al. Osteal macrophages promote in vivo intramembranous bone healing in a mouse tibial injury model. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 2011;26:1517-32.

[110] Gow DJ, Sauter KA, Pridans C, Moffat L, Sehgal A, Stutchfield BM, et al. Characterisation of a novel Fc conjugate of macrophage colony-stimulating factor. Molecular therapy : the journal of the American Society of Gene Therapy 2014;22:1580-92.

[111] Garceau V, Balic A, Garcia-Morales C, Sauter KA, McGrew MJ, Smith J, et al. The development and maintenance of the mononuclear phagocyte system of the chick is controlled by signals from the macrophage colony-stimulating factor receptor. BMC biology 2015;13:12.

[112] Lloyd SA, Yuan YY, Simske SJ, Riffle SE, Ferguson VL, Bateman TA. Administration of high-dose macrophage colony-stimulating factor increases bone turnover and trabecular volume fraction. Journal of bone and mineral metabolism 2009;27:546-54.

[113] Forbes SJ, Rosenthal N. Preparing the ground for tissue regeneration: from mechanism to therapy. Nat Med 2014;20:857-69.

[114] Schindeler A, McDonald MM, Bokko P, Little DG. Bone remodeling during fracture repair: The cellular picture. Seminars in cell & developmental biology 2008;19:459-66.

[115] Andrew JG, Andrew SM, Freemont AJ, Marsh DR. Inflammatory cells in normal human fracture healing. Acta orthopaedica Scandinavica 1994;65:462-6.

[116] Schlundt C, Schell H, Goodman SB, Vunjak-Novakovic G, Duda GN, Schmidt-Bleek K. Immune modulation as a therapeutic strategy in bone regeneration. Journal of experimental orthopaedics 2015;2:1.

[117] Sarahrudi K, Mousavi M, Grossschmidt K, Sela N, Konig F, Vecsei V, et al. The impact of colony-stimulating factor-1 on fracture healing: an experimental study. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 2009;27:36-41.

[118] Cole DJ, Sanda MG, Yang JC, Schwartzentruber DJ, Weber J, Ettinghausen SE, et al. Phase I trial of recombinant human macrophage colony-stimulating factor administered by continuous intravenous infusion in patients with metastatic cancer. Journal of the National Cancer Institute 1994;86:39-45.

[119] Guihard P, Boutet MA, Brounais-Le Royer B, Gamblin AL, Amiaud J, Renaud A, et al. Oncostatin m, an inflammatory cytokine produced by macrophages, supports intramembranous bone healing in a mouse model of tibia injury. The American journal of pathology 2015;185:765-75.

[120] Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain haematopoietic stem cells. Nature 2012;481:457-62.

[121] Greenbaum A, Hsu YM, Day RB, Schuettpelz LG, Christopher MJ, Borgerding JN, et al. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. Nature 2013;495:227-30.

Figure Legends

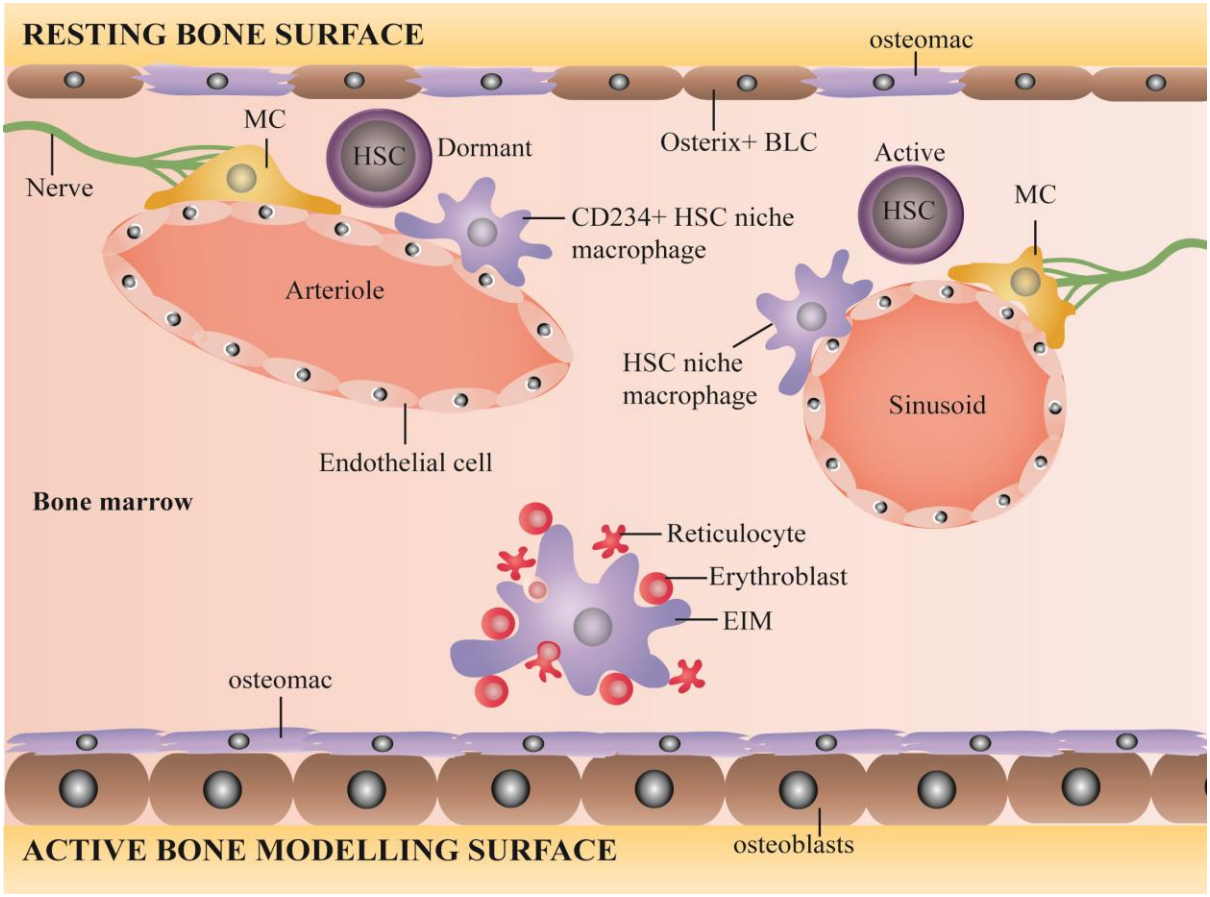


Figure 1: Schematic representations of the confirmed resident macrophage subsets within bone and BM.

Three confirmed resident macrophages subsets have been characterised within BM and bone. EIM are intermixed throughout the medullar clustering maturing erythroblasts and supporting multiple key steps in erythropoeisis. Within haematopoietic tissue within the endosteal microenvironment $CD234^{+}$ HSC niche macrophages, and potentially even osteomacs on resting bone surfaces, are an integral cell participant in dormant HSC niches. These endosteal dormant HSC niches also require association with arterioles, innervation, early mesenchymal lineage cells (MC) and $osterix^{+}$ osteoblast-lineage bone lining cells (BLC). Active HSC reside in vascular niches located adjacent to sinusoids. $F4/80^{+}$ macrophages are abundant in this location and are presumably a $CD234^{neg}$ HSC niche macrophages. Again other niche cellular constituents including nerves and mesenchymal cells help form the specific physical

requirements needed to support the self-renewing and cycling HSC within these specific niches. Finally, osteomacs are intercalated within bone lining tissues on resting endosteal surfaces or reorganise to form a canopy-like structure over osteoblasts at active bone modelling surfaces.

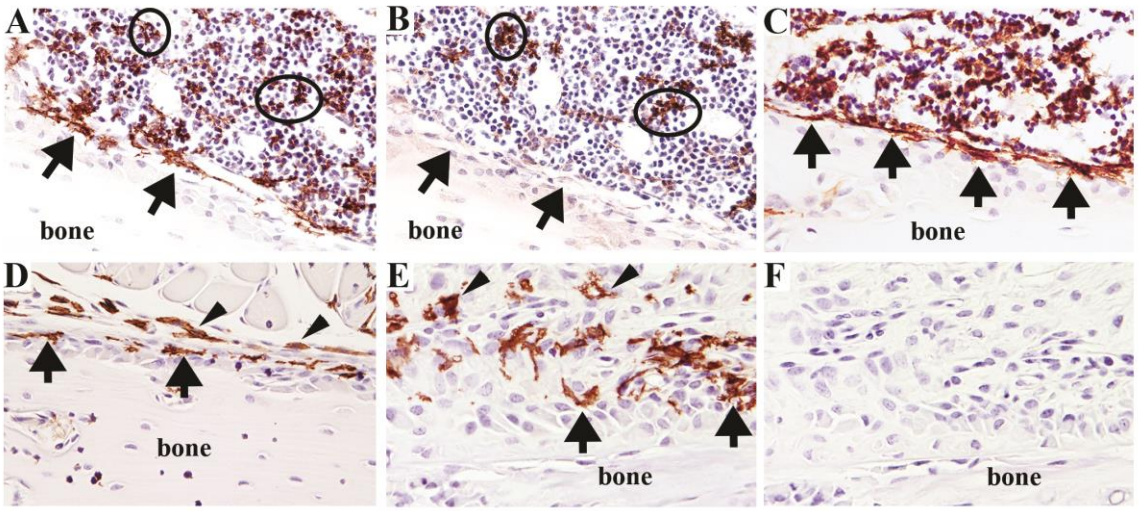


Figure 2: Bone and BM Resident Macrophage In Situ.

Hind limbs were collected from 4 week old mice, fixed and processed to paraffin block and sections stained by a chromogen based immunohistochemistry technique as previously described [30]. A) F4/80 (brown) expression within endosteum and medullar environments exemplifying the large number of resident macrophages within these physically connected environments. B) EIM ER-HR3 (brown) expression in a near serial section to (A). $F4/80^{+}ER\text{-}HR3^{+}$ EIM can be tracked (circles) whereas canopy osteomacs are clearly $F4/80^{+}ER\text{-}HR3^{neg}$ (arrows). C) Prime example of $F4/80^{+}$ (brown) osteomac canopy (arrows) at an endosteal modelling site partitioning the bone forming surface from the adjacent BM. D) Resting periosteal bone surface exemplifying its more complex structure including both $F4/80^{+}$ osteomacs within the cambium layer (arrows) and $F4/80^{+}$ capsule resident macrophages

(arrowheads). E) Periosteum within the dynamic corticalization zone that mediates bone lengthening during growth contains highly reticulated F4/80^{+} osteomacs within the cambium layer (arrows) and F4/80^{+} capsule resident macrophages (arrowheads). F) Near serial section to (E) stained with isotype matched control antibody to demonstrate specificity of staining. All sections were counterstained with hematoxylin (blue) and original magnification was 40x with digital zooming applied in (C-F).