From hurdle to springboard: the macrophage as target in biomaterial-based bone regeneration strategies

Yang-Hee Kim[1], Richard O.C. Oreffo[1], and Jonathan I. Dawson[1]*

[1]*Bone and Joint Research Group, Centre for Human Development, Stem Cells & Regeneration, Institute of Developmental Sciences, University of Southampton, Southampton, SO16 6YD, UK*

* Corresponding author
Dr J. I. Dawson
Institute of Developmental Sciences, University of Southampton, Southampton, SO16 6YD, UK
E-mail: jid@soton.ac.uk

Abstract

The past decade has seen a growing appreciation for the role of the innate immune response in mediating repair and biomaterial directed tissue regeneration. The long-held view of the host immune/inflammatory response as an obstacle limiting stem cell regenerative activity, has given way to a fresh appreciation of the pivotal role the macrophage plays in orchestrating the resolution of inflammation and launching the process of remodelling and repair. In the context of bone, work over the past decade has established an essential coordinating role for macrophages in supporting bone repair and sustaining biomaterial driven osteogenesis. In this review evidence for the role of the macrophage in bone regeneration and repair is surveyed before discussing recent biomaterial and drug-delivery based approaches that target macrophage modulation with the goal of accelerating and enhancing bone tissue regeneration.

1. Introduction

Biomaterial-based bone regeneration strategies serve to address the growing incidence of clinical scenarios in which mechanical or metabolic restrictions necessitate augmentation of natural bone repair. The biomaterial scaffold serves, in the context of bone augmentation, as a substitute for the highly effective, but severely source-restricted, autologous bone graft. The principle function of the scaffold is to provide a temporary local environment, or niche, able to foster the process of cell recruitment, proliferation and differentiation necessary for tissue regeneration to occur. One of the main strategies to achieve this is through the release of a growth-factor from a biomaterial scaffold to target endogenous cells. The clinically licensed use of recombinant bone morphogenic protein 2 (BMP-2) for spinal fusion is a prominent example of this strategy.

There is now a very large literature exploring a wide range of candidate biomolecules and, even wider, range of biomaterial scaffold and delivery technologies for bone regeneration. It is interesting to note that while early studies tended to focus principally on the responses of inflammatory cells, including macrophages, to implanted materials (see for example [1,2]), the emergence of the field of tissue engineering and regenerative medicine in the late 1990s led to a major shift in focus (Figure 1a). The vast majority of studies have since focussed predominantly on stem and progenitor cell populations as the principle target for bone regeneration. With a few notable early exceptions [3–5], the macrophage has tended to be viewed solely in terms of the host immune and inflammatory response against foreign objects and thus a hurdle to be overcome in order for stem cells to achieve their regenerative function (a 2008 review by two of the present authors [6] serves as a representative example of this tendency).

More recently however, there has been renewed interest (Figure 1b) in developing biomaterial strategies targeting immune cells – monocytes and macrophages in particular – as key effectors of regeneration. This trend has been driven not only by the long-held insight that such cells are the first to encounter and respond to an implanted biomaterial [3], but also by a growing recognition of the pivotal role macrophages play in launching and sustaining the regenerative process [7–10].

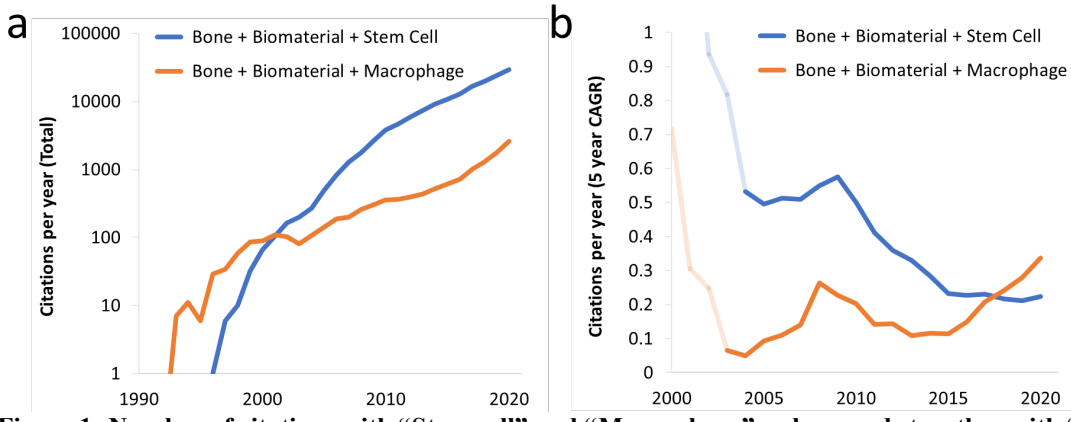

Figure 1: Number of citations with "Stem cell" and "Macrophage" as keywords together with "Bone" and "Biomaterial". (a) Total and (**b**) 5-year compound annual growth rate (CAGR) for number of citations per year. Citations to source items indexed within Web of Science Core Collection between 1970 and 2020 for the search: [TOPIC: (stem cell/macrophage) AND TOPIC: (bone) AND TOPIC: (biomaterial)]. It should be noted

that other studies using alternative terms to describe stem cell populations such as, for example, marrow stromal cells may not be represented by the search terms above.

In this review current knowledge of the role of the macrophage in bone regeneration and repair will be summarised before discussing recent biomaterial and drug-delivery approaches that target macrophage modulation to accelerate and enhance bone regeneration.

2. An overview of the role of macrophages in inflammation and bone repair

Inflammation is a protective response to damaged tissue and foreign bodies and is usually considered to be negative. Indeed, inflammation is associated with numerous diseases and cancer [11,12]. Although the resolution of inflammation was traditionally characterised as a passive process, the active anti-inflammatory, pro-regenerative role of various mediators and inflammatory cytokines from immune cells is now widely recognised [13,14]. Depending on the mediators and cytokines, the inflammation is resolved, followed by tissue repair and regeneration, and perturbation of this resolving inflammatory response leads to chronic inflammation. Macrophages play an essential mediating role in modulating inflammation and thus macrophage phenotype and function has received considerable attention.

Macrophages are found in most tissues where they play essential roles in development, homeostasis and repair [15,16]. Anatomically, in relation to bone, resident macrophages (sometimes referred to as OsteoMacs) locate proximal to bone at sites of active cell modelling [17]. The dependency of osteoblast function on bone resident macrophages has been confirmed through models of targeted macrophage depletion which result in lower osteoblast numbers and activity, and reduced bone mass [15,17]. A rather striking demonstration of this dependency (striking, particularly, for researchers who routinely work with primary osteoblast cultures) was provided by Chang et al. [17] who demonstrated that: a) standard primary osteoblast isolation protocols consistently co-isolate a not insignificant subfraction (11-16%) of F4/80 (a surface glycoprotein murine macrophage marker) expressing macrophages, and that b) purifying osteoblasts of this subfraction leads to a significant (23-fold) impairment in the ability of osteoblasts to mineralise calcium.

There is also now strong evidence for direct involvement of macrophages in repair and biomaterial mediated regeneration. This involvement extends from the pivotal role macrophages play in coordinating the initial inflammatory phase of healing, to its resolution and promotion of regenerative and remodelling phases of repair [15,18,19]. Work by Pettit and colleagues in particular on staged depletion of macrophages over the course of fracture repair has convincingly demonstrated a critical role for macrophages in supporting both endochondral and intramembranous modes of bone repair [18,19]. Similar depletion studies have confirmed the essential coordinating role of macrophages in sustaining biomaterial driven osteogenesis at ectopic [20] and orthotopic sites of bone formation [21].

2.1. Macrophages play diverse roles in inflammation and bone repair

Macrophages are very heterogeneous displaying considerable lineage diversity and phenotypic plasticity which correspond to a broad array of tissue-specific functions and states [16]. In relation to inflammation and regeneration a now conventional, albeit simplistic, distinction is to cluster macrophage functionality around two poles typically referred to as the classically activated M1 and alternatively activated M2 macrophage phenotypes.

The M1 macrophage phenotype is associated with the release of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β, the catabolic effects of which, if sustained, can result in tissue damage and lead to immunopathologies such as rheumatoid arthritis and inflammatory bowel disease [22,23]. In contrast, the activated M2 macrophage phenotype is characterised by the production of IL-10 and TGF- β1, essential in maintaining the long-term survival of stem and progenitor cells for tissue repair [24,25]. The M2 phenotype has been further subcategorised into M2a, M2b, M2c, and M2d (Table 1). Although these subtypes all describe anti-inflammatory properties, M2a and M2b designate immunoregulatory effects, whereas M2c is associated with immunosuppressive phenotypes and the anabolic phase of extracellular matrix (ECM) remodeling [26]. Moreover, M2a and M2c promote angiogenesis and tissue regeneration [1]. Somewhat distinct from these phenotypes, M2d relates principally to tumor-associated macrophages (TAMs).

Table 1. Simplified markers and biologic functions of macrophage subtypes [28–30]

Subtypes	Inducers	Surface markers	Cytokines/growth factors	Functions
M1	IFN-γ, LPS, TNF-α	CD80, CD86, iNOS, MHC-II	TNF-α, iNOS, IL-1-b, 6/8/12,	Pro-inflammation, microbicidal effect, tumor resistance, Pathogen clearance
M2a	IL-4, IL-13	CD206, CD163,CD209, Arg-1, FIZZ1	CCL17, IL-1R, Dectin-1, IL-10, Arg-1, Ym1, Fgf2, TGF-β, IGF-1	Anti-inflammatory, wound healing,
M2b	LPS+IC, IL-1β+IC	CD86, CD64, Arg-1, FIZZ1 MHC-II	IL-1, IL-10, TNF-α,	Immunoregulation, promoting infection, tumor progression
M2c	IL-10, Glucocorticoids	CD206, CD163, Arg-1, PPAR-delta, FIZZ1	IL-10, TGF-β, IGF-1, PEG2, MerTK,	Immunosuppression, tissue remodeling
M2d (TAMs)	LPS+A2R ligands, IL-6	CD163, CD68, CD206	VEGF, IL-10, TGF-β, TNF-α	Tumor progression, angiogenesis

* Arg-1: arginase-1; CCL: chemokine (C-C motif) ligand; CD: cluster of differentiation; CXCL: chemokine (C-X-C motif) ligand, FIZZ1: found in inflammatory zone 1, IC: immune complex, iNOS: inducible nitric oxide synthase, MerTK: Mer receptor tyrosine kinase, VEGF: vascular endothelial growth factor, IGF-1 insulin-like growth factor-1, A2R: Adenosine A 2 receptor (A2R).

The recognition that macrophage phenotype plays a pivotal role in the repair process has prompted investigations into phenotype modulation - particularly the switch from M1 to M2 – as an approach to enhancing tissue regeneration [31]. It is important to note, however, that while the M2

phenotype is essential for a successful repair outcome the timing and magnitude of this effect is critical. Kim et al. demonstrated that, at early (day 1) time points after biomaterial implantation, promotion of a robust M1 rather than M2 response – followed, then by promotion at day 3 of M2 – was required for an enhanced regenerative effect [32,33]. Similarly, Liu et al. in a study examining dexamethasone release from a bioglass scaffold, revealed that the number of M1 macrophages was higher than M2 at day 1, whereas the ratio was reversed by day 7 [34]. These studies suggest that the roles and functions of M1 and M2 macrophages are highly sequential and complementary. M1 macrophages facilitate the healing process by phagocytosis and proliferation of inflammatory cells and then M2 macrophage subsequently attenuate M1 activity and promote stabilisation and tissue maturation. It has been demonstrated that pro-inflammatory cytokines produced by M1 stimulate angiogenesis [35,36], whereas anti-inflammatory cytokines from M2 stabilise angiogenesis and orchestrate extracellular matrix assembly [37,38]. Taken together, sequential activation of M1 and M2 macrophages at tissue defects is crucial for modulating inflammation and tissue regeneration.

Bone repair is a complex and well-orchestrated process involving three phases: inflammation, repair, and remodelling (Figure 1). In the inflammation phase, immune cells arrive at the defects immediately after bone fracture. Neutrophils, the first line of immune cells, phagocytose bacteria and debris, and degrade damaged tissue. The M1 macrophages attenuate the function of neutrophils and phagocytosing apoptotic cells and foreign bodies. Simultaneously, ruptured blood vessels during the fracture cause a hematoma to form around the fracture site. The hematoma is characterised by local hypoxia, acidity, mechanical stresses on cells caused by deformation and movement, and increased expression of calcium, phosphorous and alkaline phosphatase [39]. This hypoxia and low pH of the micro-environment promote M2 polarisation and osteogenic/chondrogenic differentiation of stem cells [40–44].

Within the hematoma, fibrin granulation tissue begins to form, and stem cells are recruited to the area. Under the conditions of various growth factors/signals, the stem cells differentiate into fibroblast, chondroblasts, and osteoblasts. In the case of endochondral bone repair, chondroblast/chondrocytes form a cartilaginous matrix and subsequently chondrocytes undergo hypertrophic differentiation. After apoptosis of hypotrophic chondrocytes, blood vessels, macrophages, osteoclast, and osteoblasts are invaded and migrate into the cartilage matrix. The newly formed blood vessels continue to grow and spread, allowing cells to be active in bone formation [45].

At the late phase of remodelling, the centre of the callus is replaced by compact bone, whereas the edges are replaced by lamellar bone. Here, a balance between osteoblasts and osteoclasts for bone resorption and formation play an important role for regenerating normal bone tissue structure and function [46].

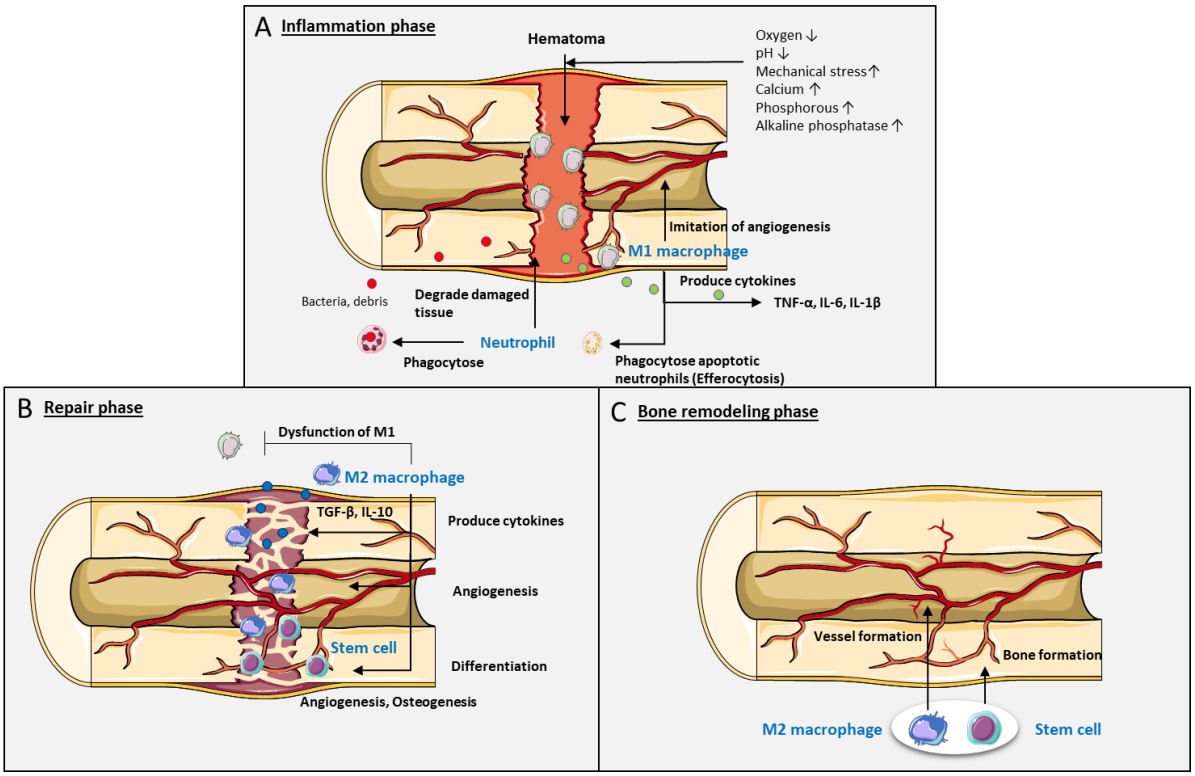

Figure 2. A complex and well-orchestrated bone repair process involving three phases; inflammation, repair, and remodeling phases.

2.2. Macrophages influence osteoblast and osteoclast behaviours

Macrophages play an important role in modulating inflammation and stimulating tissue repair including through an influence on the differentiation of osteoblasts and osteoclasts, the cells responsible for bone tissue formation and remodelling. Osteoblasts derive from skeletal stem cells (SSC) and osteoclasts and macrophages from hematopoietic stem cell (HSC) lineages. That both SSC and HSC systems originate out of the bone marrow organ is suggestive for the intimate association and co-regulation of these respective cell types. The first in depth report on the interaction between immune cells and bone cells was by Horton et al in 1972 [47]. Since then, the term “osteoimmunology” has been coined by Arron and Choi in 2002 [48], and the dynamic crosstalk between macrophage and bone cells has been a subject of intense investigation.

Macrophages themselves can be stimulated to differentiate into osteoclasts by both macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor- κB ligand (RANKL). Due to their shared ontogeny, macrophage and osteoclasts have common cytokines, receptors, and transcription factors that enable a close interaction between the two cell types. In particular, the macrophage phenotype plays an important role in promoting or suppressing osteoclast differentiation. *In vitro* studies have demonstrated that both IL-10, a typical M2 anti-inflammatory cytokine, and IL-4, an M2 macrophage inducer, inhibit the early stages of osteoclastogenesis by reducing the expression of nuclear factor of activated T cells 1 (NFATc1), RANKL, and M-CSF and by interfering with NF-κB

binding [49–51]. In addition, arginase-1, a prototypic M2 marker, is down-regulated during osteoclast differentiation [52]. Consistent with their characteristic catabolic function, M1 macrophages have been shown to promote osteoclastogenesis *in vitro*, for example through IL-6 and TNF-α which activate JAK2 and NF-κB pathways, respectively [53,54]. Interestingly, M1 signalling via IFN- γ and IL-12 has also been shown to inhibit osteoclast differentiation [55,56] however differences between the signalling profiles of macrophages from a range of sources make it hard to draw conclusions about the *in vivo* significance of these contrasting responses [57].

As well as inhibiting osteoclastogenesis M2 macrophages are known to promote osteogenesis. M2 macrophages produce transforming growth factor (TGF)-β1, bone morphogenetic protein (BMP)-2, and insulin like growth factor (IGF)-1 each of which play key roles in bone progenitor cell recruitment, differentiation and mineralisation [58–60]. Furthermore, M2 polarised macrophages have been shown to promote angiogenesis *in vivo* by downregulation of metalloproteinases (TIMP-1) [61] and IL-4 induced-M2a and IL-10 induced-M2c macrophages promote angiogenesis via placental growth factor (PIGF) and fibroblast growth factor (FGF) pathways, respectively [62].

Not only do macrophages modulate SSC regenerative functions, but SSCs correspondingly modulate macrophage inflammatory functions. SSC - macrophage cross-talk stimulates SSCs to produce prostaglandin E2 (PGE2), which in turn increases IL-10 production from M2 macrophages [63]. Regardless of cell sources, both *in vitro* and *in vivo* studies have demonstrated that SSCs dramatically suppress M1 function and enhance M2 production of anti-inflammatory cytokines via juxtacrine and paracrine signals [64–67]. These interactions underline the central significance of intercommunication between macrophage and skeletal stem cell populations and suggest new potential targets to leverage this synergy in tissue regeneration strategies.

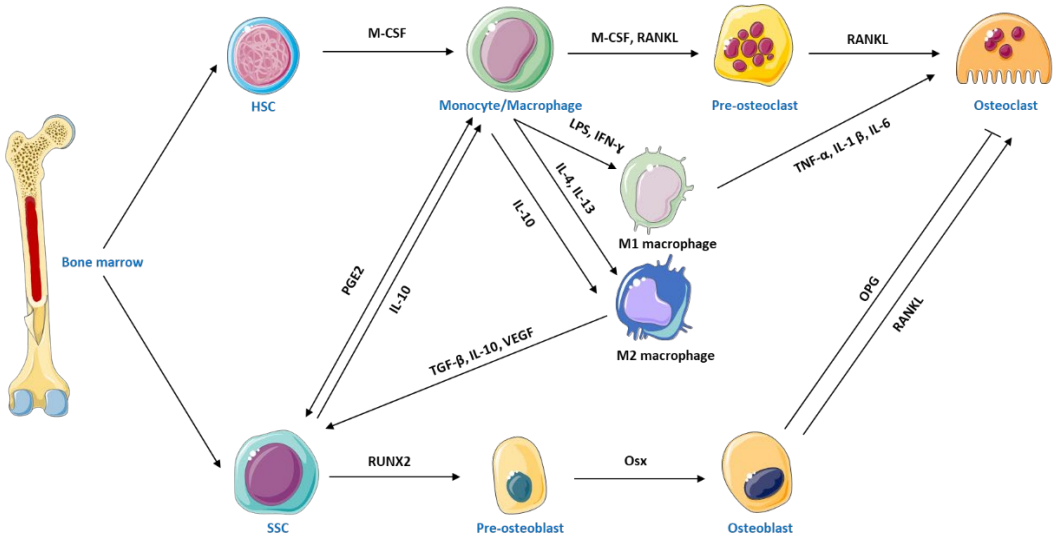

Figure 3. Regulation of BMMS differentiation plasticity. A simplified diagram for the differentiation of SSCs or HSCs into macrophages, osteoclast, and osteoblast

3. Macrophage responses to implanted materials

A wealth of data over three decades has demonstrated that implantation of a biomaterial will induce a host immune reaction to protect the host from the biomaterial and maintain homeostasis [68]. Within a few seconds of implantation, blood from the damaged vessels surround the material and host plasma components and proteins competitively and spontaneously adsorb onto the biomaterial surface [69]. This process produces a blood clot-based provisional matrix around the biomaterial [70,71] rich in cytokines, growth factors, and chemoattractants capable of recruiting immune cells [72].

Neutrophils, the first immune cells infiltrating an implantation site, characterise the acute inflammation response. The neutrophils produce extracellular traps (NETs), reactive oxygen species (ROS) and metalloproteinases to facilitate the phagocytosis of bacteria and other microbes introduced upon implantation, and to degrade damaged tissue [73]. In addition, the neutrophils also secrete CC–chemokines and cytokines responsible for monocyte recruitment and monocyte to macrophage differentiation [74].

3.1. Phagocytosis

Following the acute inflammation modulated by neutrophils, macrophages are recruited to the implants and mediate the chronic inflammation process. The macrophages (Greek: Macro – 'large'; phage – 'to eat') represent the first line of defence to infectious microorganisms through their phagocytic activities. Phagocytosis is defined as the cellular engulfment of large particles, usually over 0.5 μm, through an actin-polarisation-dependent process [75,76]. Numerous studies exploring the phagocytic response of macrophages to particles *in vitro* have demonstrated the important influence particle physicochemical characteristics, such as size, shape, charge and surface functionality play in influencing uptake by macrophages (Table 1)

3.1.1. Particle size

Controlled *in vitro* studies of macrophage phagocytosis have consistently shown that particle size across a range of scales can have a significant influence on the efficiency with which macrophages engulf particulate materials. Studies with polystyrene particles in the size range of 100 - 1100 nm, demonstrated up to a 10-fold increase in rate of uptake of particles in the larger size range compared to the smallest 100 nm particles [77]. This agrees with another study of polymer nanoparticles ranging from 130-1500 nm, which also demonstrated that larger particles permitted a higher rate of internalisation [78]. At a larger size range, studies using polystyrene micro spheres with diameters ranging from 1 to 6 μm, found that particles with diameters between 2- 3 μm were phagocytosed more readily than particles at either end of this size range [79]. Similar conclusions have been reported in a number of other studies [80,81].

While the molecular mechanism(s) at play remain unclear, a possible explanation for the significant size dependence of particle phagocytosis may be associated with actin cytoskeleton function. The cytoskeleton is responsible for the protrusion of cell membrane which forms a cup-shaped membrane fold around the particles to enable engulfment [82]. Thus, larger particles will necessitate

cell membrane receptors to extend over a greater distance resulting in the cell membrane taking a longer timeframe to envelop the particles. In contrast, smaller particles will require the recruitment of fewer signalling components before their engulfment, resulting in less time for inhibitory signals to modulate the process [83]. In the case of smaller sub-micron particles, internalisation relies on endocytosis mechanisms relying on clathrin and caveolin proteins [84].

3.1.2 Particle shape

Material particle shape is a further interesting component influencing uptake by macrophages. Microsphere particles showed enhanced phagocytosis rates compared with needle- or rod-shaped particles [85]. Two other studies observed that worm-like particles were also poorly phagocytosed [86,87]. The mechanism for shape dependence of phagocytosis is also associated with coordination of the actin cups [88]. Local shape of particles at the point of contact with the cell membrane also determines whether the actin structure is generated to allow the membrane to move over the particle [86,88]. For example, Lu et al. revealed that the influence of particle shape on phagocytosis was determined by the local orientation and geometry at the initial cell-particle contact point. The authors found macrophage-attached particles with high curvature at point of contact created a barrier to the formation of a phagocytic cup and inhibited the uptake process [85].

3.1.3 Surface chemistry

Material surface chemistry of the particle also plays an important role in influencing the phagocytic process. In particular, increased hydrophobicity results in enhanced uptake with hydrophobic microspheres considerably more susceptible to phagocytosis than hydrophilic analogues [80,89]. Similarly, phagocytosis has been found to increase with zeta potential for both the negatively charged and positively charged surfaces with the lowest uptake observed when zeta potential was zero [80,89]. Interestingly, two studies observed no significant difference between uptake efficiency of cationic and anionic particles [89,90], although for microspheres with a primary amine, sulfate, hydroxyl, or carboxyl groups, the primary amine functionalised microspheres were the most effectively phagocytosed possibly as a consequence of the surface charge density and the electrophoretic softness of the surface [81].

Functionalisation of the material surface via protein immobilisation/polymer coating can also affect macrophage phagocytosis. Polyethylene glycol (PEG) coating are widely used in the field of drug delivery to avoid phagocytosis. The hydrophilic PEG provides a shield around the coated particles, effectively preventing non-specific protein adsorption and reducing cell adhesion [91,92]. However, the uptake efficiency is dependent on the PEG chains at different grafting densities [93].

These studies demonstrate the pronounced effects specific physicochemical properties of particles can have on the rate and efficiency of macrophage phagocytosis. These insights are important, not only for informing strategies that rely on nano-/macro-sized particles for drug delivery, but also for the design of larger scaffolds, of which macrophage mediated degradation commonly entails fragmentation into particulate matter. Understanding how degradation products may impact

macrophage phagocytosis could inform new approaches to modulate and engage the chronic inflammation response following biomaterial implantation and degradation *in vivo*.

Table 2. Effect of material physicochemical properties on macrophage uptake.

Characteristics	**Parameters**	**Materials**	**Summary**	**Ref.**
Size	100 –1100 nm	Polystyrene	Phagocytosis steadily increases with increasing particle diameter.	[77]
	130 –1500 nm	Polyhexylcyanoacrylate, Polymethylmethacrylate (PMMA), Human serum albumin	A larger diameter was more easily phagocytosed.	[78]
	1– 6 μm	Polystyrene	Particles possessing diameters of 2–3 μm exhibited maximal phagocytosis	[79]
	0.5– 4.6 μm	Polystyrene	Maximal uptake in the range 1-2 μm	[80]
	0.3–10 μm	Polystyrene	Macrophages engulfed effectively polystyrene micro-spheres with 1 μm diameter	[81]
	31.7 ±14.5 nm, 2.7 ± 1.2 μm	Polyacrylamide (PA)	Micro-size particles showed higher phagocytic uptake than nano-size ones	[94]
	30 – 100 nm	Poly(ethylene glycol)-bl-poly(propylene sulfide)	Smaller particles showed significantly less uptake by the macrophages.	[90]
	0.16 –20 um	PMMA	Reduced viability of cells following phagocytosis of smaller particles	[95]
	0.5 – 4.5 μm	Polystyrene	For small particles (0.5 and 1 μm), a smaller percentage of the macrophages were phagocytic, but they internalised a relatively large number of particles.	[96]
Shape	Sphere, Rod, Needle	Cadmium telluride (CdTe) quantum dot-cystine composites	Microspheres exhibit the highest degree of internalisation and the fastest phagocytosis rate.	[85]
	Spheres, Worms	Polystyrene	Worm-like particles that exhibit negligible phagocytosis when compared to spherical particles.	[86]
	Worm-shape with 22 nm and 60 nm length	PEGylated filomicelles	Longer filomicelles exhibit reduced phagocytosis.	[87]
	Spheres, Oblate Ellipsoids, Prolate Ellipsoids	Polystyrene	Oblate ellipsoids were phagocytosed more efficiently than either spheres or prolate ellipsoids	[97]
Surface chemistries	Hydrophobic Hydrophilic Cationic Anionic	Polystyrene, Phenylated polyacrolein, Cellulose	Hydrophobic microspheres were more readily phagocytosed than hydrophilic surface ones. No significant difference in phagocytosis between cationic and the anionic surfaces	[80, 89]
	Cationic Anionic	PEGylated particles	No effect of charge on uptake.	[90]

	Primary amine Sulfate Hydroxyl Carboxyl groups	Polystyrene	Macrophages trapped microspheres with primary amine and carboxyl groups very effectively.	[81]
	PEG Brush PEG mushroom	PEGylated particles	A high PEG density coating (brush) resulted in a 200-fold and 1.5-fold decrease in clearance versus a non-coated (bare) and low PEG density coating surface (mushroom), respectively	[93]
	Fc ligands immobilisation (1:1, 1:2, 1:10, 1:50)	Polystyrene	The number of microparticles internalised per cell was significantly greater for the higher Fc density conditions of 1:1 and 1:2 than the lower Fc density conditions of 1:10 and 1:50.	[96]
	PEG coating	Silica particles	PEG coating reduced the macrophage phagocytosis of particles	[98]

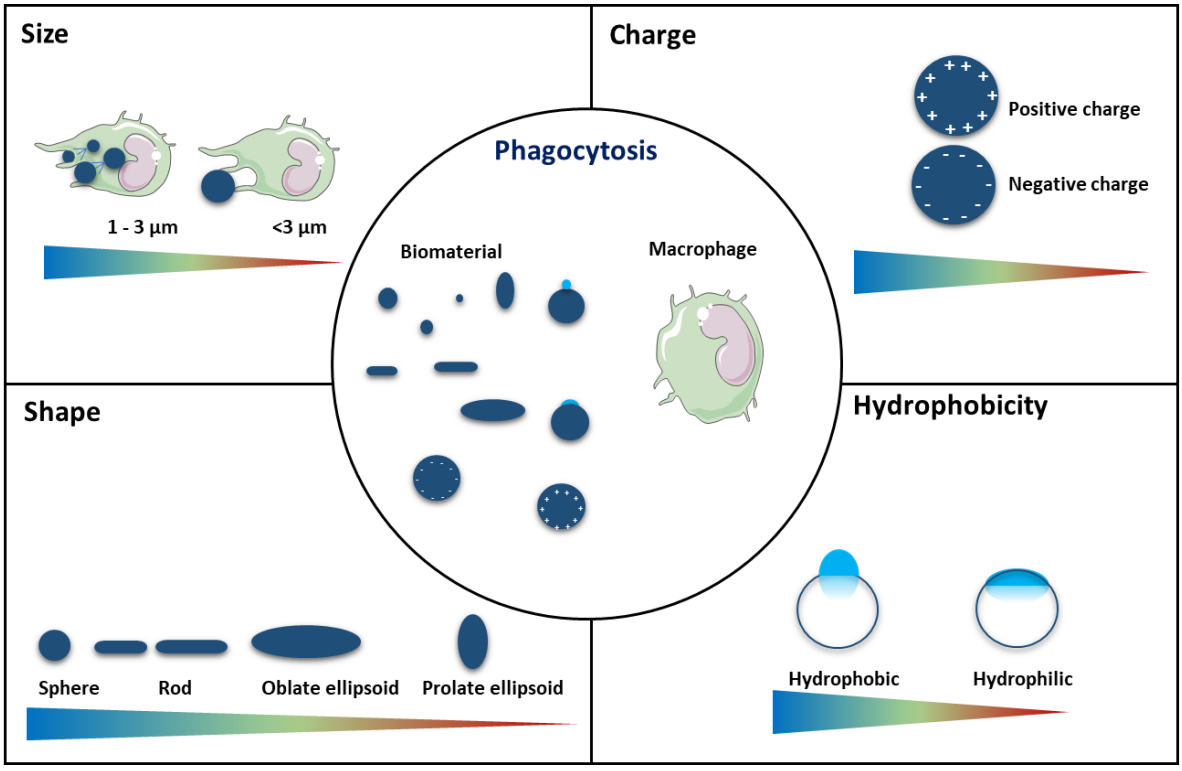

Figure 4. Overview of macrophage phagocytosis behaviour associated with biomaterial physicochemical properties.

3.2. Foreign body response to biomaterials

Central to the process of early inflammation, as indicated, is the recruitment and activation of macrophages, followed by phagocytosis of the implanted material. For particles larger than ~10 µm

macrophages will adhere to the material, fuse to form foreign body giant cells (FBGCs), and release mediators of degradation including reactive oxygen intermediates (ROIs), degradative enzymes, and acidification. Local degradation of implanted materials by FBGCs is facilitated by the formation of sealed zones between the cell membrane and the implant in a manner similar, though not identical to, osteoclast resorption of bone [99]. Due to this response being activated by particles of sizes beyond that which can be phagocytosed by macrophages this process is known as frustrated phagocytosis [100].

Biomaterial degradation via degradation mediators is dependent on the material chemistry. Thus, polyethene, typically found in artificial joints and polypropylene used as a suture material may undergo degradation by the ROIs resulting in embrittlement and fragmentation, whereas some natural and synthetic polymers are more readily degraded by catabolic enzymes and often with complete elimination [101]. In addition, as indicated above, the surface chemistry of the biomaterial can facilitate apoptosis that consequently induces dysfunction of macrophages or biomaterial.

3.3. Rejection or remodelling

Modulation of the inflammation response to a biomaterial is a crucial process for inducing tissue repair. During the process, the macrophage plays an important role in determining chronic inflammation (foreign body rejection) or inflammation resolution (tissue remodelling). As a result, the macrophages can be an inflammation mediator or/and resolution inducer depending on their phenotype and function (Figure 5).

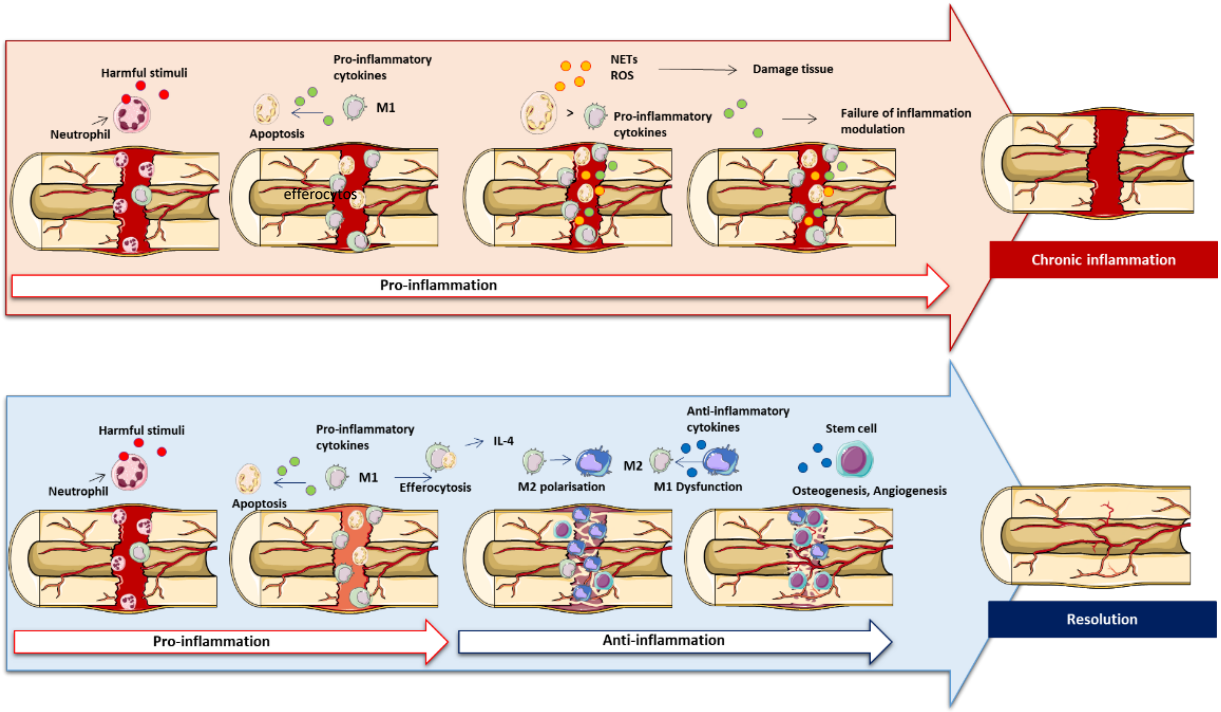

Figure 5. Crucial role of macrophages in inflammation modulation.

3. 3.1. Role of macrophages on neutrophil function

Neutrophils are the first immune cells recruited to the implant to eliminate harmful stimuli, such as debris and pathogens, and subsequently undergo apoptosis after approximately 1 day in circulation. M1 Macrophages, recruited by neutrophil chemokines, phagocytise apoptotic neutrophils, in a process termed "efferocytosis". This process is a further important determinant of macrophage phenotype and function. Voll et al. demonstrated that co-culture of macrophage and apoptotic cells increased anti-inflammatory cytokines (IL-10) and decreased pro-inflammatory cytokines [102]. Other studies have shown that efferocytosis induced suppression of TNF-α and CXCL-8 and increased TGF-β and IL-10 resulting in an anti-inflammatory microenvironment [14,49,103]. However, if apoptotic cells are not cleared, the cells progress to late apoptosis characterised by a disrupted cell membrane and loss of cell integrity called "necrosis". Damage-associated molecular patterns (DAMPs) are released from activated innate immune cells and induce a pro-inflammatory response during the process of necrosis [104,105]. These molecules cause chronic inflammation, exacerbated tissue damage, organ dysfunction and systemic autoimmune diseases [106,107]. The phagocytosis of apoptotic neutrophils by macrophages is therefore essential for resolution of inflammation.

3.3.2. Role of macrophages on Foreign Body Giant Cells (FBGCs)

The FBGC reaction is pivotal in the clearance or otherwise and eventual acceptance of a material in the body. Absence of phagocytosis can result in persistence of the material at the tissue site and FBGC generation of ROSs, degradative enzymes to generate an acidic environment at the interface of biomaterial-tissue [108] resulting in a collagenous and avascular fibrous capsule surrounding the interface and subsequent chronic inflammation. Anderson and co-workers have shown that the formation of FBGCs can be induced by IL-4 and IL-13 cytokines and subsequent cell fusion [49,109]. Interestingly, these cytokines produced from master cells, basophils and T helper (Th) cells can polarise macrophage phenotype from M1 to M2 [110]. Moore et al. demonstrated that FBGCs expressed both M1 and M2 macrophage markers [111] whilst other studies revealed that both M1 and M2 related genes were upregulated during the ensuing foreign body response [112,113]. Conceptually this implies that biomaterial-adherent macrophages initially exert pro-inflammatory influences which are subsequently down-regulated by IL-4 and/or IL-13 cytokines in the process of M2 macrophage activation. The resultant biomaterial-adherent FBGCs may therefore be immune-regulatory, host defensive, and tissue reparative in function [7].

4. Modulation of macrophage phenotype through biomaterial design

Critical-size bone defects remain a major clinical orthopaedic challenge given the inability of these large bone defects to repair spontaneously within a patient's lifetime [114]. Implantable and injectable hydrogels and scaffolds offer a promising avenue to address this unmet bone repair challenge. In essence, three-dimensional and biocompatible materials present a microenvironment to facilitate bridging of the defect gap, cell infiltration, attachment, proliferation, differentiation and subsequent generation of new bone tissue. Incorporating bioactive features such as biochemical, mechanical and

physicochemical properties into the design of biomaterials is fundamental to achieving an effective regenerative response. Thus, a plethora of studies have focussed on biomaterial modulated stem cell differentiation and implications therein for tissue regeneration – both *in vitro* and *in vivo*. With the recognition of the pivotal role played by the macrophage in the early response to the biomaterial and in mediating the transition from inflammation to regeneration, the role of the biomaterial in modulating macrophage phenotype and function has emerged as an increasingly important aspect of biomaterial design. Information on the effects of a wide range of physicochemical and mechanical properties on macrophage phenotype and behaviour and the impact on tissue regeneration is growing apace and is reviewed below.

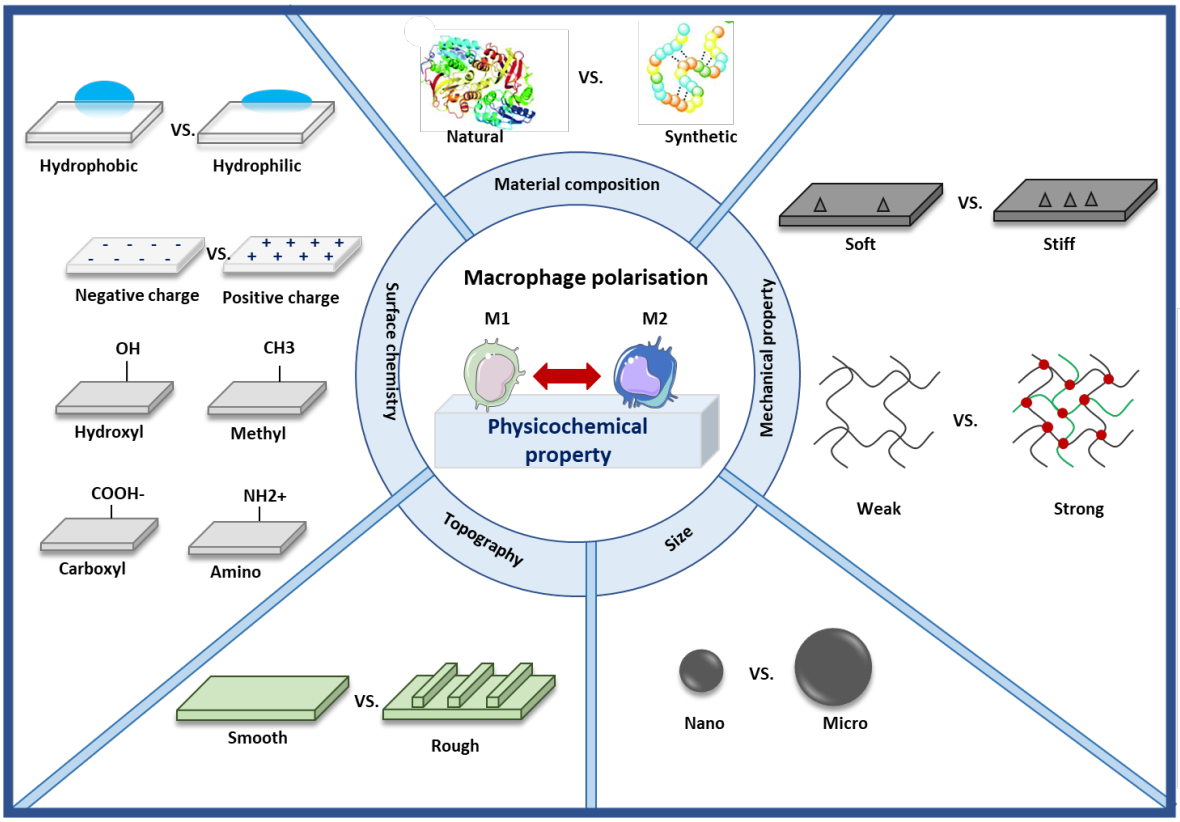

Figure 6. Physicochemical properties of biomaterial modulate macrophage polarisation

4.1.1. Surface chemical properties

Material surface properties play an important role in influencing the initial inflammatory responses. While simplistic rules tend not to apply across diverse materials, where material-specific modulation of surface properties have been investigated under comparable conditions, strong effects of such modifications on macrophage phenotype are frequently reported.

Hydrophilic and hydrophobic properties have been shown to strongly influence cell adhesion and functions across a wide variety of adherent cell types. Fibroblast (L929) [115] and mouse osteoblastic cells (MC3T3-E1) [116] displayed almost no cell adhesion and slow proliferation on hydrophobic

surfaces, whereas adipose-derived stem cells [117] displayed higher cell attachment and adipogenic differentiation on superhydrophobic substrates – though several differences in experimental conditions between these studies pertain. Similarly, surface hydrophobicity has been shown to affect macrophage adhesion, phagocytosis and inflammation modulation. For example, hydrophobic surfaces have been observed to enhance cell adhesion and phagocytosis compared to hydrophilic surfaces [80,89]. In terms of macrophage phenotype and modulation of function, macrophages were found to produce lower levels of pro-inflammatory cytokines on hydrophilic titanium (Ti) surface and produced significantly higher levels of anti-inflammatory cytokines compared to macrophages on hydrophobic surfaces [118]. Other studies have shown comparable findings with hydrophilic Ti inducing an M2 macrophage phenotype and upregulated anti-inflammatory cytokine gene levels [119–122]. Thus, surface hydrophilicity may influence macrophage phenotype and macrophage cytokine production.

Ivanovski and colleagues investigated the effect of hydrophilic/hydrophobic Ti surface on the macrophage response and osteogenic potential/bone formation potential using *in vitro* and *in vivo* studies [119,123]. When human osteoblasts were co-cultured with hydrophilic Ti surface-induced macrophage culture media, TGF-β/BMP pathway associated gene expression levels were significantly upregulated, compared to expression levels following co-culture with hydrophobic Ti surface-induced macrophage culture media. In an *in vivo* bone defect study, additional M2 macrophages were present on the hydrophilic Ti implant, resulting in enhanced bone formation compared to the hydrophobic Ti implants. Although these studies indicate hydrophilic surfaces affect M2 macrophage polarisation, this observation is not ubiquitous for all materials. For instance, hydrophobic polystyrene and methacrylate gellan gum hydrogels tend to polarise macrophages towards an M1 phenotype and increase pro-inflammatory cytokines/transcription factors [124,125]. These divergent effects of hydrophobicity in different materials, highlight the complexity of potential uncontrolled influences across different surfaces and the difficulty of drawing generalisations between diverse materials. We note also in this context that cell-surface interactions *in vivo* are mediated by the protein corona that forms rapidly upon implanted surfaces in contact with blood. Hence, at least *in vivo*, cell responses to surfaces may largely be governed by the specific composition of proteins adsorbed on various surfaces rather than directly by cell-surface interactions *per se*.

Similarly surface charge can also affect macrophage phenotype and function in diverse ways. For instance, neutral and anionic polyethylene terephthalate (PET)-based materials promoted the production of pro-inflammatory cytokines, including IL-8, IL-1β, IL-6 and TNF-α, and suppressed anti-inflammatory cytokine production [126]. In contrast, a positively charged surface material enhanced anti-inflammatory cytokine production. Similarly, a positively charged amine-terminated nanorod on PEO induced M2 phenotype polarisation, whereas carboxylic aid-terminated nanorods, with a negative surface charge, displayed M1 macrophage function [127]. In other studies on hyaluronic acid, a negatively charged material, Rayahin et al. demonstrated that the molecular weight of hyaluronic acid is important in determining the modulation of macrophage phenotype. The authors showed that low

molecular weight hyaluronic acid hydrogels induced the M1 phenotype and enhanced pro-inflammatory responses, whereas high molecule weight hyaluronic acid increased M2 polarisation [128]. The mechanism(s) underlying this effect of hyaluronic acid molecular weight remains unclear, though differences in the ability of macrophages to internalise/phagocytose gel fragments may conceivably play a role. These observations indicate the importance not only of surface chemistry but also chemical structure/composition of biomaterials in effecting divergent macrophage responses.

4.1.2. Material properties

There are a variety of hydrogels and scaffolds derived from synthetic and natural materials. As shown in Table 2, the physiochemical properties of a range of synthetic materials have been widely modified to enhance their biocompatibility and regenerative potential. A natural material is defined as a component derived from native extra cellular matrix (ECM) or other biological product, such as collagen, alginate, chitosan, elastin, fibrin, fibrinogen, silk etc. Probably as a consequence of the similarity to the ECM, it has been demonstrated that most natural polymers, in particular, chitosan, fibrin, fibrinogen, and silk, enhance anti-inflammatory cytokine production [72,129–133]. It is also possible the radical oxygen species-scavenging properties of these natural polymers induced M2 polarisation [134–136].

Among the various natural polymers, collagen is the most frequently used material for tissue regeneration, collagen being the major protein within the ECM. With regards to macrophage response to collagen, most studies to date have focused on differences in mechanical properties between various crosslinked collagen gels [137–139]. Within bone tissue regeneration, collagen has been widely used in combination with inorganic materials. Recent studies have examined the macrophage response to collagen-based composite hydrogels [140–142], in particular, collagen scaffolds incorporating different size (5 μm vs. 100 μm) and shape (needle vs spherical) hydroxyapatite particles, and extra- vs intra-fibrillar mineralised collagen scaffolds. In these studies intrafibrillary-mineralised collagen and 5 μm needle-shaped hydroxyapatite collagen scaffolds enhanced M2-associated anti-inflammatory cytokines and gene expression [140,142]. Another study showed that mineralised collagen increased anti-inflammatory cytokine gene expression, whereas hydroxyapatite in the absence of collagen, induced pro-inflammatory secretion [141]. These studies implicate a macrophage response more closely associated with deposited inorganic materials on collagen scaffolds rather than the collagen protein itself.

Decellularised extracellular matrix (decellularised ECM) is a natural composite material consisting of native ECM proteins and polysaccharides, such as collagen, elastin, proteoglycans, and glycosaminoglycans. Removal of cells from the matrix can still create a scaffold that retains the native architecture and proteins. Decellularised ECM has been found to elicit a favourable immune environment and ability to promote macrophage polarisation [143,144]. Badylak and colleagues have examined macrophage polarisation on decellularised ECM scaffolds derived from diverse tissue sources, including small intestine submucosa, urinary bladder, esophagus, skin, and colon. With the

exception of skin ECM, other tissue sources of ECM scaffolds promoted a shift toward M2 with decreased iNOS (M1 marker) expression. The mechanism by which these decellularised scaffolds shifted macrophage phenotype from M1 to M2 is currently unclear. It is possible that rheological properties and protein components in ECM scaffolds may affect macrophage phenotype, however differences in decellularisation efficiency are likely to be significant as the presence of cell remnants and mitochondria would be expected to elicit a strong pro-inflammatory response [144,145]. This effect was indicated by another study demonstrating that different decellularisation methods were associated with macrophage phenotype [146].

4.1.3. Topography

Topographical properties provide important cues in cellular responses including cell adhesion, spreading, proliferation and differentiation [147]. A wealth of studies have examined the relationship between macrophage behaviour and topographical cues derived from biomaterial and - particularly metal - implant surfaces. Rougher surfaces of titanium typically enhanced M1 macrophage function in pro-inflammatory cytokines within 24 hours [148,149]. Hotchkiss et al. showed that a rough surface promoted pro-inflammatory cytokine production after 24 hours incubation. However, macrophages on a rough surface for 72 hours of culture produced lower levels of pro-inflammatory cytokines and enhanced anti-inflammatory cytokines compared to the macrophages on a smooth surface [120]. Chen et al. reported macrophage response to nano and micro-parallel gratings (250 nm – 2 μm) imprinted on commonly used biomedical polymers, such as poly (ε-caprolactone) (PCL), poly(lactic acid) (PLA), and poly(dimethylsiloxane) (PDMS) [150] with different topographies again modulating divergent macrophage morphology and cytokine secretion profiles.

Cell shape has also been shown to be associated with macrophage phenotype [151]. For example, macrophages displaying an elongated profile displayed increased expression of the M2 phenotype marker and reduced pro-inflammatory cytokine production. In contrast, M1 polarised macrophage induced using LPS and IFN-γ displayed a round, pancake-like morphology. The effect of cell morphology on macrophage phenotype has been observed on engineered patterns on the surface of composite scaffolds. Li et al. evaluated macrophage phenotype on different surface roughness of mineralised collagen scaffolds [152]. While minerals were deposited randomly on the collagen surface, macrophage phenotype and function in the production of inflammatory cytokines corresponded to macrophage morphology indicating the control of macrophage morphology through design suggests an interesting approach to the modulation of macrophage phenotype.

4.1.4. Mechanical properties

The mechanical property of a scaffold is a key component in bone regeneration with the role of matrix stiffness garnering considerable attention given the importance of this material property in the osteogenic differentiation process [153]. Patel et al reported that macrophage elasticity is dependent on substrate stiffness and mediated by actin polymerisation and Rho-GTPase activity [154]. The authors

found when macrophages were cultured on stiff gels (150 kPa), cell filopodial projections were increased, and actin fibers were more organised in comparison to macrophages cultured on soft gels (1.2 kPa). These results indicate cell elasticity and phagocytic ability may be associated with substrate stiffness via actin polymerisation. Furthermore, Sridharan et al. showed that stiff PA gels promoted M1 macrophage function, while softer gels induced M2 polarisation [155]. In support, of such phenotype modulation, macrophages on a soft PA gel typically displayed enhanced pro-inflammatory cytokine production compared with macrophages cultured on stiff PA gels. *In vivo* studies harnessing the same gels revealed more M1 macrophages were present on the soft gel, whereas significant numbers of M2 macrophages were found on the stiff PA gel [156].

Interestingly, the influence of material stiffness appears perhaps to be a more generalisable material property than others discussed in this section. As shown in Table 2, stiffer materials generally tend to promote macrophage M2 polarisation, however, given the number of factors that can affect these results (material type, stiffness range, macrophage source and study models (*in vitro* and *in vivo*)), the effect of stiffness on macrophage phenotype remains a complex area demanding further *in vitro* and *in vivo* quantitative studies to delineate the effects of mechanical stimuli on macrophage phenotype. In contrast to static *in vitro* culture, for example, where the mechanical stimuli experienced by a cell is limited to the cell's own perturbation of its filopodia in response to materials of varying stiffness, cells *in vivo* are constantly exposed to a much wider range of dynamic mechanical stimuli and stresses, transmitted from the host by the surrounding ECM or from neighbouring cells. For this reason extrapolation of *in vivo* material performance from, particularly static *in vitro* should be undertaken with considerable caution.

Table 2. Selected studies on the effect of material stiffness on macrophage phenotype.

Stiffness	Materials	Study model	Summary	Ref.
1.4, 10, 348 kPa	PA	*In vitro*; THP-1	Macrophages secreted most TNF-a on 1.4 kPa and least on 348kPa	[157]
130, 240, 840 kPa	PEG	*In vitro;* RAW264.7 *In vivo*; Subcutaneous implantation in mice	*In vitro*; Softer hydrogel increased TNF-α, IL-6, and IL-1β expression. *In vivo;* Hydrogels with lower stiffness recruited more macrophages with a thicker FBR leading to impaired repair.	[158]
27, 58, 74, 119 Pa	Collagen *crosslinker;1-ethyl-3-3-dimethyl aminopropyl carbodiimide (EDAC)	*In vitro*; Human blood-derived macrophage	Stiffer gel resulted in a shift to M2 phenotype	[159]
0.5, 1, 1.5 kPa	Collagen	*In vitro*; THP-1	Genipin crosslinked collagen suppressed both pro-and anti-inflammatory responses, whereas	[160]

	*crosslinker, EDAC, genipin		EDAC crosslinking promoted both pro-and anti-inflammatory responses.	
11, 88, 323 kPa	PA	*In vitro*; THP-1	Stiff gels promoted M1 phenotype with upregulated pro-inflammatory cytokine genes, whereas soft and medium stiffness gel induced M2 polarisation.	[155]
0.1, 3.4, 10.3 kPa	PEG	*In vitro*; Cord blood-derived macrophage	Stiffer hydrogels exhibited M2 surface marker patterns with increased M2-associated growth factors.	[161]
2.5, 35, 63.5 kPa	PA	*In vitro*; Mouse bone marrow-derived macrophage *In vivo*; Subcutaneous implantation in mice	*In vitro*; Soft gel enhanced pro-inflammatory cytokine production, whereas stiffer gel promoted anti-inflammatory cytokines *In vivo*; More M1 macrophages were around the low stiffness hydrogels, whereas stiffer gel was surrounded by M2 macrophages	[156]
1,2, 140 kPa	PA	*In vitro*; RAW 264.7 U937, Human alveolar macrophages	With increasing substrate rigidity, production of reactive oxygen species increased, but TNF-a was decreased.	[154]

4.2. Nano/Macro particles

The application of nano-/macro-sized particles, either alone or in a scaffold composite, has been widely explored in the tissue regeneration field, particularly for targeted delivery of bioactive molecules to aid therapeutic efficacy and minimise toxicity [162,163]. Over the past few decades, a wide range of nanoparticles have been developed from different materials including gold, silica, carbon, metallic, ceramic, and polymers. Similar to biomaterial-based hydrogel materials, the physicochemical properties of such particles including size and surface modification have been found to influence not only the phagocytic activity of the macrophage, but also to have pronounced effects on macrophage phenotype. Having described above the influence surface chemistry plays in modulating phenotype here we review compositional and size effects on macrophage polarisation (Table 3).

Much work into the effects of particles on macrophages in the context of bone, has been undertaken, not directly in relation to regenerative medicine, but rather to address the challenge of wear-particle induced inflammation [8]. Prior to the improvement of bearing coupling technologies, metal or ceramic particles derived from wear which result in inflammation and osteolysis around the implant were the principle cause of joint revision surgery [164,165]. Insights from these studies have however made significant contributions to understanding acute and chronic inflammatory responses to implanted biomaterials for regenerative medicine – particularly in the context of more recent particle-based drug delivery or nanocomposite technologies [8].

4.2.1. Titanium particles

Titanium (Ti) and Ti-based alloys are widely used as dental and orthopaedic implants given their good biocompatibility and bone tissue interfacing properties. Scherbart et al. found that smaller particle sizes (25 nm) of TiO_2 at high concentration promoted iNOS pro-inflammation, whereas there was no difference of particle size and concentration on IL-β and TNF- α [166]. In addition, it has been reported that low concentrations of TiO_2 particles (10-20 nm size) did not affect macrophage polarisation and cytotoxicity, whereas higher concentration induced macrophage phenotype shift to M1 macrophage followed by oxidative damage and apoptosis [167]. These studies suggest that the size of TiO_2 particles can affect macrophage response, however, it is more likely that particle concentration is a key factor to modulate macrophage phenotype. Interestingly, Ti particles produced enhanced pro-inflammatory cytokines and co-culturing macrophages with osteoblasts resulted in pro-inflammatory cytokine generation [168]. In this regard, it has been shown that other ceramic particles, such as Al_2O_3 and ZrO_2, induced a lower pro-inflammatory cytokine than Ti particles [169] indicating that composition of nanoparticle is an important component in the macrophage response

4.2.2. Ceramic particles

Hydroxyapatite (HA) is a calcium phosphate ceramic widely used in bone tissue engineering given the osteogenic potential of this material. Lebre et al. demonstrated that HA particle shape and size significantly alter the macrophage response [170]. While small needle-shaped particles promoted macrophage pro-inflammatory cytokine production, spherical particles of the same size displayed a decrease in cytokine production *in vitro*. Furthermore, when injected *in vivo*, needle-shaped particles promoted a prolonged inflammatory response, whereas spherical parcels resolved this inflammatory response within 3 days with an enhanced anti-inflammatory response. Nano-size HA particles induced an increase in the M2 macrophage population, whereas micro-size HA increased M1 polarisation [171,172]. Mahon et al. also showed that nano HA particles enhanced M2 polarisation, activating cMaf which is in turn upregulated by the signaling molecule STAT3 [163]. Furthermore, nano-particle treated macrophages promoted osteogenesis of mesenchymal stem cells *in vitro* in an IL-10 dependent manner, demonstrating a direct pro-osteogenic role for this cytokine. From studies using an *in vivo* rat femoral defect model, nano HA functionalised scaffolds significantly enhanced tissue vascularisation and increased bone volume compared to micro-HA scaffolds. Thus, HA particle size appears important in modulating the macrophage phenotype and consequent bone regeneration.

4.2.3. Gold particles

Gold nanoparticles have been explored for tissue engineering application, in particular for their potential use in drug delivery. Gold nanoparticles alone have been implicated in the promotion of osteogenic differentiation of stem cells through p38 MARK pathway, however, differentiation potential has been reported to be dependent on charge, moiety and size of particles [173–175]. While, as above, generalisations between studies of nanoparticles is challenging, reports frequently describe pro-inflammatory effects being inversely related to particle size. For example, increased particle size (over the range 5-60 nm diameter) enhanced macrophage cellular uptake and resulted in reduced pro-

inflammatory cytokine expression [176,177] and in another study pro-inflammatory cytokine production by gold nanoparticles (3-38 nm diameter) was more pronounced in the presence of smaller particles [178]. Another study demonstrated that nanoparticles induced down-regulation of pro-inflammatory cytokine production in IL-β1 stimulated macrophages was more pronounced with smaller particles, though here the effect appeared to be mediated by an extracellular influence on IL-β1 cytokine activity/availability [179]. This observation underlines the importance of distinguishing direct macrophage-nanoparticle interactions from common extracellular sorptive interactions with soluble signalling components when interpreting immunomodulatory influences of nanoparticles, particularly in closed *in vitro* systems.

4.2.4. Silica particles

Silica nanoparticles have also attracted attention as a drug delivery system given their high specific surface area and surface functionalisation potential and widespread use in the medical arena. Bancos et al. found that smaller silica particles increased TNF-α production and greater intracellular accumulation [180]. Kusaka et al. investigated IL-1β secretion from wide range of silica particle sizes (30 – 10000nm) and reported that 30 – 3000 nm silica nanoparticles caused lysosomal damage with an increase of IL-1β. When lysosomal acidification in macrophages was blocked with bafilomycin (a specific inhibitor of vacuolar ATPase), the secretion level of IL- 1β was markedly decreased indicating macrophage polarisation is associated with size dependent-intracellular accumulation.

Table 3. The effect of nanoparticle property on macrophage function.

Materials	Size range	Cell source	Summary	Ref.
TiO_2	25 nm 250 nm	RN8383 (rat cell line)	25 nm: iNOS (pro-inflammatory marker) ↑ No difference in IL-1β and TNF- α	[166]
	10-20 nm	Mouse spleen-derived macrophage	Low concentration: no effect on macrophage polarisation and cytotoxicity High concentration:M1 polarisation	[167]
	0.9-1.6 μm <20 μm	THF-1, Peripheral blood-derived macrophage	Pro-inflammatory cytokine level in Ti: primary macrophage (blood) > cell line (THF-a) Pro-inflammatory cytokine level from primary macrophage: Ti > TiO_2	[168]
Hydroxyapatite	0.1, 5, 20, 100 μm (needle-spherical-shape	BMDM	Needle-shape (5 μm): Pro-inflammatory cytokine ↑ Spherical shape (5 μm): Pro-inflammatory cytokine ↓	[170]
	23 nm	RAW 264.7, Mouse peritoneal macrophage	M2 macrophage polarisation ↑	[171]
	1.3 μm	Human blood-derived macrophage	M1 macrophage polarisation ↑	[172]
	15-100 nm 1–2 μn	Human blood-derived macrophage	M2 macrophage polarisation ↑	[163]
Gold	12, 25, 60 nm	RAW 264.7	60 nm: cellular uptake ↑ , inflammatory response ↓	[176]
	5, 13, 45 nm	RAW 264.7	5 nm: pro-inflammatory ↑ anti- inflammatory↓	[177]

			13 nm: pro-inflammatory↓ anti- inflammatory ↑ 45 nm: pro-inflammatory↓ anti- inflammatory ↑ *BMP-2 expression level (the condition with LPS stimulation: 5<13<45 nm	
	5, 15, 20, 35 nm	THP-1 (under IL-1β condition)	5 nm: pro-inflammatory↓ 15, 20 , 35 nm: pro-inflammatory ↑	[179]
	2.8, 5.5 38 nm	J774 A1	*6 hours culture: IL-1: 2.8 <5.5 < 38 IL-6 & TNF-α: 2.8 >5.5 >38 *1-3 days culture: IL-1, IL-6, & TNF-α: 2.8 >5.5 >38	[178]
Silica	10 nm 300 nm	RAW 264.7	TNF-α: Smaller size ↑ higher particle ↑	[180]
	30, 100, 300, 1000, 3000, 10000	Mouse bone marrow-derived macrophage (BMDM)	IL-1β: 30<100<300 nm, 300> 1000>3000> 10000 nm	[181]

4.3 Macrophage responses to biomaterials – concluding comments

The physicochemical properties of biomaterials clearly play a crucial role in modulating macrophage phenotype and function and numerous studies have been undertaken to elucidate this response. Nevertheless, drawing generalised conclusions about the effects of particular parameters/factors remains challenging due, not least, to the wide diversity of experiment conditions (cell source, medium, biomaterial concentration, culture time, etc.) employed between studies. Furthermore, the majority of such investigations have been undertaken *in vitro*, often over quite short periods and the relevance of these findings to the *in vivo* situation where additional complexifying surface, cellular and bulk-mechanical effects pertain, remains to be established [182]. The nature and timing of the inflammatory state depends on the intersecting influences of the physicochemical properties of the biomaterial and, critically, its degradation profile, and the particularities of the host environment as affected by the implant site, timing post injury as well as the age and disease state of the host [32,33,183]. How these various factors influence the spatial-temporal behaviour of inflammatory and regenerative cell responses to an implanted biomaterial remains largely undefined.

Finally, it is widely recognised that inflammatory responses in rodent models correlate poorly with human conditions [184]. For example, iNOS and Arg 1, typical M1 and M2 markers in mouse, are not expressed in human macrophages, and IL-10 and TGF-β1 are regulated differently [57]. Therefore, appropriate M1 and M2 markers and relevant genes should be used, and multiple analyses (e.g. flow cytometry, quantitative polymerase chain reaction, immunochemistry staining, single cell genomics/proteomics) should be employed to provide critical information about the heterogeneity of macrophage populations both *in vitro* and *in vivo* under both physiological and pathological conditions. Such studies are essential to consolidating our understanding of macrophage responses to various biomaterials and are prerequisite for the successful translation of these technologies towards the clinic.

5. Macrophage targeting drug delivery systems

The localised delivery of inductive molecules remains a major focus in bone regenerative strategies. A wealth of material innovations have been employed for the controlled, localised delivery of growth factors and small molecules to target the recruitment, proliferation and differentiation of endogenous stem cell populations for bone repair. A growing recognition of the pivotal role macrophages play in bone repair and biomaterial response has seen a flurry of interest in drug delivery strategies that directly target macrophages as a means to enhance down-stream bone regeneration (Table 4).

Early studies exploring macrophages as potential targets for bone, developed from retrospective observations of associated immunomodulatory effects by molecules initially targeted at bone progenitor cells. For example, local delivery of a small molecule agonist of the sphingosine-1-phosphate (S1P) receptor (FTY720), was initially proposed to promote angiogenesis and recruitment of bone progenitors [185,186]. Studies in other contexts that demonstrated a role for S1P receptor signalling on macrophage phenotype [187] prompted the observation that promotion of bone repair by SIP correlated with modulation of macrophage phenotype at the tissue biomaterial interface [188,189].

The first, to our knowledge, direct attempt to modulate macrophage phenotype for bone repair applied a gelatin hydrogel-based system for dual release of another S1P receptor agonist (SEW2871) together with platelet rich plasma (PRP) [190] to increase macrophage recruitment for enhanced PRP driven osteogenesis. SEW2871 generated a dose-dependent effect on recruitment of CD68+ macrophages to the implant site, and co-delivery with the drug enhanced bone repair compared to PRP alone. Although subsequent studies have tended to focus on macrophage phenotype modulation, enhanced bone formation in this case was associated with increased pan-macrophage recruitment and thus yielded increases in both pro-inflammatory and anti-inflammatory gene expression. This result provided an early indication that down-stream regeneration depends not only on macrophage phenotype, but, critically on robust early stage macrophage recruitment to the site of tissue repair.

More evidence for the importance of a robust early inflammatory response has recently been provided through a study investigating the sequential co-delivery of BMP-2 with dexamethasone [34]. A mesoporous bioglass scaffold designed to achieve an early stage burst release of dexamethasone followed by sustained BMP2 release, enhanced ectopic endochondral bone formation. While the sequence of release was not formally controlled in the study design, dexamethasone appeared to exert an early transient attenuation of BMP-2 induced inflammation, which had the beneficial effect of reducing fibrosis around the implant. Pro-inflammatory markers were noted to recover at later time points in line with the expected depletion of dexamethasone from the scaffold. Significantly, while a modest attenuation of inflammation was beneficial, greater suppression through a higher dose of dexamethasone proved to be suboptimal. This provides further evidence for the importance of carefully balancing pro vs anti-inflammatory influences at early stages of repair. Other studies, noting the importance of a robust early pro-inflammatory M1 response for promotion of angiogenesis have targeted staged release of cytokines to enhance both M1 and M2 states in sequence, although in this

particular setup the combined strategy yielded rather modest effects on angiogenesis and macrophage phenotype *in vivo* [191,192].

Perhaps the dominant strategy targeting macrophages for bone repair to date has been the promotion or attempted development of M2 polarisation of macrophages at early stages post implantation. Several studies have demonstrated that local delivery of the M2-promoting cytokine interleukin-4 in particular, can enhance bone repair while promoting early upregulation of the M2 macrophage phenotype[193–198]. Such effects are however dose-dependent. Zheng et al. explored dose-dependent effects of local IL-4 injection into a cranial defect 3 days post-implantation of a demineralised bone matrix scaffold. As expected, increasing doses of IL-4 amplified CD206+ M2 macrophage polarisation, however higher doses also prolonged the M1 polarisation phase which correlated with suboptimal bone formation compared with lower IL-4 doses [193].

Dual release of IL-4 in combination with classic bone inductive molecules such as BMP2 is increasingly being explored to good effect. For example, sustained release of BMP2 with IL-4 in combination significantly increased bone formation in a cranial defect compared with either molecule alone. Increased bone formation was associated with reduced CD68 and iNOS positive staining and increased CD206 positive staining albeit at the relatively late (4 and 8 week) time points assessed. As with other studies, IL-4 alone was also found to produce an increase in bone formation over the cytokine free control.

As an alternative to promoting M2 polarisation, others have targeted the direct attenuation of pro-inflammatory signalling. As a well evidenced example of this approach, Julier et al. [199] combined growth factor delivery with an antagonist based immunosuppression strategy targeting interleukin-1 receptor signalling. The biological rationale for this approach was the observation that both BMP2 and PDGF-BB driven calvarial bone regeneration was significantly enhanced in IL1r1−/− mice, and that IL-1β (the main IL1r1 ligand) signalling by macrophages following injury was greatly amplified with growth-factor treatment in wild-type mice [199]. Sustained hydrogel localisation of a clinically licensed interleukin-1 receptor antagonist (IL-1Ra) was effective in overcoming the inhibitory effects of IL1r1 signalling with the result of enhanced bone repair by each the two growth factors alone and in combination [199].

Finally, in an interesting recent study, Zhao et al.[200] explored the interacting roles of innate and adaptive immune responses. The starting point here, was an observed association between the variable osteogenic activity of implanted calcium phosphate-based biomaterials and local immune cell infiltration. Using a muscle pouch ectopic bone formation assay, the authors observed that increased bone formation in biphasic calcium phosphate (BCP) versus beta-tricalcium phosphate (β-TCP) granules correlated with both a higher ratio of M2 (CD206 + and MHC II low) to M1 (CD206− and MHC IIhigh) macrophages and lower numbers and maturation of (CD86 +) dendritic cells. Further positive evidence for this association was sought through a stimulatory dual drug release approach that targeted enhanced macrophage polarisation through early (day 4) IL-4 release and suppressed dendritic

cell maturation through subsequent (day 7) dexamethasone release to further enhance BCP osteoinduction [200]. While the study design did not permit insight into the respective contributions (or timing) of these dual targets, complementary inhibitor studies of phagocytes (via clodronate liposome injection) which impeded bone formation, and dendritic cells (via a CD11c-linked diptheria toxin responsive mouse model) which enhanced bone formation, provided further indication that both the innate and adaptive immune responses interact in modulating the osteogenic response to BCP. This study thus opens up further interesting avenues for immunomodulation as a springboard for enhancing subsequent bone repair.

Table 4. Studies reporting enhanced bone regeneration *in vivo* through macrophage targeting biomolecule delivery strategies

Biomolecule[1]	*Class*[2]	*Drug delivery strategy*	*Immunomodulatory effect*[3] Mφ · M1 · M2		*Bone regeneration enhancement*[4]	*Ref*
			Down regulated	*Up regulated*		
Dex + BMP-2	CS GF	Staged local release	F4/80 · CD197 TNF-α · IFN-γ · IL-10		BMP2 ectopic bone induction	[34]
Diphtheria toxin	Toxin	Transgenic target	CD3		BCP ectopic bone induction	[200]
IL-1RA + BMP-2	SM GF	Sustained local release			BMP2 cranial defect repair	[199]
IL-1RA + PDGF-BB	SM GF	Sustained local release			PDGF-BB cranial defect repair	[199]
IL-4	Cytokine	Local injection	CD197 · IL-1β	CD206	Osseointegration of femoral implant	[197]
IL-4	Cytokine	Sustained local release	CD68 · iNOS	CD206	Cranial defect repair	[198]
IL-4 + Dex	Cytokine CS	Staged local release	iNOS · CD40 · CD3	Arg-1	BCP ectopic bone induction	[200]
IL-4	Cytokine	Continuous perfusion	iNOS · TRAP	CD206	Femoral bone repair after particle infusion	[194]
IL-4	Cytokine	Local injection	iNOS · TNF-α Apoptosis	CD206 · IL10 Angiogenesis	Cranial defect repair	[193,195]
IL-4 + BMP-2	Cytokine GF	Sustained local release	CD68 · iNOS	CD206	BMP2 cranial defect repair	[198]
Maresin 1	Fatty acid	Local release		CD206	Periodontal bone fill	[201]
Resveratrol	SM	Local release	IL-6 · TNF-α	F4/80	Periodontal bone fill	[202]
S1PRA	SM	Local release	CD197	CD163	Mandibular defect repair	[188]
S1PRA + PRP	SM BP	Local release		CD68 · TNF-α · IL10 TGF-β1	PRP ulna defect repair	[190]
S1PRA	SM	Local release	CD86	CD11b · CD206	Implant osseo-integration	[203]
SDF-1	Chemo-kine	Local release	iNOS	CD206	Mandibular defect repair	[204]
Sitagliptin	SM	Local release	CD68 · iNOS	CD163	Implant osseo-integration in diabetic	[205]

[1] Dexamethasone (Dex); Bone morphogenetic protein 2 (BMP-2); Interleukin 1 receptor antagonist (IL1RA); Platelet derived growth factor BB (PDGF-BB); Interleukin 4 (IL-4); Platelet rich plasma (PRP); Sphingosine 1-phosphate receptor agonist (S1PRA); Stromal derived factor 1 (SDF-1). [2] Corticosteroid (CS); Growth factor (GF) Small molecule (SM); Blood product (BP). [3] Associated markers colour coded for "pan" (blue), "M1" (orange) and "M2" (green) macrophage phenotypes. Non macrophage specific markers are in grey. Tumor necrosis factor alpha (TNF-α); Interferon gamma (IFN-γ); Interleukin 10 (IL-10); Interleukin 1 beta (IL-1β); Inducible Nitric oxide synthase (iNOS); Interleukin 6 (IL-6); Arginase 1 (Arg-1); Transforming growth factor beta 1 (TGF-β1). [4] Records biomolecule driven enhancement of *in vivo* bone regeneration compared against control. Biphasic calcium phosphate (BCP)

5. Conclusions & future perspectives.

Our understanding of macrophage biology and their role in tissue regeneration has developed considerably in recent years. Such insights have moved the field of biomaterial design on considerably from the long-held view of the host immune/inflammatory response to foreign objects as an obstacle limiting stem and progenitor cell activity, to a fresh appreciation of the role of the macrophage as a pivotal orchestrator of the repair process. It is increasingly clear that future success in biomaterial guided regeneration will depend on engagement with the complex but powerful inter-relationship and functions of pro-inflammatory (M1) and anti-inflammatory (M2) states and their respective contributions to launching the stem cell regenerative response. The development of our understanding of the importance of material mechanical properties, surface chemistry, topography, size and composition on modulation of the macrophage phenotype as well as the development of immunomodulatory materials offer new vistas to facilitate tissue regeneration. In particular, future work that elucidates how we harness material surface charge, topography, surface roughness and extracellular matrix biologics to facilitate the modulation of the M1 and M2 phenotype will be critical.

The future is bright for skeletal cell biology and tissue regeneration, the challenge will be to harness, at the interdisciplinary interface, our understanding of how the macrophage can orchestrate tissue regeneration. Key will be studies to integrate and understand the cellular mechanisms at play between the macrophage, the biomaterial and the cell reparative niche to facilitate bone repair, foreign body responses and phagocytosis as well as tissue remodelling and thus provide fresh perspectives. Given the considerable complexity involved we anticipate new approach drawing on state of the art molecular approaches including CRISPR-Cas9 to generate hypoimmunogenic populations for immune evasion and clinical application but also 'omics, bio-imaging to explore the macrophage phenotype during reparation as well as application of different approaches including machine learning and artificial intelligence to predict/improve our knowledge of the macrophage – cell - implant interaction. The acceptance of the fundamental importance of the macrophage as a key component in the orchestration of bone tissue repair offers a springboard to new approaches to skeletal regenerative medicine to improve the quality of life in an increasing aging population.

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