Modulating macrophage activities to promote endogenous bone regeneration: Biological mechanisms and engineering approaches

Yiming Niu[a,b,1], Zhenzhen Wang[a,b,1], Yuchen Shi[a], Lei Dong[b], Chunming Wang[a,*]

[a] State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Avenida da Universidade, Macau SAR, China
[b] State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing, 210093, China

ABSTRACT

A coordinated interaction between osteogenesis and osteoimmune microenvironment is essential for successful bone healing. In particular, macrophages play a central regulatory role in all stages of bone repair. Depending on the signals they sense, these highly plastic cells can mediate the host immune response against the exterior signals of molecular stimuli and implanted scaffolds, to exert regenerative potency to a varying extent. In this article, we first encapsulate the immunomodulatory functions of macrophages during bone regeneration into three aspects, as sweeper, mediator and instructor. We introduce the phagocytic role of macrophages in different bone healing periods ('sweeper') and overview a variety of paracrine cytokines released by macrophages either mediating cell mobilisation, vascularisation and matrix remodelling ('mediator'), or directly driving the osteogenic differentiation of bone progenitors and bone repair ('instructor'). Then, we systematically classify and discuss the emerging engineering strategies to recruit, activate and modulate the phenotype transition of macrophages, to exploit the power of endogenous macrophages to enhance the performance of engineered bone tissue.

1. Introduction: modulating, but not resisting, macrophages at the interface

Bone disorders remain a leading cause of pain, disability and death worldwide [1–3]. The past decades have witnessed the application of numerous approaches to repair bone fractures, regenerate bone tissue and restore bone health [4,5]. These methods have improved conditions in patients with non-union or delayed healing defects but still, face substantial challenges. For example, metal implants are standard tools to provide appropriate mechanical support; but they lack inherent biological functions to fully replace the lost bone [6–8]. Allogeneic/xenogeneic grafts may solve the insufficiency of autologous bone for transplantation, yet they risk immunogenic rejection and other medical and ethical issues [9–11]. Tissue engineering (TE), with or without the aid of biomaterial scaffolds, represents the future of regenerative medicine [12,13]; however, the present products of engineered bones are still very different from the real ones, both structurally and functionally.

One common challenge hindering these different approaches is the unfavourable response from the host tissue to the metal implants, transplanted cells or TE scaffolds (all referred to 'implants' hereafter). The immune system recognises these implants as 'foreign' and responds fast to them. Innate immune cells initiate phagocytosis of them – or fuse into giant cells to encapsulate the implants that are too large to internalise – and secrete inflammatory cytokines to assist this attack. Traditionally, biomaterials implants were designed to be as 'inert' as possible to minimise the immune response, but these attempts proved both unrealistic and ineffective in two aspects. First, no material is absolutely 'inert', and uncontrollable foreign body responses such as the formation of excessive fibril capsules still occur. Second, complete 'insulation' by hydrogels may largely reduce immune attack but meanwhile block the required blood vessel invasion and nutrient supply from the body to the implants, leading to poor regenerative outcomes.

The key to improving the host-implant interaction and consequently the regenerative results may lie in a better 'use' of tissue macrophages. First, these cells always and abundantly present at the host-implant interface (after the brief action of neutrophils) and are the primary cell population to exert foreign body reaction [14,15]. Second, macrophages are highly plastic, and their phenotypic change may lead to entirely different immune responses against the implants. Third, recent

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studies are increasingly uncovering the essential, underestimated roles of macrophages (and their precursor, monocytes) in tissue regeneration and particularly bone development and repair [16,17]. They can not only eliminate debris and regulate bone resorption but, more importantly, act as a powerhouse to secrete an array of cytokines under dynamical control. These cytokines mediate the proliferation, migration and differentiation of osteoblasts, endothelial cells and other building blocks of the bone tissue, stimulate angiogenesis and aid in tissue matrix remodelling [18]; they can also directly drive the osteogenic differentiation and further maturation of bone precursor cells [19–21]. After all, bone is a highly vascularised and mineralised tissue, and its regeneration is a sophisticated process involving the actions of various cells – macrophages deserve the role of headquarter that coordinate this dynamic and complex process [22].

Thus, opposite to attempting to resist macrophages action during implantation (which is also impossible), new strategies should be devised to – i) modulate the functions of the tissue macrophages locally to establish a desirable host-implant interaction and ii) harness these 'endogenous' functions to orchestrate an optimal regenerative process. In 1977, Pitt and colleagues described macrophages as 'a sweeper, a mediator and an instructor'. Forty years on, this definition has emerged to be both explicit and comprehensive to portray the role of macrophages in bone regeneration [23]. This definition is also providing insights into the design of engineering approaches to target macrophages for bone regeneration – though the second and third roles are tightly overlapped, because of the diverse actions of the macrophage cytokines under dynamic temporal control. In this review, by focusing on the sweeper-mediator-instructor role of macrophages (with some arbitral classification applied), we discuss the potential of targeting macrophages for promoting bone regeneration. First, we introduce in brief the phagocytic role of macrophages in different stages ('sweeper') of bone healing, and overview in more length the various cytokines and growth factors (GFs) secreted by macrophages. Many of these factors essentially mediate angiogenesis, cell proliferation and migration, as well as matrix remodelling ('mediator'); or directly instruct osteogenic differentiation of osteoprogenitor cells and bone formation ('instructor'). Then, we discuss the emerging strategies of designing therapeutic tools to recruit, activate, modulate and harnessing the power of macrophages for the regeneration of the human's hardest tissue.

2. Bone healing process

Bone healing is a complex and dynamic process comprising the interaction of multiple cells, molecule signals, and extracellular matrix (ECM) constituents. It possesses several common grounds with general wound healing procedures, containing inflammation, angiogenesis, and recovery of impaired mesenchymal tissue [24]. However, what makes the bone healing process different from the repair of most other tissues is that it causes no scar tissue formation. During the fracture repair process, the core players include inflammatory immune cells, endothelial cells, osteoblasts, chondrocytes, and osteoclasts.

There are two typical ossification processes during bone tissue formation, endochondral ossification, and intramembranous ossification. The mechanism of bone tissue recovery depends on mechanical influence. Highly steadied fractures heal mainly through direct intramembranous ossification, such as the fractures treated by open reduction and rigid internal fixation (ORIF) [25]. In this case, bone healing begins with inflammation, then transitions to the anabolic process, and eventually concludes with a prolonged remodelling phase, which aims to restore the original bone architecture, if possible. In other situations, the repair of mechanically non-ridged fractures is mainly through the form of endochondral ossification, in which the hallmark event is the cartilage tissue formation as a precursor for new bone to lay down. And the repair process briefly contains 4 phases: inflammatory, soft callus formation (primary anabolic), hard callus formation (late anabolic) and remodelling (Fig. 1). In the inflammatory

phase, bone fracture causes the disruption of vascular and tissue integrity, while also leading to the hematoma formation. With granulation tissue formation, the healing progress enters an initial anabolic phase. Osteoprogenitor cells and endothelial cells are mobilised to facilitate the development of cartilage tissues and the soft callus formation. Meanwhile, nascent vascular beds are amplified at the surrounding tissue reflected by the enhanced blood flow in the healing zone.

Along with the endochondral differentiation progresses, the repair process is facing a period predominated by catabolic activities. The cartilage produced by chondrocytes begins to be absorbed at the centre of ossification to leave a space for osteoblasts to perform calcium deposits and build up the primary trabecular bone. The newly generated soft callus converts to hard bony callus. Subsequently, bone healing enters a persistent remodelling stage, which characterised by a series of alternate and dynamic resorption/rebuilt events. The bony callus is progressively eroded by osteoclasts to form the medullary cavity, while osteoblasts maintain an appositional growth to increase the girth of the bone. These coupled cycles of osteoblastic and osteoclastic activities remodel the callus tissues to reinstate the marrow structure of hematopoietic tissue and original bone structure. These processes occur sequentially, yet, with substantial overlaps between each phase [26].

3. The pivotal roles of macrophages in bone regeneration

In both bone regeneration and de novo bone neo-formation, macrophages play pivotal and dynamic roles. In fracture healing, macrophages not only drive the first phase of inflammation but also secrete pro-angiogenic and mitogenic factors to promote the progress of the other three phases – fibrocartilage formation (early anabolic), hard callus formation (late anabolic) and tissue remodelling. In the de novo formation of bone tissues such as osteogenesis in scaffold-based tissue engineering, macrophages also promote angiogenesis, mitogenesis and additionally matrix mineralisation through paracrine secretion; meanwhile, they can directly instruct osteoprogenitor cells to undergo osteogenic differentiation and further maturation. In both scenarios, macrophages are also responsible for phagocytosis of cell debris and differentiate into osteoclasts to perform bone resorption.

During the bone regeneration process, the contribution of macrophages could be concluded as three aspects: 1) The clearance function of macrophages *per se*, which is responsible for phagocytizing cell debris and unifying the disordered and excessive matrix after their differentiation into osteoclasts, that is, the 'sweeper' role; 2) The indirect mediation of their paracrine cytokines on the stromal microenvironment, which is pivotal to harness the matrix mineralisation, vascularisation and others, that is, the 'mediator' one; 3) The direct guidance of the paracrine cytokines on the osteoblast accumulation and maturation, that is, the 'instructor' role. The sweeper-mediator-instructor functions of macrophages based on themselves and the secreted pro-angiogenic and mitogenic factors synergistically determine the quality and structure of bone (see Table 1). In this section, we emphasised these three aspects respectively to display their highly coordinated roles in bone repair/regeneration.

3.1. As sweepers: phagocytosis and transformation of osteoclasts

Macrophages perform phagocytosis (traditionally known as their chief role) and resorption in bone regeneration in two scenarios – 1) touching and cleansing with debris and exogenous pathogens at the injury site and 2) lineage-differentiating into osteoclasts to execute bone resorption.

3.1.1. Elimination of debris and bacteria

In the first situation, the implant-surgery damages local tissue vasculature and causes the mesenchymal cells to release injury-related biochemical signals, such as chemoattractants and cytokines. Innate
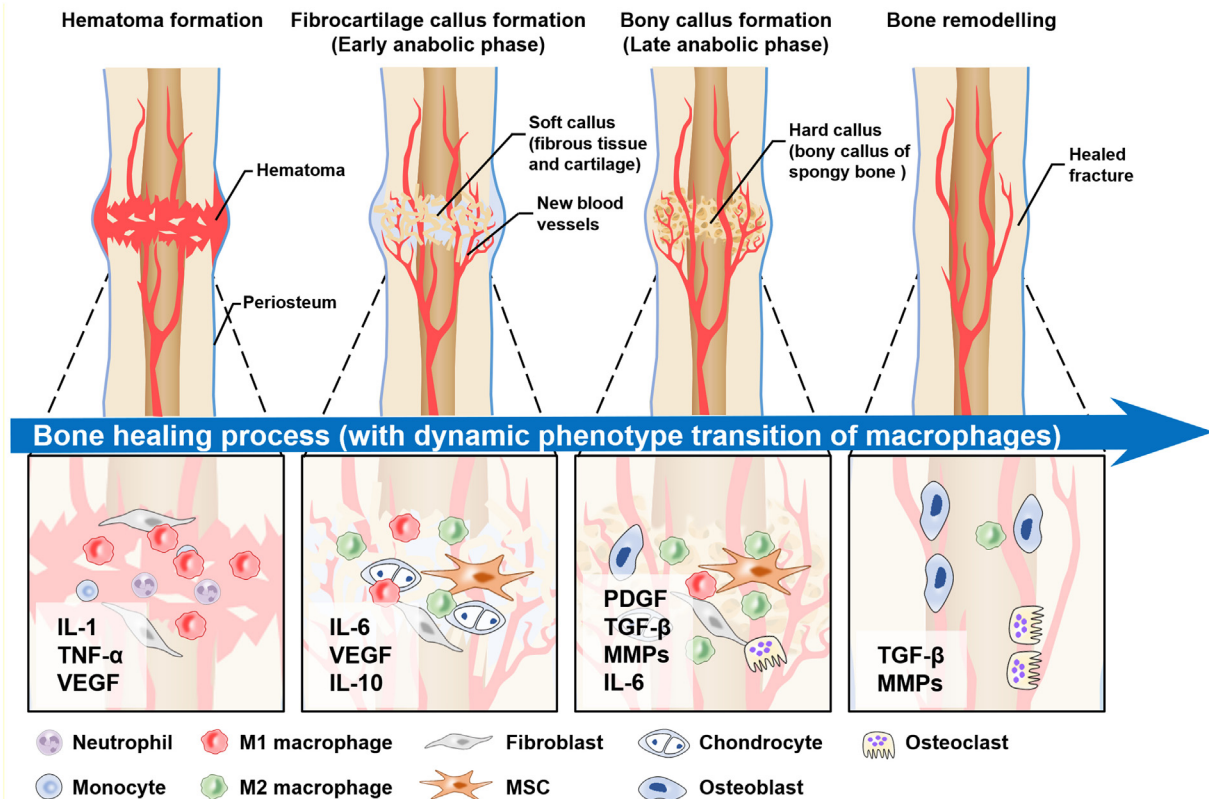
Fig. 1. Taking the femur fracture for instance, demonstration of a typical fracture healing process, as well as the relevant biological events, cellular activities and macrophage-secreted cytokines involved in these cascaded phases.

immune cells respond promptly to these signals. In particular, macrophages arrive on the scene and become the primary soldiers to phagocytise foreign objects [27,28]. Their combating with infection and phagocytizing apoptotic cells as well as tissue/cell remnants provided the favourable immune microenvironment for subsequent osteogenesis.

3.1.2. Formation of osteoclasts

In the second scenario, macrophages regulated the balance of bone microenvironment by wiping out the superabundant stromal matrix after their differentiation into the osteoclasts [29]. Osteoclasts are derived from hematopoietic cells of the monocyte/macrophage lineage; while osteoblasts are from multipotent mesenchymal stem cells (MSCs) [30,31]. Generally, excess of pro-inflammatory mediators in periarticular bone erosions can be directly induced by osteoclast differentiation and/or indirect through enhanced receptor activator of NF-kB ligand (RANKL) production, the key osteoclastogenic cytokine [32].

RANKL is defined as a membrane-residing protein, whose receptor, RANK, on the surface of marrow macrophages, prompts macrophages to change the phenotype to multinucleated osteoclasts [33,34]. Together with osteoprotegerin (OPG), RANK and RANKL serves as the essential component of RANK/RANKL/OPG signalling pathway that regulates osteoclast differentiation and activation] As a decoy receptor, soluble OPG molecules are combined with RANKL to prevent RANKL binding to RANK, sequentially to inhibit the process of osteoclasts formation and maturation. Therefore, the ratio of OPG, RANKL and RANK plays an important role on the polarisation and activation of osteoclasts [35]. After adhesion on bone matrix, osteoclasts resorb hydroxyapatite through releasing hydrochloric acid, while degrading collagen and other bone matrix proteins by secreting proteases like cathepsin K [36]. Apart from macrophages themselves, some cytokines they secreted are also important determinants of bone resorption, which will be talked in followed section [37,38].

3.2. As mediators: secreting cytokines to create a pro-regenerative niche

Macrophages secrete a plethora of cytokines that play major roles in shaping up a pro-regenerative niche. In such paracrine regulation, macrophages promote cell mitogenesis, matrix mineralisation or blood vessel formation, leading to the formation of the microenvironment required for bone repair and regeneration [39,40]. Based on their phenotypes, macrophages are commonly categorised in an over-simplified way into pro-inflammatory M1 (or 'classically activated') and anti-inflammatory M2 (or 'alternatively activated') types of polarisation. Both types of macrophages are important for bone regeneration: at the early bone healing stage, the main cytokines to create the regenerative niche are largely pro-inflammatory, such as interleukin 1 (IL-1), IL-6, tumour necrosis factor (TNF) and macrophage colony-stimulating factor (M–CSF)–1 [27,41,42]. Nevertheless, a prolonged pro-inflammatory reaction may delay healing, and M2-type macrophages are required as they secrete growth factors (e.g. platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) families) and enzymes to accelerate angiogenesis and fracture healing [43–45]. So, regulating macrophage phenotypes under dynamic control has emerged as a promising direction for engineered approaches for bone regeneration.

3.2.1. Stimulating matrix mineralisation

Bone is unique in that it is a highly calcified tissue. The mineralisation of bone matrix is a complicated process involving the action of osteoprogenitor cells and blood vessel invasion. Macrophages act as a key mediator of matrix mineralisation during bone regeneration, and they can reside in either bone or associated tissues. First, bone resident macrophages ('osteal-macrophage'), such as the CD169$^+$ macrophage, which are required for osteogenesis and fracture repair, are shown to provide vital pro-anabolic support to osteoblasts and induce matrix mineralisation *in vitro* and *in vivo* during both bone homeostasis and

Table 1

The cytokines secreted by macrophages and their functions.

Cytokine	Function	Macrophage Phenotype	Macrophage Role	Ref.
RANKL	Key osteoclastogenic cytokine. • Regulating osteoclast differentiation and activation; • Balancing bone metabolism.	M1-like	Sweeper (3.1.2); Mediator (3.2.3).	[32–35,61]
TNF-α	Pro-inflammatory cytokine. • Creating the regenerative niche; • Promoting recruitment and differentiation of mesenchymal progenitor cells to promote bone healing; • Guiding bone formation and remodelling.	M1-like	Mediator (3.2); Instructor (3.3).	[80,81,104]
IFN-γ	Interferon cytokine. • Regulating osteoblast differentiation and mineralisation through inducing bone morphogenic protein 2 (BMP-2) in MSCs; • Controversial bi-directional role in the osteoclastogenesis: improving or aggravating bone mass.	M1-like	Mediator (3.2.2; 3.2.3).	[59,65,66]
PDGF VEGF	Angiogenic growth factor; • Stimulating matureness of fibroblast and angiogenesis; • Accelerating formation of blood vessel and fracture healing.	Both M1-like and M2-like	Mediator (3.2.4).	[43–45,78]
bFGF	Aangiogenic growth factor; • Stimulating matureness of fibroblast and angiogenesis at early healing.	M1-like	Mediator (3.2.4).	[39]
MMP-9	Proteinase. • Cleaving extracellular matrix; • Affecting chondrocyte proliferation and differentiation, osteoblast recruitment and survival, angiogenesis, osteocyte viability and function; • Increasing macrophages' infiltration.	M1-like	Mediator (3.2.2).	[50,56,57]
CCL3 CXCL10 CXCL12	Chemokines. • Affecting osteoclastogenesis, including osteoclast differentiation and osteoclast ability to resorb the bone matrix.	M1-like	Mediator (3.2.3).	[62–64]
IL-1β	Cytokine belongs to interleukins. • Directly inducing MSC differentiation into osteoblasts; • A strong osteotropic function at the early phase of bone formation.	M1-like	Mediator (3.2); Instructor (3.3).	[85–87]
IL-4	Cytokine belongs to interleukins. • Inhibiting osteoclast formation from macrophages; • Attenuating the inflammatory level elevated by the excessive infiltration of M1-like macrophages; • Overturning TNF-α-involved osteocyte apoptosis progression.	M1-like	Mediator (3.2.3).	[73,122–124]
IL-6	Cytokine belongs to interleukins. • Attenuating bone resorption; • Increasing ALP activity and accelerates MSC osteogenesis in combination with dexamethasone.	Both M1-like and M2-like	Mediator (3.2.3); Instructor (3.3).	[68,82,90–92]
OSM	IL-6 subfamily cytokine. • Inducing osteoblast differentiation and matrix mineralisation; • Stimulating osteogenic commitment; • Attenuating adipogenic differentiation.	Both M1-like and M2-like	Instructor (3.3).	[69,93–96]
IL-11	IL-6 subfamily cytokine. • Positively mediating osteogenic differentiation mechanical stress and PTH stimulation; • Inducing osteogenic differentiation of mesenchymal progenitor cells; Supressing adipogenesis.	M1-like	Instructor (3.3).	[88,89]
IL-18	Mitogenic interleukin. • Promoting the proliferation of both osteoblastic and chondrogenic cells; • Inhibiting osteoclastogenesis via GM-CSF.	M1-like	Mediator (3.2.3); Instructor (3.3).	[97]

repair [46,47]. Ablation of macrophages, nevertheless, impaired the osteogenic differentiation of osteoprogenitor cells in the bone marrow towards osteoblasts [38]. In detail, bone formation and parathyroid hormone-dependent bone regeneration could be mediated by osteal-macrophage, which contributed to bone homeostasis. Macrophage depletion inhibited parathyroid hormone (PTH) anabolic actions in bone [48]. The role of osteal-macrophage in bone repair is primary healing through intramembranous ossification and progresses through all major phases of stabilised fracture repair. The related researches show that both F4/80$^+$ Mac-2$^{-/low}$ TRACP$^-$ osteal-macrophage and F4/80$^+$ Mac-2^{hi} TRACP$^-$ inflammatory-macrophages, present within the bone injury site and persist throughout the healing time series, including the periosteal osteogenesis [49]. Alexander et al. found that inflammatory macrophages and osteal-macrophages were totally different, although both of them exist in tissues associated with a bone fracture. The point is, raising osteal-macrophages, but not inflammatory macrophages, during the early anabolic and inflammatory stages of bone repair accelerated the deposition of bone matrix [46]. *In vivo* experiments on the tibial-injury model manifested that osteal-macrophages are required for deposition of collagen type I (Col I) matrix and bone mineralisation.

Coordinately, during the early anabolic phase, macrophages are present in adjacent tissues contributing to endochondral ossification, whereas many macrophages potentially sustained present within repair-associated tissues during the rigidly stabilised fixation [46,50,51]. The co-culture of macrophages with calcifying vascular cells [52] or human vascular smooth muscle cells [53] enhanced alkaline phosphatase activity and mineralisation potential [54].

3.2.2. Mediating matrix remodelling

Bone remodelling involves the removal of mineralised bone by osteoclasts followed by the formation of bone matrix through the osteoblasts that subsequently become mineralised. Macrophages, especially those of M1 polarisation, produce proteinases, such as matrix metalloproteinase 9 (MMP-9) [50], that cleave extracellular matrix, which is an integral part of tissue remodelling and development [55]. As an indirect testimony that macrophages influence anabolic outcomes during stabilised fracture repair, Céline Colnot et al. indicated that MMP9 regulates crucial events during adult fracture repair [56]. Pivotal functions of MMPs during development and bone regeneration have been already expounded, such as chondrocyte proliferation and

differentiation, bone modelling and remodelling, osteoblast recruitment and survival, angiogenesis, osteocyte viability and function [57]. Besides, MMP9 also be associated with increased infiltration of macrophages [50]. In addition, exogenous VEGF enhanced ossification and callus maturation had been proved [58]. Another report by Rifas demonstrated that interferon gamma (IFN-γ), induce bone morphogenic protein 2 (BMP-2) in MSCs and therefore may regulate their differentiation and mineralisation [59].

3.2.3. Acting on bone resorption and regulating metabolism balance

Beyond impacts on bone regeneration, macrophages also play an important role in bone resorption, providing ingredients for novel bone formation and keeping a dynamic equilibrium. Upon formation, immature osteoclast undergoes osteoclastogenesis, a complicated procedure that includes commitment, differentiation, multinucleation, and fusion [60]. The regulation of osteoclastogenesis by cytokines has been extensively studied. In osteoclast precursor cells, RANKL induces the IFN-beta, which constitutes a critical aspect of the negative feedback regulation mechanisms of RANKL signalling to suppress excessive osteoclastogenesis [61]. Other than RANK/RANKL, both *in vitro* [62] and *in vivo* studies [63,64] demonstrated CXC chemokines receptor and ligand regulate the osteoclast differentiation and maturation: CC-chemokine ligand 3 (CCL3) induces osteoclastogenesis; CXCL10 and CXCL12, as well as of their specific receptors CXCR3 and CXCR4 involve in a differential autocrine/paracrine mechanism during the process of osteoclast differentiation; CXCL12 stimulates MMP-9 production and increasing osteoclast ability to resorb the bone matrix. IL-18, secreted by both macrophages and osteoblasts, inhibits osteoclastogenesis via granulocyte-macrophage colony-stimulating factor (GM-CSF). IFN-γ has also been shown to play an important role in the regulation of osteoclastogenesis. Interestingly, IFN-γ acts as a bi-directional factor as shown *in vivo* studies. It either decreases osteoclastic bone resorption, leading to an improvement of bone mass [65], or to increase osteoclastic bone resorption, leading to a decrease in bone mass [66]. *In vitro*, IFN-γ has a marked inhibitory effect on osteoclast formation in receptor activator of RANKL–stimulated bone marrow monocyte precursors [67]. In addition, cytokines mediating osteoclast function during bone resorption has also been studied. IL-6 affects the osteoclast formation as indicated *in vivo* knock-out study which suggested that osteoclast formation appeared more severe in the IL-6 (−/−) mice, showing exogenous addition of recombinant IL-6 could attenuate bone resorption [68]. Also, its subfamily protein oncostatin M (OSM) inhibits bone resorption, proved in the cultures of primary neonatal murine and fetal rat calvarial osteoblasts [69]. IL-4 reversibly inhibits osteoclast formation from macrophages, via inhibition of TNF signalling and block of RANKL-dependent activation of NF-κB.

3.2.4. Mediating the formation of blood vessel inserted in bone injury

The process of angiogenesis is an indispensable event during skeletal and soft-tissue regeneration, enabling the recruitment of key cellular participants. The capillaries emerging from wound surrounding tissue are required to supply the hypoxic blemish site. Perivascular cells are on the surface of vessels, forming new blood vessels by integrating into the existing vascular network. After the reformulation of oxygen and growth factors delivery, the osteogenesis processes could be insured, including differentiation of osteoprogenitor and of stem cells [70,71]. Evidence indicates that the growth of blood vessels in bone and osteogenesis are coupled [72,73], the main reason is about underlying cellular and molecular mechanisms. A new capillary subtype was found in specific locations of the murine skeletal system, which has the capability of mediating the growth of the bone vasculature and generating molecular microenvironments, to maintain perivascular osteoprogenitors and contact angiogenesis with osteogenesis [74].

A small fraction of macrophages presenting in the repair early stage strongly expresses vascular endothelial growth factor (VEGF)-A which has nonredundant functions for the induction of vascular sprouts. As the prevailing VEGF source during the early phase of repair, these macrophages possess properties of both M1 and M2 activation [75–77]. The experiment shows that exogenous VEGF enhanced blood vessel formation, while a soluble, neutralizing VEGF receptor treatment could decrease angiogenesis. On account of the crucial ability of VEGFs to promote angiogenesis in fracture repair [58,78], it definitises that macrophages have a direct effect on vascularisation within the granulation tissue during bone repair.

The study of Faith H. Barnett etc. Shows that macrophages could structurally form primitive, non-endothelial "vessels" or vascular mimicry channels in angiogenesis [79], consistent with another earlier discovery that macrophages contribute to building neo-vessels in active multiple myeloma through vasculogenic mimicry. Indeed, Faith H. Barnett, etc. also found that hypoxia may be an important mediator of vascular mimicry formation which is also conditionally associated with myeloid-specific hypoxia-inducible factor 1 alpha (HIF-1α).

Inflammatory cells in damaged tissue, also possess functions including the promotion and determination of inflammation and the support of cell proliferation and tissue restoration following trauma, especially macrophages. They contribute to fracture healing in late phases by releasing cytokines, which are also essential for guiding other immune cells and regulating new bone formation and remodelling. Messengers such as VEGF, PDGF and fibroblast growth factor (FGF) could stimulate matureness of fibroblast and angiogenesis [78], which initiate the proliferative phase. During early healing, inflammatory processes encourage bFGF release from macrophages when the oxygen tension is low and lactate is high from anaerobic metabolism [39].

3.3. As the instructor towards maturation of osteoblasts and differentiation of mesenchymal stem cell

After the complex and coordinated synthesis and trafficking of cytokines to the cell surface, released cytokines mediate bone regeneration via different approaches. Like interleukin, TNF-α and inducible nitric oxide synthase, these kinds of pro-inflammatory mediators, only be produced in quantity by macrophages when being activated [80]. In murine models, TNF-α and IL-6 are produced at the fracture site within 24 h of injury and are implicated in the recruitment and differentiation of mesenchymal progenitor cells to promote bone healing [81,82]. Interestingly, low-dose TNF-α administered to the fracture site at the time of surgery and 1 day post surgery improved fracture healing in mice [81], suggesting that TNF and potentially macrophage activation, has complex dose- and time-dependent outcomes on bone healing. Besides, the inflammatory response helps establish dynamic homeostasis of reparative-relating cytokines, which is also essential for guiding bone formation and remodelling [83].

Some indispensable osteoinductive signals, BMP-2, BMP-4, BMP-5 and BMP-6 could also be synthesised by macrophages [41,84]. IL-1β has been well evidenced to play an essential role in, and act in the early phase of bone formation. It directly induces MSC differentiation into osteoblasts *in vitro*, via the Wnt-5a/receptor tyrosine kinase-like orphan receptor 2 (Ror2) signalling pathways. However, for the MC3T3-E1 cell line that is already committed to osteoblasts, IL-1beta has less significant effects – it could enhance BMP-2- and BMP-4-induced alkaline phosphatase (ALP) activity but has no effect when used alone [85]. Inhibition of IL-1beta signalling with IL-1 receptor antagonist suppresses tissue growth and bone formation in rabbit models [86]. Furthermore, two different groups reported that IL-1beta could even stimulate *in vitro* cultured cartilage explants to express osteogenic protein-1 (OP-1) – an osteogenic marker, revealing a strong osteotropic function of IL-1beta in tissue development [87].

IL-11 is another interesting cytokine that positively mediates osteogenic differentiation. First, as a major effector to mechanical stress and PTH stimulation, IL-11 potentiates Wnt signalling to induce osteogenesis and to suppress adipogenesis; IL-11 can be down-regulated by aging and excess glucocorticoids, and thus account for impaired

osteoblastic differentiation by these two factors [88]. IL-11 also directly induces osteogenic differentiation of mesenchymal progenitor cells. In *in vitro* cultured C3H10T1/2 cell line, IL-11 increases ALP activity and up-regulates the level of other osteogenic markers such as bone sialo-protein (BSP) and osteocalcin (OCN), but does not influence the level of chondrogenic, adipogenic, or myogenic markers; combined use of IL-11 and BMP-2 does not only increase ALP activity but also suppresses the undesirable adipogenic effects of BMP-2, indicating that IL-11 be involved earlier than BMP-2 in bone development. In an *in vivo* rat ectopic model, recombinant IL-11 plus BMP-2 loaded with gelatin sponges accelerated bone repair as compared to the treatment of BMP-2 alone [89].

IL-6 members have increasingly been indicated to play constructive roles in maintaining bone homeostasis. An excellent review by Franchimont and co-workers has discussed the status of IL-6 as an osteotropic factor [90], and more evidence has emerged to support such a definition. *In vitro*, IL-6 and its soluble receptor (sIL-6R) are often in combined use for testing their osteogenic effects. IL-6 alone fails to induce osteogenesis in MSC without dexamethasone treatment, possibly because the cells lack the specific receptors. Nevertheless, once the cells are activated by dexamethasone, sIL-6R or sIL-6R/IL-6 complex triggers the gp130-STAT-3 pathway, and consequently increases ALP activity and accelerates MSC osteogenesis [91]. IL-6 also has a synergetic effect with OP-1 in stimulating the ALP activity in fetal rat calvaria cells [92].

OSM and leukaemia inhibitory factor (LIF) are two members of IL-6 family and share the gp130 receptor. Classically activated macrophages (M1), both in mouse and human, secret the major cytokine OSM inducing osteoblast differentiation and matrix mineralisation from MSCs through binding to OSM receptor or a signaling-competent receptor complex [93]. OSM has been identified to have strong osteotrophic effects. In the cultures of primary neonatal murine and fetal rat calvarial osteoblasts, OSM promotes IL-6 secretion [69]. The study by P Guihard revealed a close relevance of OSM signal with macrophages which inducing osteogenesis of mesenchymal stem cells. Through a cyclooxygenase-2 and prostaglandin-E2 regulatory loop, OSM was produced in M1 macrophages rather than M2 macrophages, when the TLRs were activated by lipopolysaccharide (LPS) or several endogenous ligands. However, using neutralizing antibodies or siRNA to OSM, OSM receptor subunits gp130 and OSMR, or to the downstream transcription factor STAT3, the stimulation of osteogenesis was prevented. These results figured out an essential status of OSM in promoting bone formation, which is uncoupled from bone resorption [94].

An interesting study in mice further reveals that selective binding to different receptors underlies the biological function of OSM – when OSM acts via the OSM receptor, it induces RANKL production and osteoclast formation; but, when OSM binds LIF receptor (LIFR) as it does in the *in vitro* and *in vivo* Osmr−/− model, it inhibits the secretion of sclerostin – a BMP antagonist with anti-anabolic effects on bone formation. Therefore, to develop a treatment based on OSM-LIFR signalling has the potential to be an efficient anabolic approach to enhance bone formation independent of bone resorption [95]. In addition, OSM also stimulates osteogenic commitment and attenuates adipogenic differentiation of human adipose mesenchymal stem cells (hADSCs) *in vitro*, as characterised by the lesser accumulation of lipid droplets and enhanced matrix mineralisation [96].

There are other cytokines than ILs that mediates osteoblast formation. IL-18 is mitogenic to both osteoblastic and chondrogenic cells. Its role in influencing the differentiation of mesenchymal stem cells is still unclear [97]. Besides, the low-density lipoprotein receptor-related protein 1 (Lrp1) produced by young macrophage plays a role in bone homoeostasis, osteoblast function and even rejuvenate fracture repair in old mice [98]. Fu and co-workers reported that CCL3 inhibited osteoblast function and suppressed proliferation and osteogenic potential of osteoblasts in patients with myeloma bone disease. The osteoblast inhibition induced by CCL3 is associated with the Runx2/Osx pathway and the suppression of mineralisation activation and OCN expression

[99]. IFN-gamma in bone formation both *in vitro* and *in vivo* is less explored. A contrary study by Duque and co-workers reported that IFN-gamma is required for the osteogenic differentiation of MSCs *in vitro* [99] and its signalling reduces the bone formation *in vivo* [100].

Overall, macrophages are appropriately positioned to directly influence events during the early anabolic phase of healing, suggesting an extension of their contributions beyond early inflammatory events. However, soft callus is far from enough to support the structure to baring mechanical load, so that they need to be converted to hard bony callus. Remodelling of new bone enation is necessary to reinstate the original bone structure, which generally occurs simultaneously with the early anabolism process within the fracture zone, are interdependent. We could not assert the specific cellular and molecular contributions to the individual processes. Both bone resorption and bone formation are basilica remodelling events, and the quality and speed of fracture healing depend on the homeostasis of these two activities. There are mounting attempts on accelerating bone healing by regulating macrophages in different ways, which proclaims a new trend on bone repair.

4. Harnessing the power of macrophages for enhanced osteogenesis

Macrophages have come into prominence with increasing understanding of their multifaceted contributions in osteogenesis and fracture repair. Thus, the significance of macrophage contribution is a clear motivation for researchers to develop diverse methods to evoke this autogenous power in promoting bone regeneration and neovascularisation.

4.1. Stimulation of macrophages with therapeutic treatment

Macrophages, with a hallmark of heterogeneity and plasticity in response to outer stimulations, are indispensable for osteogenesis, which turn them into an ideal target for regulation of the immune microenvironment surrounding bone defects. Yet there are few studies focused on the traditional therapeutic treatment stimulating macrophages to facilitate osteogenesis. As a model biostimulation, CO_2 laser treatment is widely believed to contribute to osteoconduction and has been generally used in the clinical dental regeneration area. The outcome of CO_2 laser processing on macrophage behaviour is revealed in a recent study [101]. Following CO_2 laser stimulation, the amount of BMP-2 released from macrophages was significantly up-regulated. Subsequently, the osteogenic differentiation condition of human periodontal ligament cells (hPDLs) was significantly enhanced when applied with the conditioned medium of laser-activated macrophages, indicating that CO_2 lasers could stimulate the cytokine secretion of macrophages to modulate the activities of hPDLs. These helpful insights may partly explain the mechanism of CO_2 laser-induced osteoblastic differentiation, providing us a non-invasive, painless and thermal therapy for restoring bone tissue function.

4.2. Scaffold-mediated drug delivery strategies for modulating macrophage behaviours

Compared to external physical stimulation, local delivery of active small molecular substances from a reservoir or a coating layer of scaffold is a straightforward way to influence the performance of monocytes/macrophages in bone healing. In consideration of accumulating evidence in support of the important functional inputs of macrophages during multiple stages of fracture healing, researchers developed diverse methods of bioactive substance delivery to regulate the function of macrophage in different phases of bone regeneration. Current immunomodulatory delivery approaches could be simplified as the following: 1) enhancing monocyte/macrophage recruitment at the injured site; 2) activating pro-inflammatory phenotype at the initial healing stage; 3) tuning the macrophage polarisation switching to an M2-like

phenotype and inhibiting osteoclastic differentiation; 4) or sequentially guiding M1-to-M2 macrophage phenotype transition to enhance bone regeneration.

4.2.1. Enhancement of monocyte/macrophage recruitment at the injured site

Along with the accumulating literature supporting the substantive and prolonged contributions of macrophages in tissue regeneration, a number of researchers attempt to mobilise these immune cells moderately located within the injured site as a therapeutic approach.

Direct delivery of macrophages for cell therapy may encounter potential risks, such as detrimental immune responses and the transmission of adventitious agents. Hence, there are few paradigms of macrophage therapy in tissue repair, except an attempt of delivering bone marrow-derived macrophages for improving liver fibrosis condition [102]. More researchers tend to mobilise the endogenic monocytes/macrophages to facilitate bone repair. For this purpose, a series of recent studies focused on an agonist of sphingosine-1-phosphate type 1 receptor (SEW2871). Through employing the micelle formed with L-lactic acid oligomer-modified gelatin, SEW2871 molecule was incorporated into a gelatin hydrogel drug delivery system and enhanced macrophage recruitment *in vivo* [103]. Thereafter, the researchers reported the simultaneous effect of SEW2871 and platelet-rich plasma (PRP) incorporated in hydrogel system on enhanced macrophage enrichment. This synergistic effect was further demonstrated to be rewarding on the macrophage-involved microenvironment immunomodulation for osteogenesis, which evoked pro-inflammatory cytokines (like TNF-α) at the early stage after application, while coupled with a significant increase in the secretion of anti-inflammatory cytokines (such as OPG, IL-10 and TGF-β1) at 10 days postoperatively [104]. In the following research, MSC cells, as a crucial component in the anabolic process, were also designed to be enriched in the bone defects along with macrophages by co-delivery stromal cell-derived factor 1 (SDF-1) and SEW2871-micelles. This dual release strategy was proven to be a promising approach for the recruitment of MSC and macrophages, which further provided a suitable repair environment and enhanced bone regeneration [105].

Except for the administration of chemical compounds, growth factors were also investigated for their facilitation of macrophage infiltration and contribution to bone defect recovery. For instance, in a clinical study, M-CSF was observed to be enriched in fracture hematoma. And the M-CSF level in fracture site and in the patient's peripheral serum were both dramatically elevated compared to the M-CSF concentrations measured in serum of healthy controls, suggesting that M-CSF is an important macrophage-attractive molecule may participate in bone repair process [106]. Furthermore, appropriately timed injecting M-CSF in the fracture tissue was reported to facilitate the enrichment of mononuclear phagocyte progenitor cells and their differentiation into macrophages. And the anabolic progress during endochondral callus formation was indeed boosted by both inflammatory and resident macrophages [107]. Besides, James K Chan et al. described a strategy by the addition of recombinant human TNF (rhTNF) during the early repair phase to augment fracture healing effects in a murine tibial fracture model. Supplement of rhTNF enhanced both recruitment of neutrophils and monocytes through CCL12 pathway, while systemic performing anti-TNF treatment conversely compromised fracture healing [108]. Overall, these results indicate the impairment of innate immune response could not be ignored during the fracture healing process.

Another recent study revealed that VEGF, as a chemotactic factor for macrophages, was critical for a tight coupling between angiogenesis and osteogenesis during the repair of small bone defects [109]. In VEGFα CKO mice, the behaviours of F4/80+ macrophages were revealed to be suppressed in both defect sites and adjacent marrow niche, suggesting that VEGF is required for the macrophage mobilisation during the premier inflammatory stage. Together with the evidence that macrophages releasing angiogenic factors during bone healing process, the positive correlation between the density of vascular plexus and the quantity of macrophage, implied that vascular invasion is connected with macrophage recruitment. Although the beneficial effects of VEGF on bone repair have been confirmed in many studies, there are plenty of investigations failed to achieve positive results in an application with VEGF gene therapy or exogenous recombinant VEGF. The requirements of VEGF amounts are diverse in different bone defect healing situations. Excessive amount of VEGF could inhibit osteoblast maturation and impair PDGFR activation [109,110]. Therefore, appropriate delivery strategies for controllable and temporal administration of physiological amounts of growth factors are needed. And the application of *ex vivo* growth factors may require careful consideration especially when there is lacking evidence of the insufficient levels of specific growth factors in treatment objects.

4.2.2. Activation of macrophages to switch into an M1-like phenotype and release pro-osteogenic factors

Inflammation and inflammatory-invoked foreign body response following implantation of a medical device have long been regarded as a perniciousness to mineral deposits and appropriate integration between engineered scaffolds and the adjacent tissues. Although extended inflammation is unwelcome, there is a newly born concept recognizing the essential biological requirement of inflammation as a motivator of osteogenic differentiation and neovascularisation. And as important mediators of inflammatory, macrophages/monocytes were demonstrated to play an apex regulatory role in driving the early anabolic process of bone formation [93,111–113]. Yet there are still few related researches on the application of pro-inflammatory macrophages in tissue repair, especially bone regeneration.

It is a common strategy to deliver drugs or cytokines with nanoparticles to directly facilitate the osteoblastic differentiation of stem cells. However, restrictions, such as high expenditures, complex preparation process and especially side effects caused by the leaking risk of supraphysiological concentrations of delivery molecules, hamper their further clinical application in the near-term future. Shi et al. developed an *in situ* one-pot synthesis approach to fabricate mesoporous silica nanospheres doped with hypoxia-inducing copper ions (Cu-MSNs). These mesoporous nanospheres had uniform spherical morphology (~100 nm), ordered mesoporous channels (~2 nm) and homogeneous Cu distribution. These Cu-MSNs could be ingested by macrophages and tune a beneficial immune milieu by stimulating the secretion of pro-inflammatory factors, which led to robust osteogenic differentiation of MSC via OSM paracrine pathway [114].

4.2.3. Promotion of the anti-inflammatory phenotype switching or inhibition of the osteoclastic differentiation

As mentioned above, the transient pro-inflammatory environment at the early stage and angiogenesis initiated by the inflammatory factors are a boon for bone regeneration, but turn to be a bane if not controlled. After scaffold embedding, the persistent chronic inflammation induced by macrophage-involved host immune response could severely impair the regeneration process and finally result in implant failure. The optional outcomes, positive or negative, due to macrophage involvement depend to a great degree on the polarisation towards specific functional phenotypes (M1-or M2-like phenotypes) and the capacity of proper transition and resolution of inflammatory polarised activation in the late stage of tissue repair. Hence, inflammatory response should be suppressed in due course contributing to the subsequent functional maturation of osteogenic cells and stability maintenance of new vessels.

Bisphosphonates have been widely employed in the therapies of metastatic bone disease, with the effect of suppressing bone adsorption by suppressing the osteoclast behaviours. Lee et al. described a bisphosphonate delivery strategy using gold nanoparticles (GNPs). Alendronate (ALD), a common bisphosphonate for postmenopausal

osteoporotic patients, was grafted to the GNPs surface (GNPs-ALD) to develop a drug-delivery system, which could slowly release ALD avoiding the side effect of bone formation suppression induced by excessive inhibition of bone resorption. *In vivo* study proved that GNPs-ALD were effective for preventing osteoporosis in the ovariectomised (OVX) mouse model [115]. In another research, zoledronate, another kind of bisphosphonate, was locally injected during implantation of alpha-tricalcium phosphate/collagen sponge (α-TCP/CS) to promote critical size calvarial defect repair. As an active intervener of inflammatory reaction initiated by macrophages, zoledronate remarkably reduced excess inflammatory factor secretion and attenuated severe osteoclast formation, benefiting for considerable bone mass increases [116].

Sphingosine-1-phosphate (S1P) is a sphingolipid growth factor with the ability to modulate macrophage phenotypes. It was noteworthy that the exogenous addition of S1P stimulates macrophages to choose the M2-like phenotype over a pro-inflammatory subset [117]. Based on this discovery, Das et al. developed fused nanofibers comprising poly(DL-lactide-co-glycolide) (PLAGA) and polycaprolactone (PCL) to deliver a S1P synthetic analog, FTY720. Animal experiments indicated that the composite nanofibers could locally deliver FTY720 to direct macrophage polarisation towards M2-like phenotype and promote microvascular remodelling, leading to significant osseous repair in a mandibular bone defect model [118]. Das et al. proceeded to evaluate the *in situ* regulation capacity of FTY720-coated allografts towards multiple marrow-derived cells in a rat critical size segmental tibial defect model. The bone allografts, coated with a mixture of FTY720 and PLGA, achieved improved integration with the surrounding tissue, new bone formation and neovascularisation, via local immune regulation enhancing the angiogenesis–osteogenesis coupling in bone repair [119]. In another study, researchers locally delivered FTY720 using murine basement membrane-based hydrogel (Matrigel) and human trabecular bone allografts to improve bone regeneration conditions in both models of endochondral and intramembranous ossification. It was revealed that the FTY720-laden injectable Matrigel could facilitate the bone repair in a murine tibial fracture model. Similarly, FTY720 directly coated on human trabecular bone grafts could also accelerate new bone deposition in a rat critical-size cranial defect model [120].

Not only depend on M2 polarisation inducer, composite strategy seems to be a potential therapeutic method to eliminate pro-inflammatory cytokines and modulate the immune microenvironment. Yin et al. designed a "cytokine blocker" (BANC) constructed with LPS-stimulated macrophages cell membranes enveloping gold nanocages, to neutralise pro-inflammatory cytokines by the overexpressed cytokine receptors on cell membrane (Fig. 2A and 2B) [121]. Besides, resolvin D1, an anti-inflammatory drug, was pre-loaded into the nanocages to facilitate M2 polarisation. This BANC system was revealed to efficiently attenuate the inflammatory response, induce M2 phenotype and at last promote the bone repair in the femoral defect (Fig. 2C).

Local delivery of cytokines for tuning the macrophage polarisation and making phenotypic contributions is another important strategy to improve bone healing condition and the indispensable vascularisation in the defect site. For instance, Wu et al. described the inflammatory suppression effect of IL-4 injection on the prevention of steroid-triggered osteonecrosis. IL-4 intervention could attenuate the inflammatory level elevated by the excessive infiltration of M1-like macrophages and overturn TNF-α-involved osteocyte apoptosis progression [122]. Based on the anti-inflammatory effect of IL-4, various immunomodulatory scaffolds have been developed. Nanofibrous heparin-modified gelatin microsphere (NHG-MS) was fabricated through emulsion and phase separation process (Fig. 2D), in which IL-4 was incorporated and stabilised via its binding domains with heparin component on NHG-MS (Fig. 2E) [123]. The prolonged release of IL-4 could efficiently elevate the M2/M1 ratio, resolve the chronic inflammation aroused by diabetes mellitus and enhance osteogenic differentiation and bone repair (Fig. 2F). In another study, researchers found that high-stiffness

transglutaminase crosslinked gelatins (TG-gels) could promote osteoblastic differentiation of bone progenitor cells but induced the M1 polarisation of macrophages. Hence, IL-4 was incorporated into this TG-gels together with SDF-1α to tune macrophage phenotype and direct the mobilisation of endogenous osteoprogenitor cells respectively [124].

Besides maintaining the viability of engineered bone tissue, the infiltration of neovascularisation is essential for achieving successful osteogenesis as well. To enhance microvascular network remodelling, Krieger et al. designed a poly(ethylene glycol) diacrylate (PEG-DA) hydrogel incorporated with N-desulphated heparin for delivery of SDF-1α [125]. Based on the high affinity of heparin towards cytokines and the supplement of albumin as a stabilizing factor, SDF-1 could be delivered and sustainably released for over 3 days, maintaining its bioactive chemotaxis. The released SDF-1α was proved to be beneficial for promoting the spatial orientation of anti-inflammatory monocytes, which express a higher level of CXCR4 on their cell surface. And the tuned innate immune response was in connection with the status of vascular remodelling, implying a promising strategy to improve vascular remodelling in a space regulation manner.

4.2.4. Delivery of "living drug" for regulating macrophage behaviours

The immunomodulatory effects of MSCs have drawn the growing attention of researchers, along with the in-depth understanding of the coordinated interaction between macrophages and MSCs, as well as its dominated effects on successful bone regeneration [126]. The MSC therapy is demonstrated to facilitate *in situ* recruitment of macrophages. A recent study revealed that transplanted hMSCs associated with biphasic calcium phosphate granules could mobilise circulating monocytes to facilitate bone formation [127,128]. Similarly, another research group adopted fibrin hydrogel as an attractive carrier of MSCs to support host macrophage recruitment and immunomodulation at the early stage of implantation [129]. In these applications, rather than an effecter, the delivered MSC acted as an inducer to trigger the innate immune response and improve the mobilisation of macrophages to the implantation area.

Besides immunocytes recruitment, MSCs application is proven to be helpful for attenuating the host inflammatory response and building up an optimal environment for osteoblastic maturation [126]. It was reported that encapsulated MSCs could attenuate the fibrous capsule formation invoked by foreign body reaction (FBR) in contrary to acellular hydrogels, via paracrine down-regulation of the classically inflammatory-activated macrophages [130]. Based on the anti-inflammatory properties of MSCs, Tour et al. proposed an approach to regulate the local FBR via loading MSCs on bionic architecture fabricated with hydroxyapatite (HA), to achieve highly efficient outcomes of osteogenesis [131]. Subsequent studies confirmed the crucial role of MSC-derived "extracellular vesicles" (EVs) or "exosomes" in educating macrophages and shifting their polarisation towards an anti-inflammatory phenotype [132,133]. This finding provides us a potential strategy to employ EVs as an alternative cell-free method to conduct regenerative processes.

4.2.5. Guiding of M1-to-M2 macrophage phenotype transitions

As mentioned above, bone regeneration and tissue vascularisation are complex and dynamic processes, during which macrophages exert diverse functions in sequence by switching from a pro-inflammatory state to a pro-reparative state. Thus, it requires precise control strategies to sequentially release immunomodulatory molecules and guide the phenotype transition. Indeed, the overlapping delivery of macrophage phenotype stimuli (IFN-γ and IL-4) has been reported failed to achieve an effective regenerative outcome [73]. Because this dual delivery results in a synchronous, but not successive, M1-/M2-polarisation. Moreover, the transition should be triggered under precise regulation. Too early augment of M2 phenotype macrophages is also detrimental for tissue repair as shown in a cutaneous healing model
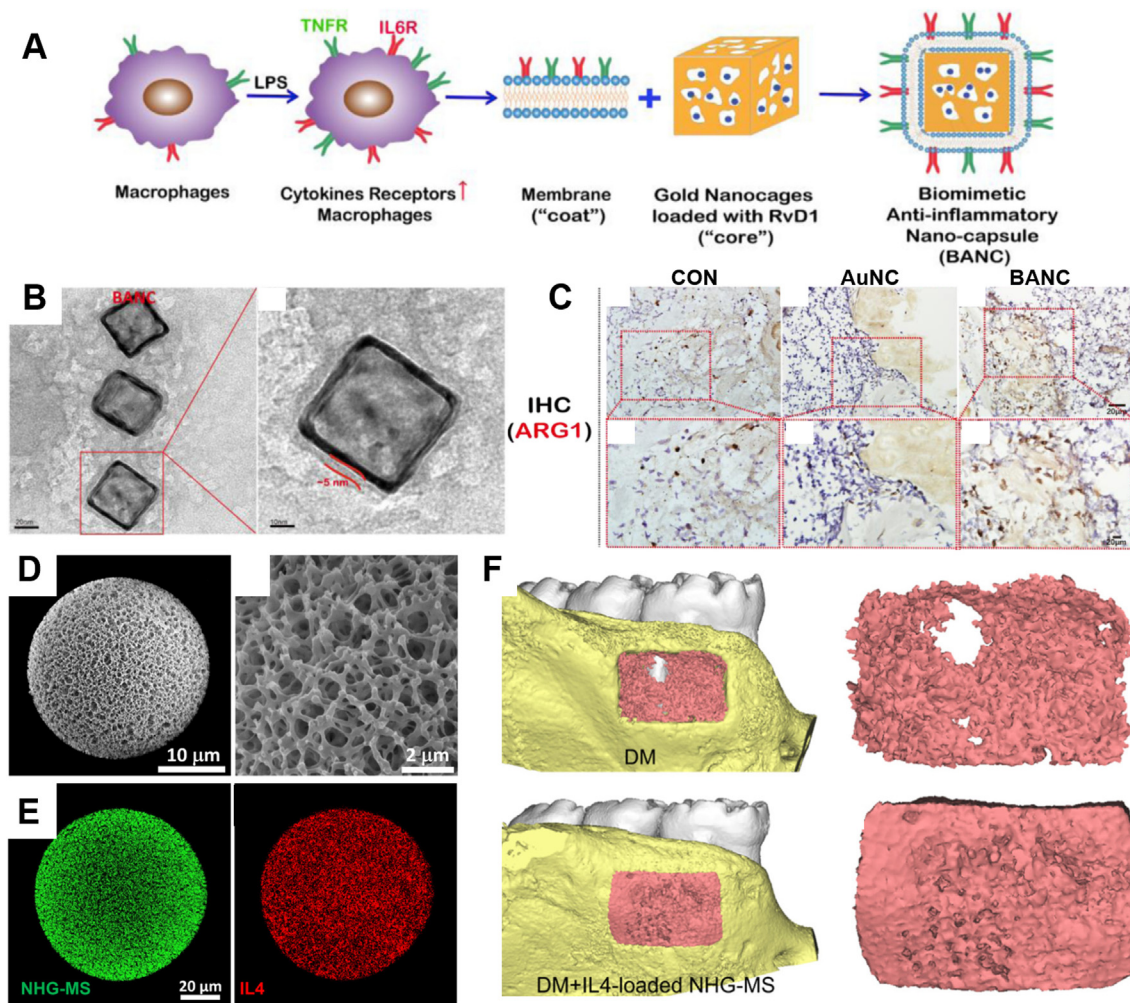
Fig. 2. Strategies of delivery macrophage stimuli to resolve inflammation and enhance M2 polarisation. A) Scheme of the fabrication of BANC system; B) TEM images of BANC; C) IHC staining of ARG1 to assess the M2 polarisation-inducing effects of BANC during femoral bone defect repair; D) SEM images showing the microporous architecture of NHS-MS; E) Fluorescent images showing IL4 (red) evenly distributed in the NHG-MS (green); F) The defect was completely repaired under the diabetes mellitus condition in IL-4-loaded NHG-MS group. Reprinted with permission from Ref. [121,123], Copyright (2020) Elsevier and Copyright (2018) American Chemical Society.

[134]. Therefore, modulating macrophage phenotypes via sequential delivery of M1-/M2-activated molecules along with the repair process would be a promising strategy for bone tissue regeneration.

To achieve this goal, Gao et al. designed a dual cytokine delivery system based on TiO$_2$ nanotubes (TNTs) (Fig. 3A and 3B) [135]. In this system, IFN-γ, as a pro-inflammatory stimulus, was mixed into the hydrogel coated on the outer surface of TNTs to benefit rapid release, while anti-inflammatory IL-4 was loaded into the TNTs for delayed release (Fig. 3C). *In vitro* test demonstrated that this cytokine sequential delivery tool can augment the M1 population in the early stage and promote the regression of inflammation in the late period. Besides cytokines, anti-inflammatory drugs have been also adopted to trigger the switch of M1 to M2 macrophage phenotype. A biomimetic calcium phosphate (bCaP) coating layer was designed to cover and separated the pro-M2 molecule simvastatin (SIMV) from IFN-γ to delay the stimulation of pro-reparative state following a pro-inflammatory phenotype (Fig. 3D) [136]. The successive M1 to M2 activation on this dual drug delivery system was observed with both THP-1 and bone marrow macrophages (Fig. 3E and F).

Research has demonstrated that temporal regulation of inflammatory milieu conditions to initiate tissue angiogenesis followed by M2 phenotypic polarisation of macrophages in time sequence would be also critical for the ideal vascularisation of the engineered implant

[137]. Towards this end, Spiller et al. subcutaneously implanted a glutaraldehyde cross-linked collagen sponges that achieved well vascular infiltration, associated with the presence of considerable M1 and M2 macrophages. It was in contrast to the poorly vascularised porous collagen sponges inundated with M2 macrophages, and the lipopolysaccharide-coated collagen scaffolds hastily degraded by inflammatory macrophages, suggesting the coordinated contributions of both M1 and M2 macrophages in vascularisation. Thereafter, the researchers proceeded to validate this hypothesis by developing a biodegradable scaffold with sequential delivery of IFN-γ and IL-4 cytokines to tune the M1/M2 polarisation transition of macrophages [138]. The rapid release of IFN-γ was realised through adsorption onto the scaffolds, whereas IL-4 release was sustained over 6 days via biotin-streptavidin binding onto scaffolds (Fig. 3G and H) [73]. Though the sequential release of cytokines from scaffolds was able to promote M1/M2 phenotypic transition of macrophages *in vitro*, there were no obvious changes in polarisation of host macrophages during subcutaneous implantation. It suggested that better temporal control of cytokine delivery would be necessary. But indeed, it provides a potential strategy to temporally control cytokine release for bone defect healing by implanting scaffolds.
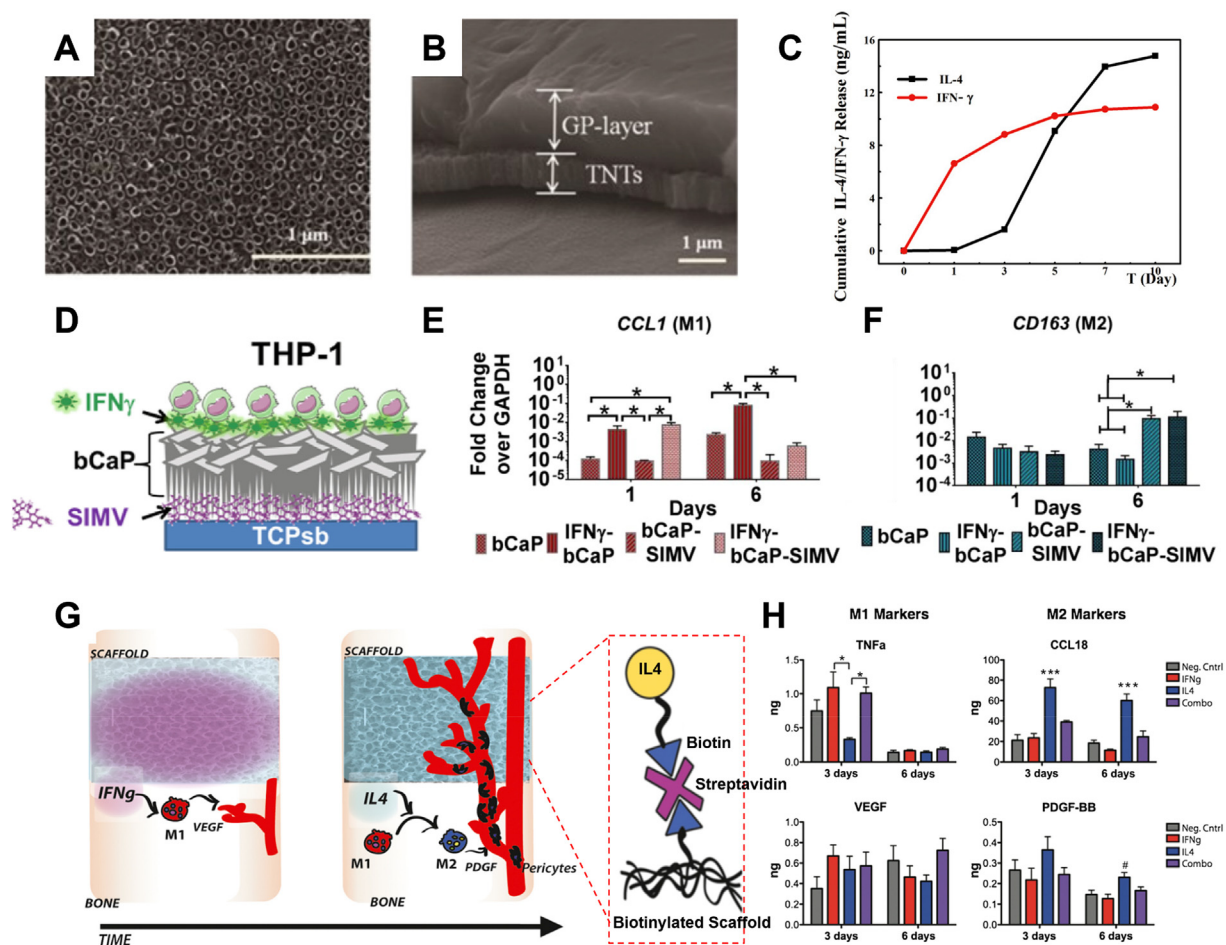
Fig. 3. Strategies of sequential delivery macrophage stimuli to guide M1 to M2 macrophage phenotype transition. SEM images of A) surface of TiO$_2$ nanotubes (TNTs) and B) cross-section of the TNT with carboxymethyl-chitosan hydrogel coating layer; C) Cumulative release profiles of IL-4 and IFN-γ from TNT with hydrogel coating; D) Scheme of biomimetic calcium phosphate (bCaP) barrier layer separated pro-inflammatory IFN-γ from the M2-promoting simvastatin (SIMV); E) M1 and F) M2 macrophage marker expression for THP-1 cultured on bCaP surface over 6 days; G) decellularised bone scaffold with IFN-γ physically loaded on scaffold while IL-4 attached through biotin-streptavidin binding, to achieve sequential release profile; H) Cytokine secretion by macrophages activated on this decellularised scaffold. Reprinted with permission from Ref. [73,135,136], Copyright (2014) Elsevier, Copyright (2018) Wiley and Copyright (2018) Elsevier.

4.3. Bioactive scaffolds to tune macrophages for osteogenesis

Considering the obstacles of growth factor delivery or cell therapy, such as burst release-induced supraphysiological dose administration or unintended immune reaction, a mounting number of researchers recently focus on the properties of biomaterials in regulating the immune environment of bone regeneration.

4.3.1. Use of immunomodulating inorganic materials

It is reported that elevated calcium level in extracellular milieu has been demonstrated for the enhancement of the chemotactic effects towards multiple cell types [139], including macrophages [140], mediated by calcium sensing receptor (CaSR). Based on this theory, a recent research developed a complex membrane constructed with PLA electrospun nanofibers comprising calcium phosphate (CaP) ormoglass (organic modified glass) nanoparticles, to facilitate the neovascularisation during bone regeneration. It is demonstrated that this approach elevated the local secretion of pro-angiogenic cytokines, induced by the calcium ions-triggered macrophage recruitment, and promoted blood vessel sprouting in the biomaterial [141]. Similarly, Chen et al. investigated the role of macrophages on biomaterials-regulated osteogenesis, using β-TCP extract [142]. Based on the activation of CaSR pathway, the phenotype of macrophage transferred to M2 in response to β-TCP extracts, while bone morphogenetic protein 2 (BMP2) was

remarkably elevated by the β-TCP stimulation. Subsequently, when apply the supernatants of macrophages cultured with β-TCP extract to bone marrow mesenchymal stem cells (BMSCs), their osteoblastic differentiation was dramatically improved.

Magnesium (Mg) is biodegradable metal possessing many potential advantages compared to current scaffold materials. Mg could be doped in calcium phosphate cement (CPC) to improve the performance of CPC in terms of its rapid setting and high early strength [143]. More importantly, the doped Mg ions contributed to modulate the inflammatory activation of macrophages and suppress the TNF-α and IL-6 expression, while promoting osteogenesis related cytokine TGF-β1 up-regulation [144]. In another study, Mg particles were mixed in a PDLLA matrix to enhance MSC viability and osteogenic differentiation. The dissolution of Mg ions from composites was proven to be able to regulate the macrophage-induced inflammatory response evoked by PDLLA degradation components [145,146]. However, micron-size magnesium particles could be engulfed by macrophages, resulting in the necrosis of macrophages and the release of inflammatory cytokines [147].

Silicon is one of the major trace elements in the human body, which benefits for bone homeostasis and healing process [148,149]. *Wu* et al. adopted clinoenstatite (CLT, MgSiO$_3$) as a tough coating material, exceedingly twice the HA coatings in bonding strength. Mg and Si ions released from CLT coatings could trigger conductive osteointegration, by down-regulating pro-inflammatory cytokines and suppressing

osteoclastogenesis [150]. Sun et al. described a silicified collagen scaffold (SCS), fabricated by collagen matrices incorporated with intrafibrillar amorphous silica [151]. Silicic acid continually leached from this SCS system stimulated the differentiation of peripheral blood monocytes into TRAP-positive cells that expressed diverse chemotactic cytokines, including SDF-1α, TGF-β1, VEGFa and PDGF-BB. These cytokines further promoted homing of BMSCs and endothelial progenitor cells benefiting for the subsequent angiogenesis and the stabilisation of microvascularisation [152].

Besides inorganic ions mentioned above, other ions such as strontium (Sr) [153–156], copper (Cu) [114,157,158], Zinc (Zn) [159], have also been incorporated into bone scaffolds to modulate inflammatory cytokine secretion and enhance their osteogenic/angiogenic properties.

Carbon nanomaterials, comprising graphene, carbon nanotubes (CNTs) and carbon nanohorns (CNHs), fabricated by a rolled graphene structure and formed into a cone, are prevalent nowadays in biological and medical applications. Hirata et al. demonstrated that CNHs were tended to be phagocytosed and localised in the lysosome of macrophages and stimulated macrophages to release OSM. The secretion of OSM could accelerate the osteogenic differentiation of co-cultured mesenchymal stem cells, indicating the therapeutic potential of nanomaterials for bone regeneration [160]. All the above discoveries provided a deeper understanding of the mechanism of inorganic material-mediated bone regeneration.

4.3.2. Use of ECM-inspired components

Tissue repair scaffolds that mimic or directly use ECM components are like to contribute to the regeneration process by setting up an optimal environment for immunomodulation and osteogenic differentiation.

Fibrinogen, classically considered as a pro-inflammatory protein, mediates different cellular interactions and activates major members of the inflammatory reaction, like neutrophils and monocytes/macrophages. When adsorbed to chitosan scaffolds, fibrinogen stimulated the polarisation of adherent macrophages to express a significant number of cytokines involved in bone homeostasis and vascularisation, such as MIP-1δ, PDGF-BB, BMP-5 and BMP-7 [161]. The researchers proceeded to explore the mechanism of fibrinogen for regulating inflammation. The research data indicated that fibrinogen could trigger the pro-osteogenic effect of monocytes through TLR-4-mediated interaction and subsequent secretion of BMP-2. Besides, MAPK activation in ERK 1/2 and JNK pathways was also involved in this process [162]. Based on the previous study, Vasconcelos et al. designed a porous scaffold entirely constituted of fibrinogen to promote bone regeneration in a femoral rat bone defect model (Fig. 4A). Fibrinogen invoked higher levels of pro-inflammatory cytokines, such as IL-6 and IL-8, in the inflammatory stage, while reduced plasma levels of IL-1β and amplified the expression of TGF-β1 at 8-week postoperatively. It suggested that fibrinogen scaffold could provide provisional support for 3D tissue growth and also construct a pro-regenerative milieu (Fig. 4B and C) [163].

In another study, chondroitin sulphate, a glycosaminoglycan believed to tune inflammation, was functionalised on a collagen scaffold (CSCL) [164]. Its effects on macrophages in physiological and LPS-stimulated conditions were separately investigated. Based on the experiment data, CSCL was revealed to be able to regulate macrophage phenotypic polarisation through obstructing the LPS/CD44/NFκB cascade activation, which indicated the immunomodulatory potential of this biomimetic materials to modulate macrophage-specific activation for bone repair.

ECM hydrogels, composed of endogenous ECM proteins, are biocompatible porous bioactive materials, which facilitate the recruitment of progenitor cells and macrophages, perform immunomodulatory functions and drive tissue regeneration. A periosteum extracellular matrix (PEM) hydrogel was fabricated from porcine decellularised periosteum (Fig. 4D) [72]. Its porous mesh inner structure benefited for the cell permeability and adhesion (Fig. 4E). Moreover, it improved M2

phenotype transition, angiogenesis and bone tissue mineralisation *in vivo* (Fig. 4F).

4.4. Modulation the activity of macrophages at the host-scaffold interface

The interaction between scaffolds and surrounding tissue was initiated immediately upon implantation and influence the fate of the scaffolds throughout the entire healing process. Therefore, it is crucial to design an appropriate scaffold with proper surface attributes [165–167], aiming at modulating the host cell into a right phenotype at the host-scaffold interface.

4.4.1. Modulation of macrophage phenotypes through physical properties of interfaces

Recent research has demonstrated that surface engineering of biomaterials, including tuning the stiffness, hydrophilicity and topographical properties (roughness, pore size, nano/microtopography, etc.), could modulate the critical events in the immune response to biomaterials.

Macrophages could perceive biomechanical cues and respond in the phenotypic switch during the host-biomaterial interactions. A recent study adopted a transglutaminase cross-linked gelatin (TG-gel) as the stiffness-tuneable matrix and investigated the cellular effects of matrix stiffness on macrophage polarisation [168]. The encapsulated macrophages in high-stiffness TG-gel could be modulated to a pro-inflammatory phenotype and indirectly influenced the bone progenitor cell fate and osteogenic outcomes. Another study discussed the responses of macrophages cultured on mechanically loaded polycaprolactone electrospun substrates [169]. It was revealed that macrophages displayed a sensitive response to extrinsic mechanical stimuli and shifted towards an M2-like phenotype, suggesting that biomechanical niche could guide macrophage activation and benefit for tissue repair.

To assess the influence of surface roughness towards immune response, Hotchkiss et al. evaluated the effect of surface modifications on macrophage activation and cytokine secretion. Results suggested that Ti with the smooth surface could stimulate inflammatory macrophage activation to express elevated levels of IL-1β, IL-6 and TNFα, while rough and hydrophilic titanium surface triggered an M2-like anti-inflammatory state of macrophage polarisation that was associated with the secretion of IL-4 and IL-10 [170]. In another study, researchers demonstrated that macrophage-derived osteoclasts were sensitive to specific surface roughness, formed distinct phenotypes and further affected osteogenic differentiation [171]. Compared to rougher surfaces, smoother surfaces promoted the cell fusion process of attached osteoclasts showing larger size with more nuclei. In addition, the conditioned medium from osteoclasts on the smooth surface, containing clastokines such as CTHRC1, exerted more obvious anabolic effects on osteogenic differentiation.

In addition, the macrophage immune response invoked by nanotopographic surfaces and the culture microenvironment should be taken into account during the assessment of nanotopography-mediated osteogenesis [172]. It was reported that micro-/nano-topographical characters could effectively determine the morphology of macrophages and polarised orientation (Fig. 5D–F) [173]. Based on micro-patterning techniques, macrophages with elongated cell shapes could directly elevate their expression of M2 markers and decrease inflammatory cytokine secretion, without exogenous stimulation of cytokines [174]. Moreover, macrophage elongation could also enhance the IL-4 and IL-13 involved M2-induction, while it weakened the cell response towards M1-like stimuli, such as IFN-γ and LPS [175]. Similarly, Chen et al. demonstrated that nanoporous structures have significant modulatory effects on macrophage responses (Fig. 5A and B) [176]. The transduction of the nanoporous physical cues into intracellular biological cues was relied on varying the shapes and invoking the autophagy of macrophages. It led to an anti-inflammatory response and the secretion of
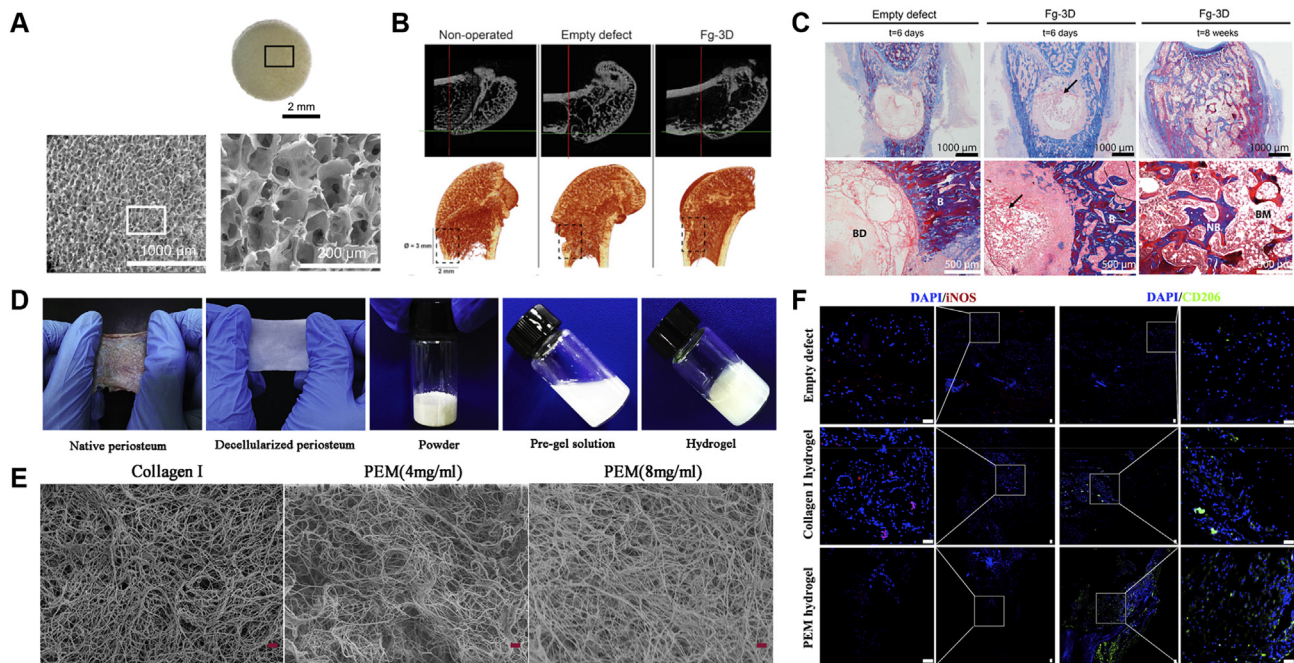
Fig. 4. Hydrogel derived from extracellular matrix components. A) Gross view and SEM images of Fg-3D scaffolds; B) X-ray images and 3D reconstruction of Fg-3D implants at defect sites; C) Masson's trichrome staining of bone defects with/without Fg-3D implanted for 8 weeks; D) Images of periosteum decellularisation and preparation of hydrogel. E) SEM analysis of the nanofibrous structures of the PEM. F) Assessment of the PEM hydrogels ability to promote M2 macrophage polarisation *in vivo*. Reprinted with permission from Refs. [72,163], Copyright (2016, 2020) Elsevier.

pro-osteoblastic factors, such as BMPs and Wnt 10b (Fig. 5C). In another study, researchers demonstrated that macrophages responded to the surface topographic characteristics of biomaterials (such as smooth, rough and rough-hydrophilic), *via* the activation of Wnt signaling pathway [177]. It suggested a novel strategy of immunotherapeutic nanotopographies for bone biomaterials applications. To further achieve dynamic modulation of macrophage phenotypes, researchers designed a shape memory film consisting of polycaprolactone doped with gold nanorods [178]. Responded to near-infrared irradiation, the flat surface of this film could transform into a microgrooved pattern. Meantime macrophages on the film surface were sequentially induced to elongate their cell outline and express anti-inflammatory cytokines, suggesting a dynamic immunomodulatory strategy for optimised tissue healing outcomes.

To assess the effects of pore size and fibre density of a nanofiber scaffold on the activation of mouse bone marrow-derived macrophages, Garg et al. seeded macrophages on varying polydioxanone electrospun scaffolds. It was shown that large fibre/pore size could conduct to up-regulated expression of Arginase 1 (M2 marker), together with higher amount of angiogenic cytokines VEGF, bFGF and TGF-β1 [179]. This study provided some inspiration to design implantable scaffolds for enhanced *in situ* angiogenesis and tissue regeneration.

4.4.2. Tuning macrophage behaviour by a bioactive interface for bone regeneration

Biological properties of scaffold surface play an important role in modulating the immune response *via* the interaction with macrophages at the tissue-scaffold interface. In order to investigate the influence of ECM components modified on polymer substrate towards macrophage differentiation. Correia et al. tested a series of proteins from ECM modified on poly(L-lactic acid) (PLLA) films for macrophage polarisation [180]. Upon differentiation, monocyte-derived macrophages (MDM) on all surface modified films exhibited lower levels of IL-6 and IL-10 expression in contrary to non-modified films. After challenging MDMs with the robust pro-inflammatory stimuli LPS, pPLLA, poly(L-lysine) and fibronectin-modified films presented a remarkable decrease

in IL-6 secretion, yet a reverse trend in IL-10 expression. It implied that surface-modification on PLLA films could break the balance of macrophage polarisation towards an anti-inflammatory profile, especially when suffering the inflammatory stimuli.

Besides adopting ECM ingredients to produce biomimetic surfaces, other natural polymers were also used to construct immunoregulatory interfaces. In a recent study, enantiomeric polylysine was grafted on the surface of poly(propylene fumarate) polyurethane films (PPFU) to investigate the polarisation-regulatory effects on macrophages [181]. According to the expression analysis of paracrine cytokines and polarisation related genes, the poly-d-lysine grafted PPFU film exhibited a more effective induction of *in vivo* M2 polarisation and a thinner fibrous capsule wrapping implants. The restricted inflammation and M2 polarisation process involved CD44 and integrins, as well as the activation of focal adhesion kinase (FAK), Rho-associated protein kinase (ROCK) and downstream PI3K/Akt1/mTOR signalling pathway.

Natural polysaccharides were also attractive materials for designing bioactive scaffold surfaces to activate host macrophages into a pro-osteogenic phenotype. A bioactive surface was prepared using a macrophage-affinitive glucomannan polysaccharide (acBSP) coated on the exterior surface of hydrogels to enhance osteogenesis of the delivered therapeutic MSCs (Fig. 6A and 6B) [182]. A series of *in vitro* cell adhesion, gene expression and signalling analyses suggested that the acBSP polymer effectively facilitated the adhesion and activation of macrophages and specifically induced them to express abundant pro-osteogenic/-angiogenic cytokines (OSM, VEGF and PDGF-B) (Fig. 6C and D). And this coating layer strategy has been proven as an effective, convenient and open technology platform to be applied for bone tissue regeneration (Fig. 6E). Based on the macrophage-modulating activities of this acetyl glucomannan, an anisotropic open porous scaffold was further fabricated with the single-component (acBSP biomaterial) *via* gradual solvent-diffusion technique for coordinating the entire bone regenerative cascade during calvarial bone defect regeneration (Fig. 6F and G) [183]. The anisotropic and porous architecture conducted the adhesion and activation of macrophages on the outer surface, yet guided the inward migration and settlement of osteogenic progenitors
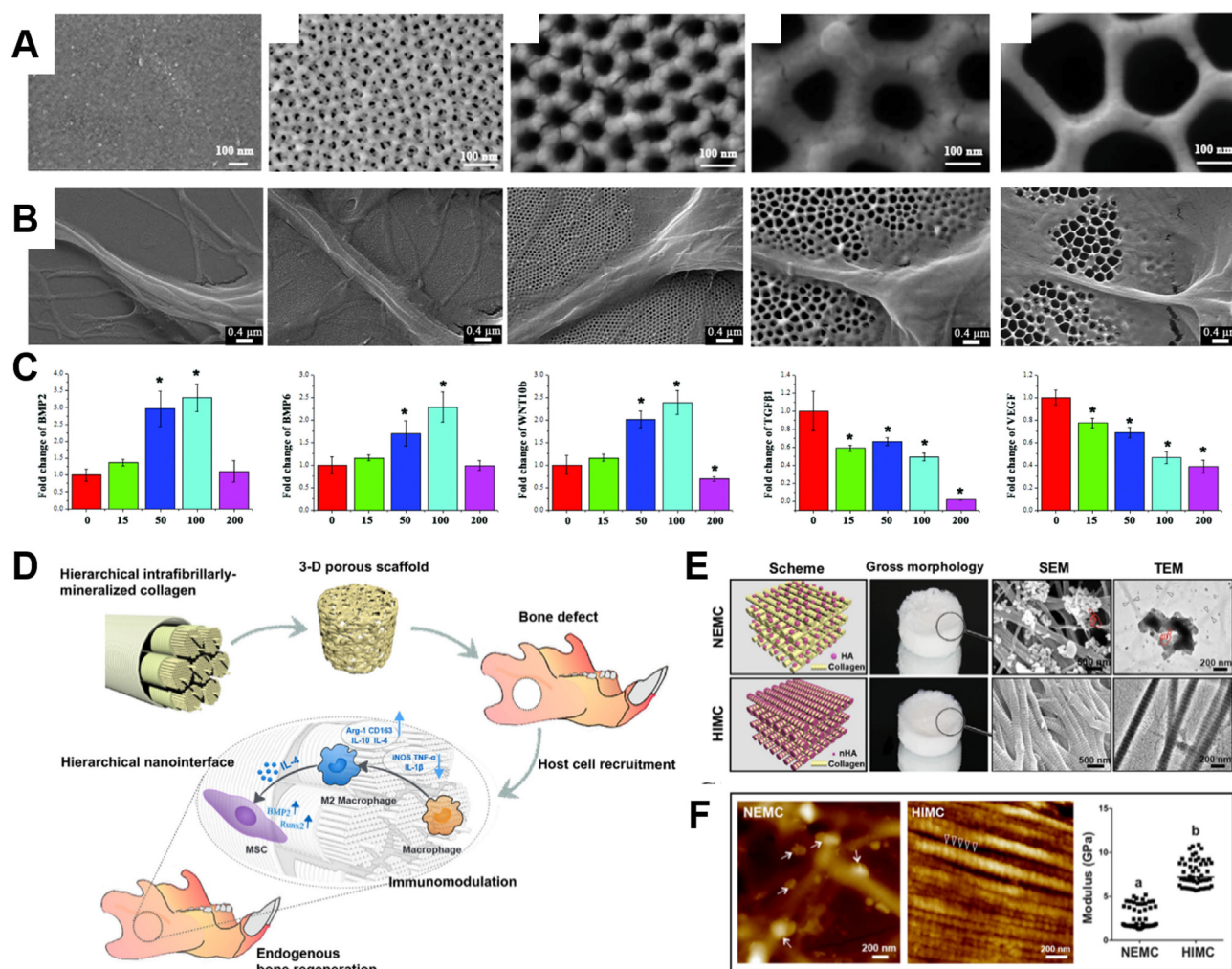
Fig. 5. Strategies of tuning surface topographic characteristics to modulate macrophage phenotypes. A) SEM images of alumina surfaces with diverse nanopore sizes and B) morphologies of macrophages adhered on different surfaces; C) The osteogenic cytokines (BMP2, BMP6 and Wnt10b) and fibrosis related factors (TGFβ1 and VEGF) expressed in macrophages activated on different sized nanopore surface; D-F) The nanometer-scale interface of biomaterials regulates osteogenic differentiation of stem cells and local immune response through independently physical signals to cells. Reprinted with permission from Refs. [173,176], Copyright (2017) Royal Society of Chemistry and Copyright (2019) American Chemical Society.

in the interior of scaffolds (Fig. 6H). The bioactive acBSP component of this scaffold could stimulate macrophages adhered on the external surface to transform into a moderate inflammatory phenotype, whose cytokine secretion would directly or indirectly assist the mobilisation of osteoprogenitor cells, as well as the subsequent endogenous *in situ* bone regeneration of calvarial defects (Fig. 6I).

5. Outlook

Bone is a richly vascularised dense connective tissue which shaped through an elaborate and dynamic process. Each stage during bone formation, remodelling, and fracture healing is precisely modulated by a spectrum of biochemical signals and physical cues, which can hardly be imitated by current therapeutic tissue engineering strategies with artificial blends of a few types of signal molecules or cytokines. Thus, a much better idea is to develop live cell-guided approaches following the natural rhythms of bone repair.

Macrophages have come into prominence with accumulating understanding of their multi-layered contributions in ossification and fracture healing. The inappropriate polarisation status or insufficient enrichment of specific macrophage subpopulation possibly implicated in compromised fracture healing. Thus, macrophage is a convincing target to be manipulated as an apex regulatory cell, which provides a

clear motivation to develop diverse methods of evoking this autogenous power in promoting osteogenesis and neovascularisation. However, these attempts also confront challenges. Macrophages are the principal source of pro-inflammatory mediators, thus impertinent macrophage-activation may impair the host immune homeostasis. In addition, as the progenitors of osteoclasts, improperly polarised macrophages in the bone tissue may also be involved in osteoclast formation and subsequent osteolysis. All of these remind us a delicate control of the balance between beneficial and unfavourable macrophage functions in the development of novel bone regeneration approaches. These strategies depending on the precise modulation of macrophage behaviours open an avenue to harnessing the power of host immunity to enhance the *in situ* bone regeneration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence this paper.
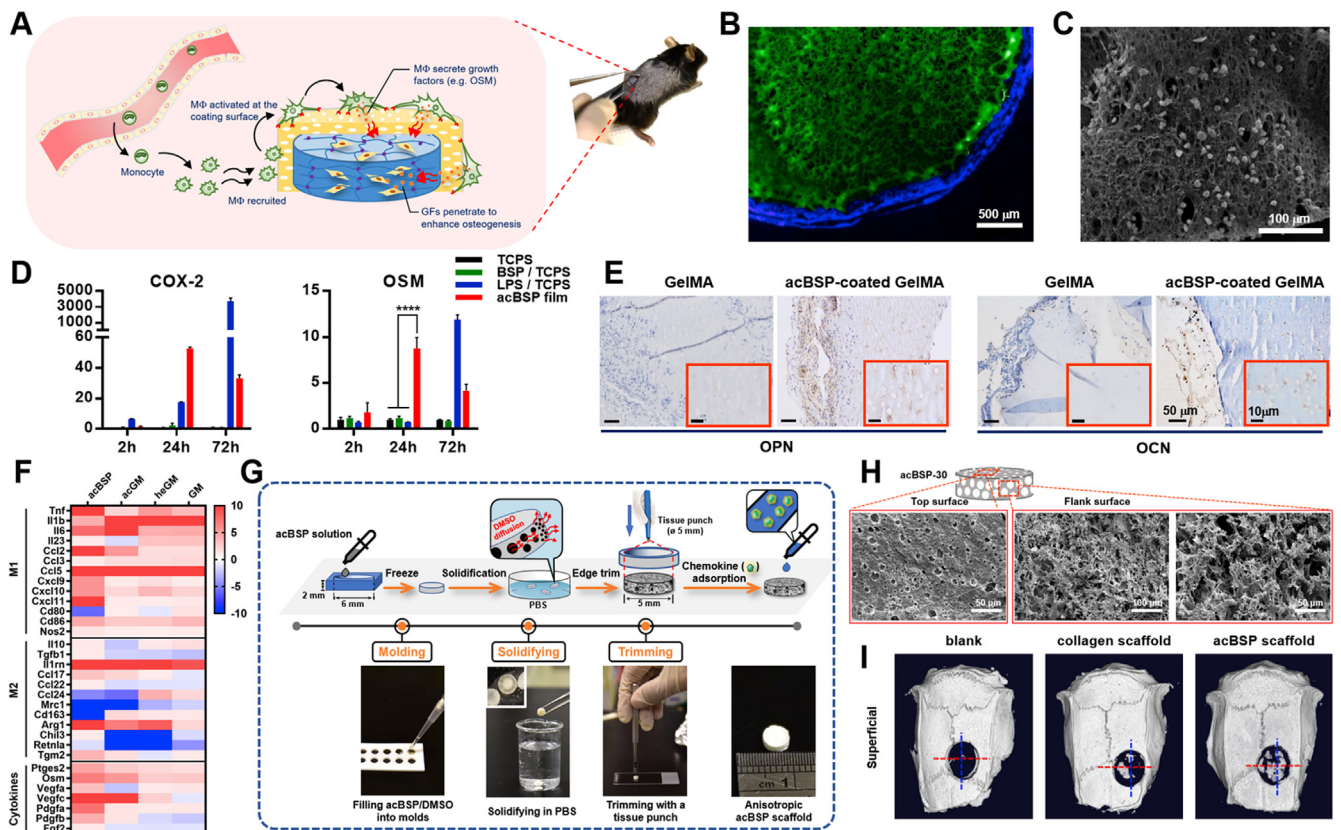
Fig. 6. Strategies of constructing a bioactive interface to tune macrophage behaviour. A) Illustration of the MSC-laden GelMA scaffold coated with acBSP polymer as a bioactive interface; B) Cross-section fluorescent image of GelMA (green) coated with acBSP material (blue); C) SEM images of monocytes adhering on acBSP interface; D) The expression of pro-osteogenic cytokine in primary macrophages (BMDMs) adhering on acBSP substrate; E) Bone sialoprotein (OPN) and osteocalcin (OCN) staining of cell-laden gels implanted in mice; F) Microarray analysis of selected gene expression in BMDMs treated with acBSP and other alkyl modified glucomannan; G) Preparation process of the anisotropic porous acBSP scaffolds; H) SEM images of the top surface and flank surface on acBSP scaffolds; I) Micro-CT images of treated calvarial defects taken 12 weeks after surgery. Reprinted with permission from Ref. [182,183], Copyright (2017, 2020) Elsevier.

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