Engineering immunomodulatory hydrogels and cell-laden systems towards bone regeneration

Sara Nadine[1]*, Clara R. Correia[1], João F. Mano[1]*

[1]CICECO – Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

*E-mail: saranadine@ua.pt and jmano@ua.pt

Abstract

The well-known synergetic interplay between the skeletal and immune systems has changed the design of advanced bone tissue engineering strategies. The immune system is essential during the bone lifetime, with macrophages playing multiple roles in bone healing and biomaterial integration. If in the past, the most valuable aspect of implants was to avoid immune responses of the host, nowadays, it is well-established how important are the crosstalks between immune cells and bone-engineered niches for an efficient regenerative process to occur. For that, it is essential to recapitulate the multiphenotypic cellular environment of bone tissue when designing new approaches. Indeed, the lack of osteoimmunomodulatory knowledge may be the explanation for the poor translation of biomaterials into clinical practice. Thus, smarter hydrogels incorporating immunomodulatory bioactive factors, stem cells, and immune cells are being proposed to develop a new generation of bone tissue engineering strategies. This review highlights the power of immune cells to upgrade the development of innovative engineered strategies, mainly focusing on orthopaedic and dental applications.

Keywords: Osteoimmunomodulation; Cell encapsulation; Bone tissue engineering; Macrophages; Hydrogels.

1. Introduction

Immunomodulation is a hot topic in tissue engineering and regenerative medicine (TERM). The immune system plays essential regulatory roles during tissue repair. Depending on the type of tissue, organ, or life stage, the immune response can impact positively or negatively during the healing process.[1] When the subject is bone regeneration, there is a well-known collaboration between the immune and skeletal systems. Indeed, immunocompromised patients often experience delayed or ineffective

healing following a fracture.[2,3] Thus, understanding the biology of bone tissue is crucial when designing biomimetic bone niches aimed for complete integration of the biomaterial and regeneration of the damaged tissue.[4] However, developing an appropriate biomaterial for bone regeneration is still challenging, and several clinical trials using TE strategies are not yet applicable on a large scale for clinical practice. One of the major challenges is the significant difference between the complexities of the proposed bone TE strategies and their actual clinical applications.[5] A successful TE strategy applicable for clinical translation requires cost-effectiveness, scalability, and simplicity, without using sophisticated and expensive instrumentations. For a long time, various scaffolds were designed to avoid the initial inflammatory reaction and prevent immune responses against biomaterials after implantation. Nowadays, we know that inert biomaterials, such as bioceramics and biomedical metals, only replace the function and structure of hard tissue, and the absence of bioactivity and biodegradability make them unattractive for long-term implantation.[6] Thus, safer and smarter biomaterials, with a combination of cell adhesion sites, bioactive factors, and controlled degradation have been lately emerging.[7] Additionally, the importance of inflammatory cell recruitment in the initial stages of bone healing and the polarization of the immune cells to a more pro-regenerative profile led to envisioning a new generation of bone-engineered approaches. These biomaterials promote bone-to-implant osteointegration without neglecting the local immune microenvironment. In an effort to modulate the host's cells around bone implants while harnessing the power of immune cells in modulating osteoprogenitor cells, various strategies have been incorporating bioactive biomolecules[8,9] and metal ions[10,11] or optimizing surface physical properties[12–14], such as topography, porosity, wettability, and surface charge. Such important findings have been already summarized in detail, focused on bone regeneration.[15,16] Currently, several hydrogels and cell encapsulation systems have also been designed for osteoimmunomodulatory purposes. Due to a number of appealing features, such as their injectability into confined spaces with specific geometries, their ability to create a controlled microenvironment for encapsulated cells, and their ease of adding multiple compartments or components with diverse functions, such strategies bring several advantages for bone tissue engineering.[17] In particular, innovative strategies are being proposed to overcome one of the major drawbacks of TE strategies aiming for bone regeneration: the lack of vascularization and integration with the host's environment after biomaterial's implantation. Developing fully functional hydrogels and cell-laden

structures to replace damaged tissues by resembling the bone native scenario is highly demanded.

This review mainly discusses hydrogel-based strategies that specifically influence a favourable immune response for improved bone regeneration. First, a short overview of bone biology and the importance of the immune system during bone regeneration and vascularization will be addressed. Then, different scenarios, ranging from the delivery of bioactive factors to those incorporating immune cells, will be analysed. While discussing fundamental aspects of the bone regenerative process, practical examples of the latest and most exciting research contributions of hydrogels and cell encapsulation systems with osteoimmunomodulatory properties will also be presented.

2. The bone tissue regenerative process

The successful regeneration of large bone defects remains a significant challenge in orthopaedic research. Bone is a highly dynamic tissue able to adapt its structure to mechanical stimuli. Characterized by its bone-forming osteoblasts and bone-resorbing osteoclasts, bone tissue has a remarkable capacity to self-repair and to heal without scar formation following a traumatic injury. However, critical defects resulting from severe non-union fractures and some pathological disorders, with average diameters larger than 2 cm, require a therapeutic intervention to facilitate bone regeneration.[18] The approach that remains the current gold standard for osseous reconstruction is the implantation of bone grafts. Although autologous bone grafts are immunocompatible and osteoconductive, they are associated with a considerable donor site morbidity risk. Moreover, despite allogenic grafts appearing to resemble autologous grafts' properties in terms of biomechanical stability and elasticity, concerns about disease transmission and immunogenicity are an undeniable challenge.[19,20] On the other hand, the use of synthetic bone-substitute materials may result in poor integration, excessive inflammatory reactions, and eventual bone resorption.[21] In the following sections, different cell-laden engineering strategies are discussed, alongside the native bone's physiological phenomena, to elucidate about their role during bone regeneration.

2.1. Bone physiology and biology

Natural bone presents a complex hierarchical structural organization composed of an organic phase, mainly constituted by collagen fibrils from type-I collagen molecules (ca. 90% of the organic mass), and an inorganic phase, comprised predominantly of

nanocrystals of carbonated hydroxyapatite (HAp) that are distributed along the collagen fibrils.[4,22] More than 200 different types of non-collagenous matrix proteins, such as glycoproteins, proteoglycans, and sialoproteins, provide physical and biochemical support and contribute to the abundance of signals in the immediate extracellular environment.[7]

Macroscopically, bone tissue is arranged either in a compact pattern (cortical bone) or a trabecular pattern (cancellous bone), and its functional units consist of osteons (**Figure 1A**). Cortical osteons comprise concentric circles with vertical Haversian canals containing blood vessels and nerves and are surrounded by concentric lamellae (ca. 3 μm). Trabecular osteons are composed of a honeycomb-like network of trabecular plates and rods filled with red bone marrow.[23] Osteocytes, representing 90-95% of all bone cells, reside in the fluid-filled cavities (lacunae) within the mineralized bone.[24] The cortical bone is covered by a fibrous connective tissue layer – the periosteum - onto which muscles, ligaments, and tendons attach. In contrast, the inner surface of cortical bone, cancellous bone, and blood vessels canals are surrounded by the endosteum.[23]

The development of bone tissue is achieved via intramembranous (mainly flat bones) or via endochondral (mainly long bones) ossification (**Figure 1C-D**). During the intramembranous bone formation, mediated by the inner periosteal osteogenic layer, capillaries from surrounding tissues invade the mesenchymal zone, differentiating mesenchymal stem cells (MSCs) directly into mature osteoblasts. On the other hand, the development of bone via endochondral ossification is more complex, requiring an initial mobilization and hypertrophy of cartilage cells, the chondrocytes. After directing the mineralized matrix formation and attracting blood vessels, the hypertrophic chondrocytes undergo apoptosis. Then, the cartilaginous matrix functional properties coupled with infiltrating blood vessels contribute to osteoblasts invasion, longitudinal bone growth, and hematopoietic marrow replacement.[25]

The modelling and remodelling processes are vital to maintain bone strength, integrity, and mineral homeostasis during a bone lifetime. Bone modelling is an anabolic process that forms and adapts the spatial distribution of the grown tissue, optimizing its geometry in response to physiological influences or mechanical forces until skeletal maturity is achieved.[26] Otherwise, bone remodelling is a continuous process where osteoblasts and osteoclasts work sequentially in the same remodelling unit to resorb old bone (termed as osteoclastogenesis) and form new tissue (termed as osteogenesis) (**Figure 1B**). There are four sequential remodelling phases, namely activation,

resorption, reversal, and formation, where old bone packets are removed and replaced with a newly synthesized extracellular matrix (ECM), followed by matrix mineralization.[23,27] Briefly, the activation phase involves the detection of biochemical or physical signalling, such as mechanical strain or parathyroid hormone (PTH) action by osteocytes, which leads to the recruitment and activation of mononuclear cells, and subsequently, their fusion and differentiation into multinucleated preosteoclasts.[28] Afterwards, the resorption phase is characterized by the release of C-C motif chemokine ligand 2 (CCL2) by osteoblasts in response to PTH signalling, increasing the recruitment of preosteoblasts to the bone surface.[29] Additionally, the expression of osteoprotegerin (OPG) is decreased by osteoblasts, while colony-stimulating factor (CSF-1) and receptor activator of NF-κB ligand (RANKL) are enhanced, leading to osteoclast proliferation and maturation, followed by degradation of the mineralized bone matrix.[30,31] Meanwhile, metalloproteinases (MMPs) produced by osteoblasts enable the degradation of unmineralized osteoid, exposing the arginyl-glycyl-aspartic acid (RGD)-binding sites for osteoclasts attachment. Then, mature osteoclasts anchor to RGD-binding sites via αvβ3 integrin molecules, generating an acidic microenvironment able to degrade the organic and inorganic components of bone.[32] During the reversal phase, a cleaning process involving mononuclear and mesenchymal-bone lining cells begins to remove and engulf matrix debris remaining in the bone resorption cavity, preparing the surface for the subsequent bone formation.[33] Ultimately, during the formation phase, the formed cavity starts to be replaced and filled with osteoblastic cells. Derived from pluripotent MSCs through the master runt-related transcription factor 2 (RUNX2), osteoblasts rapidly deposit ECM, composed mostly of collagen I, in densely packed columns.[34] This process is completed after gradual mineralization by osteoblasts, which release membrane-bound matrix vesicles that concentrate calcium and phosphate while enzymatically destroying mineralization inhibitors such as pyrophosphate or proteoglycans.[35] A portion of osteoblasts become engulfed by unmineralized osteoid, undergoing terminal differentiation into osteocytes and forming a network extending throughout the mineralized bone.[24] Osteocytes are sensitive to mechanical stimulus and have been described as having a role in spatial organization control and bone remodelling cycle during their long lifetime (compared to osteoblasts and osteoclasts). This remodelling process is ended when an equal amount of resorbed bone has been replaced.[36]

2.2. Mechanisms of bone repair and regeneration

Nowadays, more than 10% of fractures involve delayed or compromised healing.[37] In humans, fractures are the most common large-organ traumatic injuries. The healing process resembles the ontological events that take place during embryonic skeletal development. Such a postnatal process can restore the damaged skeletal organ in terms of cellular composition, structure, and biomechanical function, reproducing the pre-injured tissue.[38] After tissue damage, a dynamic and well-regulated interplay between cellular, humoral, and molecular mechanisms is required to restore the native tissue architecture. The healing process occurs in three distinct stages which overlap in time and space: inflammation, soft and hard callus formation, and remodelling.[39,40] More recently, it has been shown that immune cells significantly impact the differential capacity for healing. For instance, depletion of T-lymphocytes decreases osteoblast maturation and bone mineralization, contributing to delayed repair and remodelling.[41] Macrophages were shown to induce progenitor cells to undergo osteogenesis and to secrete mineralization-related proteins such as alkaline phosphatase (ALP) and collagen type I.[42,43] In fact, immunocompromised HIV patients were shown to have impaired healing or non-union fractures, and grafts of bone marrow in immunocompromised animals strongly enhanced their bone healing ability.[44,45] Additionally, both osteoblasts and osteocytes were shown to release immune mediators to recruit immune cells when responding against a pathogenic bone infection.[46,47] Cells from the innate immune system are the first components to arrive at the injury scene. The inflammatory phase is characterized by the development of a hematoma resulting from the bleeding at the fracture site and the activation of the plasma coagulation cascade. The formation of a provisional fibrin matrix is followed by the influx of inflammatory cells, attracted by platelet-derived factors, endogenous molecular patterns, and local tissue macrophages.[39] Short-lived neutrophils, usually the first inflammatory cells to arrive at the fracture site, express several proteases and antimicrobial substances essential to degrade potential pathogens. Importantly, neutrophils also secrete inflammatory and chemotactic mediators, such as IL (interleukin)-6 and CCL2, to recruit the second wave of cells, namely monocytes and macrophages.[48,49] During the clearing of damaged areas, T-lymphocytes and natural killer (NK) cells were shown to have an active role by producing RANKL and inducing the differentiation of myeloid progenitors into osteoclasts.[50,51] While osteoclasts resorb necrotic bone fragments and necrotic edges of

bone tissue, macrophages phagocyte the provisional fibrin matrix and necrotic cells. Simultaneously, macrophages release a repertoire of mediators in a spatially controlled manner, such as tumour necrosis factor-α (TNF-α), IL-1β, IL-6, and CCL2, that initiate the recruitment of fibroblasts and osteoprogenitor cells from their local niches.[52–54] Under the influence of stromal-derived factor-1 (SDF-1) and bone morphogenetic proteins (BMPs), MSCs are recruited and migrate into the injured site. At the same stage, local strategies such as members of the transforming growth factor-β (TGF-β) family, as well as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor-2 (FGF-2) are vital for angiogenesis and redevelopment of the vascular network.[55,56] As result, the fracture is replaced with fibrinous/granulation tissue composed of an unorganized extracellular collagen matrix full of MSCs and new blood vessels. The callus formation is identified during the replacement of the formed granulation matrix. In the peripheral regions of bone, the production of periosteum occurs via intramembranous bone formation by the differentiation of progenitor cells into osteoblasts (**Figure 1C**). In contrast, the repair of the inner part of the fracture occurs via endochondral ossification with the differentiation of progenitor cells into chondrocytes and following synthesis of the cartilaginous matrix (**Figure 1D**).[57] The cartilaginous template is produced by chondrocytes and results from the synthesis of a combination of type-I and -II collagen to provide mechanical support to the fracture for the subsequent mineralization. Fibroblasts replace gaps where cartilage is insufficient, generating fibrous tissue. All these well-coordinated events that mediate cell differentiation and proliferation are stimulated by the expression of growth factors, including TGF-β2 and -β3, as well as BMPs.[39] Within the soft callus construct, chondrocytes are able to synthesize considerable amounts of collagen type II.[58] Afterwards, these cells undergo hypertrophy, releasing collagen-X and calcium, mineralizing the surrounding cartilaginous matrix. T- and B-lymphocytes also emerge during the mineralization of cartilaginous callus and support endochondral differentiation. By releasing TNF-α and IL-17, both lymphocytes trigger mature chondrocytes apoptosis and suppress the differentiation of MSCs into chondrocytes.[59,60] Simultaneously, it is observed the differentiation of perichondrial cells into osteoblasts and further deposition of woven bone on the cartilage scaffold, while the primary soft cartilaginous callus is resorbed.[61] This stage of bone healing is commonly recognized as the formation of hard callus, typically irregular and under-remodelled. Macrophages have been shown to have a key

role during cartilage degradation by producing MMPs. Any dysregulation in the activity of MMPs has been related to bone fracture non-union.[62,63] Overall, the transition of soft into hard callus is highly controlled by immune cells, showing the continued significance of immune-bone interaction even after the end of the inflammation phase. Ultimately, the woven bone-hard callus remodelling is the final step that can last for several years. Essentially, the woven hard callus is gradually replaced by lamellar bone, and the process is sustained with a balance of hard callus resorption by osteoclasts and lamellar bone deposition by osteoblasts.[64]

2.3. The impact of immune cells during bone regeneration

Several years ago, it was established that soluble factors produced by antigen-stimulated immune cells induced osteoclast formation and stimulated bone resorption. This activity was named osteoclast-activating factor.[65] Subsequently, accumulating evidence has indicated that physiological bone formation and remodelling processes require a well-established network between skeletal and immune cells. Indeed, immune cells regulate bone resorption via cytokine release, namely CSF-1, RANKL, and OPG.[66,67] The RANKL/RANK signalling promotes the differentiation of multinucleated osteoclasts from their precursors and their activation and survival in normal bone remodelling. Alternatively, OPG protects bone from excessive resorption by binding to RANKL and disrupting its interaction with RANK.[67] RANKL is mainly sourced by osteoblasts, activated T-lymphocytes, and neutrophils, while OPG is secreted by various MSCs, as well as by B-lymphocytes.

B- and T-lymphocytes are major components of the adaptive immune system and are responsible for humoral and cell-mediated immunity, respectively. Depending on the subtype, these immune cells have been shown to dynamically influence skeletal homeostasis. For instance, regulatory T cells $CD4^{+}CD25^{+}$, a subset of T-lymphocytes, support osteoblastic differentiation and negatively impact osteoclast formation. Such subset inhibits osteoclastogenesis through direct cell-cell contact via CTLA-4.[68] On the other hand, it was also observed that the lack of an adaptive immune system in knock-out mice significantly enhanced fracture healing.[69] In fact, the $CD8^{+}$ T-lymphocytes subset was shown to impact endogenous bone regeneration negatively.[70] Lymphocytes do not appear to affect the initial stages of fracture healing; however, their absence impacts matrix organization and formation, resulting in stiffer bones deficient in

elasticity and dysregulations in collagen deposition. Thus, the lack of lymphocytes makes bone tissue more susceptible to fractures.[69,71] The release of IL-17 by T-lymphocytes is another essential feature during osteogenic differentiation. IL-17-releasing T-lymphocytes stimulate the proliferation and osteoblastic differentiation of MSCs.[72] IL-17 also supports osteoclasts differentiation by inducing the expression of M-CSF and RANKL by MSCs.[73] Furthermore, B- and T-lymphocytes are crucial mediators during callus remodelling. Both cells were shown to infiltrate the bone callus during the mid-to-late bone regeneration and release OPG, inhibiting osteoclastogenesis.[74] The crosstalk between B- and T-lymphocytes, via CD40/CD40L co-stimulation, also increase OPG production.[75] Interestingly, decreased ratios of $CD8^+$ T-lymphocytes and regulatory T lymphocytes are correlated with enhanced healing outcomes.[76] Compared to naïve immune phenotypes, the experience of the immune system was shown to have a direct influence on bone formation capability, disturbing the structural properties of trabecular and cortical bone, as well as overall mechanical competence.[77] Thus, the potential of the adaptive immune system to promote successful bone healing after fracture is highly dependent on the polarization balance of immune cells.

Alternatively, NK cells belong to the lymphoid lineage but mediate innate immune responses against viral-infected and tumour cells. The role of NK cells during fracture healing is not yet fully understood. Besides their function as cleaners of damaged cells, NK cells were shown to release chemokines, such as (C-X-C motif) ligand (CXCL7), which can induce MSCs migration.[78] Moreover, NK cells are known to express RANKL and M-CSF, and they can trigger the differentiation of monocytes into osteoclasts.[51]

After a fracture, neutrophils seem to be involved in the secretion of multiple cytokines and chemokines essential to initiate downstream responses leading to bone regeneration. These innate immune cells are the first to invade the callus, being responsible for recruiting monocytes and macrophages to the injury site.[79,80] Macrophages tend to exhibit the highest regulatory activity in both repair and fibrosis stages among all immune cells.[81]

Owing to their significant potential for therapeutic strategies, the study of different macrophage populations in different stages of tissue repair, fibrosis, and regeneration has increased in the past years. Also known as osteomacs, resident macrophages were notably located immediately adjacent to mature osteoblasts.[82] Osteomacs contribute to

tissue homeostasis, tissue-specific physiology, and innate immune surveillance, being present within virtually all types of tissues throughout life. Macrophages were also shown to be required for hematopoietic stem cells (HSCs) niches maintenance.[83] The absence of macrophages may lead to loss of endosteal osteoblasts and egress of HSCs into the bloodstream. Murine osteomacs were found to express the F4/80 marker; however, it is still unclear if all resident macrophages are positive for this marker.[84] The recruited monocyte-derived macrophages are in charge of interstitial immune surveillance. These derived macrophages penetrate infected tissues to coordinate innate and adaptive immune responses, promoting host antimicrobial defence, antitumor immunity, and inflammatory responses.[85] Based on distinct receptor expression, cytokine production, effector function, and chemokine repertoires, macrophages have been broadly characterized into M1 and M2 phenotypes. M2 macrophages can also be classified into different sub-populations, namely M2a, M2b, M2c, M2d, and M2f, depending on the nature of the differentiation biomolecules and expressed markers.[85–87] Once activated, the classically activated M1 macrophage subset is characterized by high production of pro-inflammatory mediators (e.g. TNF-α, IL-6, and IL-1β), whereas alternatively activated M2 macrophages exhibit ECM synthesis and remodelling functions, releasing anti-inflammatory, angiogenic, and growth factors (e.g. IL-10 and TGF-β).[85]

There is factual evidence that macrophages play fundamental regulatory roles in essential aspects of the bone healing process. The critical role of macrophages during bone repair was convincingly proved by a complete failure in bone mineralization in fracture *in vivo* models depleted of macrophages.[42,88] After an injury, the paracrine signalling between MSCs and macrophages is pivotal for osteogenic differentiation. The *in vitro* culture of MSCs with a macrophage cell line conditioned medium was shown to increase the MSC's ALP activity. The osteoinduction induced by macrophages was mediated by BMP-2 and TGF-β.[89] Additionally, MSCs co-cultured with macrophages showed overexpression of osteocalcin and osteopontin, two well-known osteogenic differentiation markers.[90] Macrophages can also induce the differentiation of MSCs into osteoblasts by releasing oncostatin M (OSM), and by activating the MSCs signal transducer and activator of transcription 3 (STAT3). The MSC-mediated bone formation was established by the expression of CCAAT/enhancer-binding protein δ (C/EBP δ), core-binding factor subunit alpha-1 (Cbfa1), ALP, and bone sialoprotein (BSP) markers. The OSM production by macrophages depends on a prostaglandin E2

(PGE2) and cyclooxygenase 2 (COX2) regulatory loop and requires direct cell-cell contact with MSCs.[91,92]

Moreover, the polarization state acquired by macrophages can have a considerable influence on osteogenic stem cell differentiation. After tissue damage, the inflammatory response is mediated mainly by M1 macrophages, vital for the acceleration of fracture repair. In later stages of bone remodelling, M2 macrophages control the bone formation process by releasing pro-regenerative cytokines. For instance, the anti-inflammatory factor IL-10, mainly secreted by M2 macrophages, has a critical function in bone tissue, affecting bone stability. IL-10 depleted mice presented features of osteoporosis, including a reduction in skeletal mass, suppressed bone formation, and lack of biomechanical strength compared to wild-type counterparts.[93] IL-10 also has an effect on osteoclastogenesis by up-regulating OPG expression, which inhibits osteoclasts formation.[94] The controlled and timely switch of macrophages between M1 and M2 phenotypes was already observed in a mouse osteotomy model during endochondral ossification.[95] Nevertheless, an excessive inflammatory response in the initial phase leads to impaired bone healing, while prolonged M2 phenotype in the later stages can lead to the formation of detrimental foreign body giant cells (FBGCs) and chronic inflammation.[96] Although *in vitro* studies demonstrated that macrophages are highly plastic and oscillate between different polarization states, it is still unclear if macrophages switch individually from M1 to M2 phenotypes or if different phenotypes of macrophages emerge and vanish during the distinct stages of tissue repair. In fact, the exact function of the diverse polarization states of macrophages during bone repair remains undetermined.[97,98]

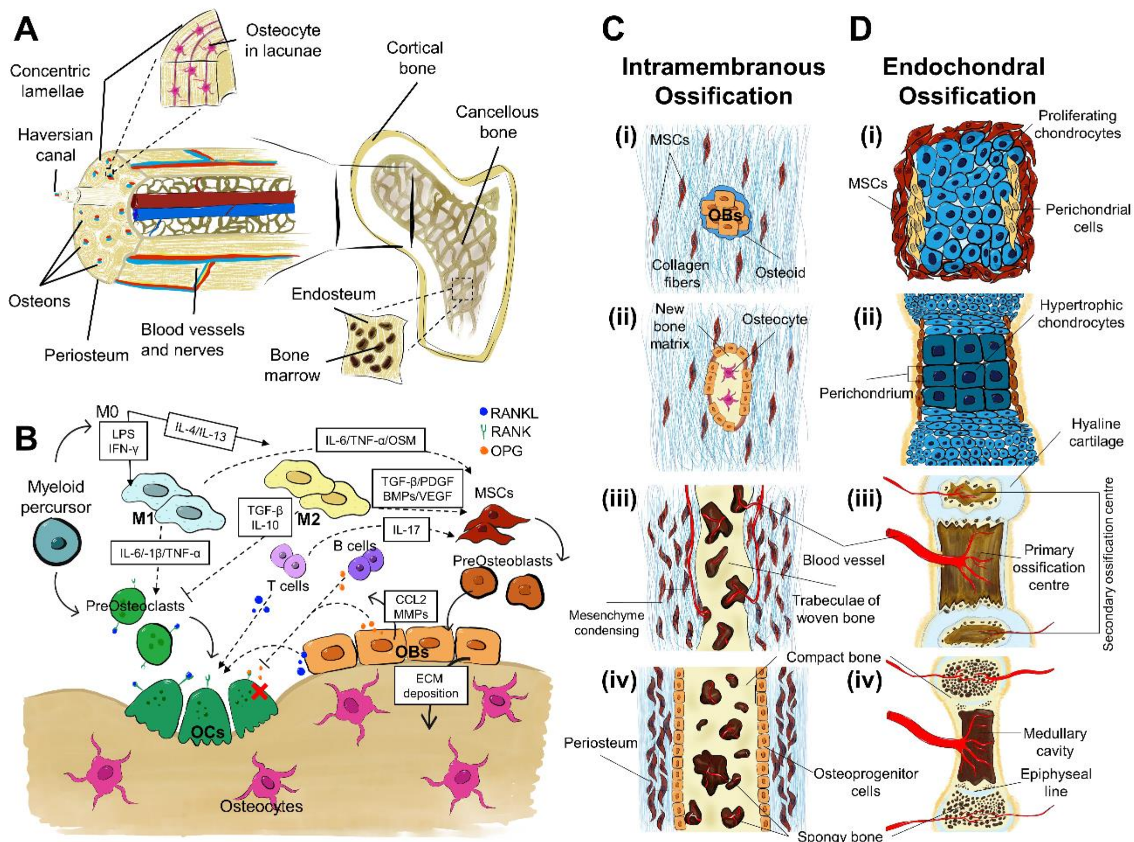


Figure 1 – A. Schematic illustration representing the hierarchical organization of bone tissue, from macro- to nano- and ultra-structures. **B.** Role of bone and immune cells during the bone remodelling process. **C.** Intramembranous ossification stages: (i) formation of an ossification centre in the fibrous connective tissue, (ii) secretion and mineralization of bone matrix (osteoid), (iii) development of trabecular bone, (iv) formation of compact bone and periosteum and emerging of red bone marrow **D.** Endochondral ossification stages: (i) chondrocytes undergo differentiation and condensation, (ii) maturation and hypertrophy of chondrocytes to develop the calcified hyaline cartilage surrounded by the perichondrium (primary ossification centre), (iii) cavitation of the primary ossification centre and invasion of blood vessels of periosteal bud followed by the formation of the secondary ossification centres, (iv) ossification of the epiphyseal plates to form epiphyseal lines. Nomenclature: BMP – bone morphogenetic protein; CCL – C-C motif chemokine ligand; ECM – extracellular matrix; IL – interleukin; IFN – interferon; LPS – lipopolysaccharides; M0 – unpolarized macrophages; MMP – metalloproteinases; MSCs – mesenchymal stem cells; OBs – osteoblasts; OCs – osteoclasts; OPG – osteoprotegerin; OSM – oncostatin M; PDGF – platelet-derived growth factor; RANK - Receptor activator of nuclear factor kappa-B; RANKL – RANK ligand; TGF – transforming growth factor; TNF – tumour necrosis factor; VEGF – vascular endothelial growth factor.

2.4. The impact of immune cells on bone vascularization

Bone is a highly vascularized tissue, being thus of paramount importance to re-establish a proper blood flow after a fracture. Dysfunctions in the blood flow may result in defective angiogenesis and osteogenesis.[99] Usually, following a fracture, the lack of oxygen, nutrients, and trophic factors promote the repair via endochondral ossification, whereas in non-hypoxic cases, the intramembranous ossification is triggered.[100] During bone healing, the release of angiogenic growth biomolecules in a spatiotemporally well-regulated manner is required to stimulate and stabilize the development of blood vessels.[101] Macrophages are critical regulators of inflammatory angiogenesis and vascular remodelling. These innate immune cells are believed to release and express a plethora of pro-angiogenic and cell recruiter factors, including interleukins, TNFs, MMPs, CSFs, CXCLs, CCLs, TGFs, VEGFs, FGFs, and insulin-like growth factors (IGFs), to initiate vascular repair.[102–104] A study in murine models showed that by decreasing the number of macrophages through a clodronate liposome treatment, the number of blood vessels diminished in the intramembranous bone area when compared to controls.[95] However, an appropriate bone vascularization during bone regeneration is dependent on the timely polarization from pro-inflammatory M1 macrophages to the anti-inflammatory M2 phenotype since prolonged inflammatory signalling is correlated with delayed bone healing.[98,105] While M1 macrophages release VEGF and are implicated in the initial stages of blood vessels formation, M2 macrophages secrete PDGF-BB to induce vascular anastomosis.[106,107] Spiller *et al.* demonstrated that M1 macrophages might work simultaneously with M2c macrophages in the early stages of tissue repair. The later stages of regeneration seem to be controlled by the M2a macrophage sub-population.[108] Additionally, distinct anti-inflammatory macrophage sub-populations may control the angiogenic behaviour of endothelial cells in different ways. M2a macrophages appear to control the endothelial cells-pericytes interactions; M2c macrophages are involved in cell sprouting; whereas vessel maturation seems to be regulated by M2f macrophages.[87] In fact, macrophages were shown to be involved in cell sprouting through the directed filopodia extension towards a higher VEGF concentration.[109] Other immune cells may also be involved in the vascularization of bone tissue. For instance, immature myeloid cells appear to affect neovasculature following a bone fracture. Besides increasing the migration and motility of endothelial cells, these progenitors of the myeloid lineage were shown to enhance endothelial tube

formation.[110] Mast cells are also a potent source of pro-angiogenic factors and were shown to mediate vascular regeneration in an ischemia mouse model.[111] Regulatory T-lymphocytes are highly tissue- and context-specific and thus, may exhibit contrasting behaviours in angiogenesis. However, most studies demonstrate their effectiveness in promoting new vessels formation through VEGF signalling or by modulating the response of other immune cells.[112,113] VEGF can also recruit MMP-9-delivering neutrophils, which consequently promotes angiogenesis.[114] Also, neutrophils were shown to migrate and accumulate in a directional way around the nodes where endothelial sprouting begins.[115]

Therefore, the design of different engineered strategies should modulate the local immune environment in an effort to improve bone regeneration, angiogenesis, and osteointegration. Additionally, the crosstalk between immune and stem cells requires a complete elucidation since the use of cell-laden TERM strategies usually involves the encapsulation of MSCs.

3. Osteoimmunomodulatory hydrogels and cell encapsulation strategies for bone regeneration

Since TERM principles were defined,[116] significant progress has been achieved. The concept relies in the bioengineering of living tissues that could substitute or aid specific biological processes by combining cells and biomaterial-based scaffolds, which can be further functionalized with biomolecules of interest.[117] The aim is to develop customizable and immune-compatible constructs able to restore, maintain, or improve injured or diseased tissues instead of replacing them with inert constructs.[117]

Metallic-based scaffolds, widely used to displace bone fractures, provide physiological load-bearing functionality to the implant site, and facilitate healing. Although possessing good mechanical strength, such strategies are bioinert and cannot fully integrate with the host's surrounding tissues.[118] Along with TERM field evolution, plenty of other strategies aiming for bone substitution has emerged. The incorporation of cells in instructive biomaterials to ultimately regenerate damaged tissues is a crucial element in TERM. Seeded cells deposit ECM to produce the foundations of a tissue. Since various cell types are available in the skeletal structure, the use of primary and multipotent stem autologous cells has been shown to improve the multifunctional-engineered systems to regenerate damaged tissues.[119] Ideally, to prevent a clinical

intervention from removing the implant, bone regeneration technologies should be able to replace the bone ECM without necrosis or scar in parallel with scaffold degradation.

Hydrogels are the majority of cell encapsulation systems used for tissue regeneration. Due to a number of appealing features, such as resemblance with native ECM, biotolerability, suitability for incorporation of molecules of interest, and high-water environment, hydrogels are a promising strategy to substitute the gold standard grafts.[120] Furthermore, hydrogels can be implanted by minimally invasive procedures (injectability) with the ability to match irregular defects. Although the lack of osteoconductivity is an issue for natural hydrogels, multiple strategies have been used to improve their mechanical properties, including the reinforcement with bioactive glass and hydroxyapatite crystals or interpenetrating double networks to increase their strength and toughness.[121,122]

Overall, one of the current trending topics in TERM is the immunomodulatory perspective on how bone biomaterials are envisioned. Herein, we will overview the immunomodulatory power of hydrogels and cell-encapsulation systems, which are able to improve the bone regeneration process through the control of the polarization of macrophages during the inflammatory reaction.

3.1. Hydrogels with osteoimmunomodulatory potential

Besides playing critical roles during osteogenic differentiation, macrophages also determine the success or failure of bone substitute implants.[123] The biomaterial-tissue interaction usually activates a foreign body response (FBR), which can lead to chronic inflammation, tissue damage, and fibrosis.[124] Following implantation of a biomaterial, a provisional matrix interface between the surrounding tissues and material is immediately created. The biomaterial-tissue interaction is initiated by the absorption of plasma proteins, such as fibrinogen and complement, onto the surface. Then, similarly to the natural healing process, acute inflammation is triggered and driven by neutrophils, mast cells, and other immune cells. At the same time, multiple bioactive compounds are released in a sequential fashion to regulate the inflammatory response.[125] Monocytes are also recruited and differentiated into macrophages to act as antimicrobial and phagocytic agents.[126] The prolonged presence of mononuclear cells, including monocytes and lymphocytes, gives rise to the chronic inflammatory phase. When the biomaterial is biocompatible, the next step relies on fibroblasts recruitment, granulation, neovascularization, and new tissue formation.[127] However, if

the inflammation phase persists, usually beyond a three-week period, it implies that the implanted biomaterial is not biocompatible and an indicator of infection. The presence of macrophages and FBGCs, which are multinucleated cells that arose from fused adhered macrophages, are indicative of FBR.[124] Additionally, when the biomaterial's surrounding microenvironment fails to polarize inflammatory macrophages into an M2 phenotype and control the inflammation, it may lead to fibrous capsule formation.[123] However, the prolonged presence of M2 macrophages is as well demonstrated to induce the formation of FBGCs.[96] The fibrous capsule separates the implant from the host's tissue, impairing the biomaterial's integration and compromising its function to fill the fracture with fully functional bone.[15] Through specific interactions between a repertoire of receptors, macrophages recognize several proteins, saccharides, lipids, and nucleic acid ligands. These interactions may elicit a diverse range of cellular responses, which may be supportive or detrimental to bone healing.[128] Thus, it is important to design osteoimmunomodulatory biomaterials to stimulate immune cell activation toward a favourable regeneration profile and biomaterial integration without inducing an FBR. To achieve full integration, it is imperative to understand the immunological profile of biomaterials.

Lately, great examples of advanced hydrogels that improve bone regeneration while enhancing the host's immunomodulation have been studied. However, the regulation of macrophages polarization by natural biomaterials remains limited. For that, an injectable alginate/sericin/graphene oxide hydrogel was envisioned to promote bone regeneration while controlling macrophage polarization (**Figure 2A** (**i**)).[129] The proposed hydrogel was crosslinked via HRP-catalysed reaction and exhibited controlled degradation, releasing sericin and graphene oxide. The controlled release of sericin was shown to promote macrophage migration and polarization into an M2 phenotype via the NF-κB and MAPK pathways (**Figure 2A** (**ii**)). On the other hand, the enhanced osteogenic differentiation and mineralization of encapsulated rat MSCs were associated with the graphene oxide release and macrophage polarization. When injected into rat distal femoral defects, the inflammatory host response to the biomaterial remained low, while the bone defect was quickly repaired.[129] In a different approach, electrostatic reinforced hydrogels composed of alginate/gellan gum matrix and amino-functionalized 45S5 bioactive glass induced the polarization of macrophages to an M2 phenotype while up-regulated the osteogenic gene expression of rat bone marrow MSCs.[130] An injectable periosteal ECM (PEM) hydrogel was shown promising for bone healing when

implanted in a rat critical-sized calvarial defect model. Results confirmed that the PEM hydrogel facilitated the transition from M1 to M2 macrophages, promoted blood vessel migration, and matured bone formation in large bone defects compared to collagen I hydrogels.[131] An interesting strategy to immunomodulate the local bone environment during scaffold implantation is proposed by Chengcheng and colleagues.[132] Briefly, the idea relies on the fabrication of a biomimetic anti-inflammatory nano-capsule coated with LPS-treated macrophage cytokine receptors to neutralize the inflammatory cytokines. Additionally, the nano-capsules deliver in a controlled manner resolvin D1, which promotes M1 to M2 macrophage polarization. The biomimetic nano-capsules were implanted with boron-containing mesoporous bioactive glass scaffolds. Scaffolds alone or nano-capsules without resolving D1 were used as controls. When implanted into a mouse femoral defect model, the results confirmed a decreased inflammatory response and enhanced newly formed bone compared to controls.[132]

Gelatine methacryloyl (GelMA) has been widely used for bone tissue engineering. More recently, besides incorporating inorganic compounds in GelMA to improve bone regeneration, these constructs have also been evaluated for their immunomodulatory impact.[133,134] The incorporation of nanosilver and halloysite nanotubes in GelMA hydrogels was suggested as an osteoimmunomodulatory biomaterial.[133] While nanosilver possesses potent anti-inflammatory and antibacterial effects, halloysite nanotubes exhibit bone regeneration capacity. After being studied *in vitro* and *in vivo*, the hybrid hydrogel demonstrated a good ability to modulate the immune environment toward improved bone healing, with the extra of exhibiting antibacterial activity against both Gram-negative and Gram-positive bacteria.[133] When nano fishbone (NFB) was incorporated in GelMA, similar results were obtained. The presence of NFB within the hydrogel resulted in the polarization of macrophages to an M2 phenotype. The NFB-containing hydrogel was also an effective strategy for the osteogenic differentiation of human dental pulp stem cells (hDPSCs) *in vitro*. After implantation in rat cranial bone defect model, the NFB-hydrogels enhanced new bone formation and osteointegration with a thinner fibrous layer, compared to animals without material implantation or implantation of GelMA without NFB (**Figure 2B**).[134]

Interestingly, hydrogels were also envisioned as adhesive strategies while regulating bone metabolism to avoid loosening implant osteointegration under osteoporotic conditions. For that, a double-adhesive hydrogel composed of MA hyaluronic acid with alendronate, commonly employed as the first-line therapy for osteoporosis, was

integrated with aminated bioactive glass modified by oxidized dextran. The hydrogel bonded to both inorganic calcium phosphate and organic collagen on trabecular bone. The double-adhesive hydrogel could inhibit the osteoclast differentiation of macrophages induced by RANKL and promote osteoblasts differentiation. Improved fixation of the orthopaedic implants (titanium screws) was also observed when screws were injected with the hydrogels before implantation, promoting cancellous bone reconstruction by regulating the local metabolism.[135]

3D-printed hydrogels with immunomodulatory properties have also been proposed for bone regeneration. The fabrication of chitosan/silk fibroin/cellulose nanoparticles (CS/SF/CNPs)[136] and ECM/polyethylene glycol diacrylate (PEGDA)[137] hydrogels are examples that 3D printing can be combined with osteoimmunomodulation. After 1 day of culture, Raw 264.7 macrophages in contact with the CS/SF/CNPs hydrogel exhibited iNOS and CD68, characteristic of inflammatory M1 macrophages. However, a polarization to regenerative M2 macrophages was quickly observed through enhanced expression of CD86 and CD163 markers 3 days after culture. Furthermore, the osteogenic potential of CS/SF/CNPs hydrogels was tested in rats. Results showed their ability to trigger bone regeneration, with increased density of newly generated cells and mature osteocyte cells compared to CS and CS/CNPs scaffolds and negative groups (without treatment).[136]

Overall, the study of the immunomodulatory properties of hydrogels is necessary and is expanding since the immune system plays a significant role in bone regeneration. The bioengineered strategies should not only present osteogenic properties but also modulate the immune environment for enhanced tissue integration.

3.1.1. Impact of mechanical signals in macrophage immunobiology

The physical properties of hydrogels can positively or negatively influence osteogenesis and immunomodulation. It is well-known that osteoprogenitor cells are sensitive to mechanical cues, and the lack of biophysical forces in native environment can affect significantly the formation, resorption, and adaptation of bone tissue.[138,139] Thus, the selection of an appropriate biomaterial is indispensable, since the mechanical properties of the matrix effectively impact the behaviour of osteoprogenitor cells. For instance, stiffer matrices have been shown to increase the osteogenic differentiation of MSCs.[140,141] Although biomaterials with mechanical properties close to the native bone

tissue increase mineral deposition, the implantation of these stiffer engineered approaches might have a negative effect on immunomodulation and biomaterials integration.[142,143] In fact, the physical properties of hydrogels, including the stiffness[142–144], pore size[145,146], and surface modification[147,148], can control the proliferation, differentiation, and function of macrophages. However, the mechanobiology of these immune cells is still poorly understood, since most of the studies just correlate the biomaterials design with macrophage polarization, without exploring the molecular signalling pathways.[149]

The stiffness of hydrogels is one of the most explored physical factors since macrophages were shown to be highly mechanosensitive.[150] Zhuang and colleagues found that stiffer GelMA hydrogels (29.2 ± 1.5 kPa) induced macrophage polarization into a more pro-inflammatory phenotype, presenting more focal adhesion staining and releasing significantly higher amounts of IL-6 and TNF-α. On the other hand, softer GelMA hydrogels (1.9 ± 1.8 kPa) showed a more elongated shape, releasing significantly more TGF-β and CCL17 anti-inflammatory factors. When softer hydrogels were subcutaneously implanted in mice, Arg-1 and iNOS staining revealed more anti-inflammatory M2 macrophage infiltration with thinner fibrotic capsule formation than stiffer hydrogels (**Figure 2C**).[142] Contradictory findings were observed when mice bone marrow-derived macrophages were cultured in polyacrylamide hydrogels with different substrate stiffnesses. The designed hydrogels with low (2.55 ± 0.32 kPa), middle (34.88 ± 4.22 kPa), and high (63.53 ± 5.65 kPa) substrate modulus were comparable to collagen fibres, osteoid and pre-calcified bone, respectively. After 3 and 5 days in culture, results evidenced that macrophages seeded in low stiffness hydrogels expressed more CD86, produced higher amounts of reactive oxygen species (ROS), and released more pro-inflammatory cytokines than the other groups, suggesting their polarization into a M1 phenotype. After subcutaneous implantation in C57/BL6 mice, hydrogels with low substrate stiffness were infiltrated mainly by pro-inflammatory $CD86^+$ macrophages while stiffer substrates were surrounded by anti-inflammatory $CD206^+$ macrophages. Moreover, researchers concluded that macrophage polarization was mediated through the NF-κB signalling pathway.[144] The pore size of hydrogels also influences macrophage polarization. For instance, poly(2-hydroxyethyl methacrylate) (pHEMA) hydrogels with distinct pore sizes were implanted subcutaneously in a mouse model. Non-porous hydrogels were used as control. After 3 weeks of implantation, the infiltration of M2 macrophages was more evident in the

large pore size hydrogels (160 μm) than in the small pore size hydrogels (34 μm). However, results suggested that the small pore size hydrogel increased vascular density. Additionally, the non-porous hydrogel induced the formation of a thicker fibrous collagen capsule containing a denser cell infiltration, compared to the other conditions.[145] Interestingly, the spatial confinement of macrophages has been shown to downregulate the LPS-induced late-responsive genes, decreasing the expression of pro-inflammatory markers, such as IL-6, CXCL9, and iNOS, by mechanomodulating chromatin compaction and epigenetic alterations (HDAC3 levels and histone 3 lysine 36 dimethylation). These results were obtained regardless of whether the cells were confined by micropatterning, microporous substrates or cell crowding.[146] Importantly, the phenotype of macrophages-laden hydrogels can also be programmed through integrin-mediated interactions. Incorporating cell-binding domains within hydrogels, such as α2β1, has driven encapsulated macrophages towards a natural healing phenotype. When the integrin α2β1 was blocked, encapsulated macrophages polarized to the M1 phenotype.[147] Undoubtedly, macrophages recognize the architecture and spatial information of the surrounding environment. The phenotype of these immune cells is regulated and adapted by the microenvironmental niches in which they reside due to macrophage's vast array of sensing molecule.[151] Thus, it is crucial to understand the mechanotransduction pathways of macrophages when designing smart hydrogels for bone TE, in order to direct endogenous macrophages to promote tissue regeneration and biomaterials integration.

3.2. Biological factors delivery

Different bioactive molecules are known to be crucial for bone homeostasis and regeneration. However, direct signal factors and cytokines delivery requires a large amount due to the fast inactivation and clearance, which is associated with a high risk of adverse effects.[152] On the other hand, the controlled and sequential delivery of cytokines was shown promising to harness the angiogenic behaviour of host macrophages. A decellularized scaffold prepared from trabecular bone was designed to release a short amount of interferon γ (IFN-γ), known to drive macrophages to classically activated M1 macrophages, followed by a sustained release of IL-4, known to induce a M2 phenotype. Accordingly, results show that the polarization of macrophages from M1 to M2 phenotypes occurred. Additionally, after implantation in a

murine subcutaneous model, scaffolds releasing solely IFN-γ showed increased vascularization compared to negative controls (scaffolds without IFN-γ).[98]

Within this scope, hydrogels have emerged as a promising technology for bone tissue engineering, acting as bioactive molecule carriers while conferring support for tissue growth. The molecules of interest can be protected and delivered in a controlled and targeted way, recruiting endogenous stem and immune cells to the defect site to mediate osteoimmunomodulation and healing of bone tissue.[153] The main examples of signal factors used in biomaterials to modulate the immune response while inducing bone osteogenesis and angiogenesis are anti-inflammatory cytokines, such as IL-4 and IL-10, growth factors, including TGF-β, PDGF-BB, and FGF, or even ions and exosomes.[154–159]

IL-4 and SDF-1α were incorporated in high-stiffness transglutaminase crosslinked gelatines (TG-gels) to decrease the inflammatory response that this hydrogel typically triggers.[156] The presence of IL-4 effectively polarized macrophages to a M2 phenotype, with increased immunostaining for CD163 and CD206, compared to hydrogels without IL-4. Additionally, when MSCs-laden TG-gels were indirectly co-cultured with macrophages using transwell inserts, the Alizarin Red S staining and ALP activity demonstrated that the presence of M2 macrophages enhanced the osteogenic differentiation of MSCs. When TG-gels were implanted in rat periodontal defects, the tissue biopsies revealed the same tendency of *in vitro* studies, with a higher amount of recruited M2 macrophages, while SDF-1α guided endogenous MSCs to the defect site.[156] Another interesting concept explored by Fan and colleagues was the fabrication of blood clot gels loaded with BMP-2. Also, a mild localized laser-induced hyperthermia treatment was performed to accelerate bone repair. The BMP-2 blood clot gels were implanted in mice with large bone defects, and results indicate a certain degree of inflammation caused by local trauma. However, macrophage polarization into a pro-regenerative M2 phenotype was observed after the fourteenth day, reinforced with a high release amount of IL-4 and IL-10. At the same time, the healing of bone tissue was significantly accelerated.[160] Wang *et al.* developed hydrogels composed of hyaluronic acid grafted with quaternary chitosan loaded with IL-4 and BMP-2 to modulate macrophages polarization and accelerate osteoblasts differentiation. The loaded hydrogel was complexed with a 3D-printed mesoporous titanium alloy scaffold due to its excellent mechanical support and osteoconductivity. The release of the signal factors boosted the polarization of macrophages to a M2 phenotype (**Figure 2D**), and

the osteoblastic differentiation of MSCs.[161] IL-4 and BMP-2 cytokines were also incorporated in graphene oxide, and then embedded in carboxymethyl chitosan/PEGDA hydrogels for osteoimmunomodulation purposes.[162] This combination of factors is widely explored to create effective osteoimmunomodulatory biomaterials, obtaining similar results both *in vitro* and *in vivo*.

TGF-β1 is a growth factor known to stimulate alternatively activated M2 macrophages differentiation. An injectable lithium-heparin hydrogel containing gelatine-heparin microspheres loaded with TGF-β1 was envisioned for bone repair and modulation of the early-stage osteoimmune response. After 4 weeks of implantation in a rabbit femoral defect, M2 macrophages were activated, and a significant increase of osteogenic and angiogenic factors, including TGF-β1, BMP-2, and VEGF, was observed. After 12 weeks, the bone defects filled with the TGF-β1-loaded hydrogel were almost fully healed, with a considerable amount of new-formed bone matrix and a high number of newly formed vessels (**Figure 2E**).[155]

Besides cytokines and growth factors, biomaterials can release other agents to regulate the early-stage osteoimmune response. The dual release of SEW2871 and platelet-rich plasma (PRP) from gelatin hydrogels was envisioned for bone repair.[163] SEW2871 is a sphingosine-1 phosphate agonist able to recruit macrophages. In contrast, PRP was already associated with improved osteogenesis and vascularization, since it contains multiple growth factors, including TGF-β, PDGF, VEGF, SDF-1, and EGF. SEW2871 was solubilized through micelle formation with L-lactic acid oligomer-grafted gelatine micelles to overcome its insolubility limitation. Results show that the migration of macrophages was significantly higher when hydrogels released both SEW2871 and PRP *in vitro* and *in vivo*. Additionally, the presence of both agents significantly increased the mRNA expression of TGF-β1 and OPG in the biopsied tissues after 3 days of implantation, compared to controls. The implantation of SEW2871-PRP gelatine hydrogels into rat bone defects resulted in more extensive bone tissue formation than hydrogels incorporating either SEW2871 or PRP (**Figure 2F**).[163]

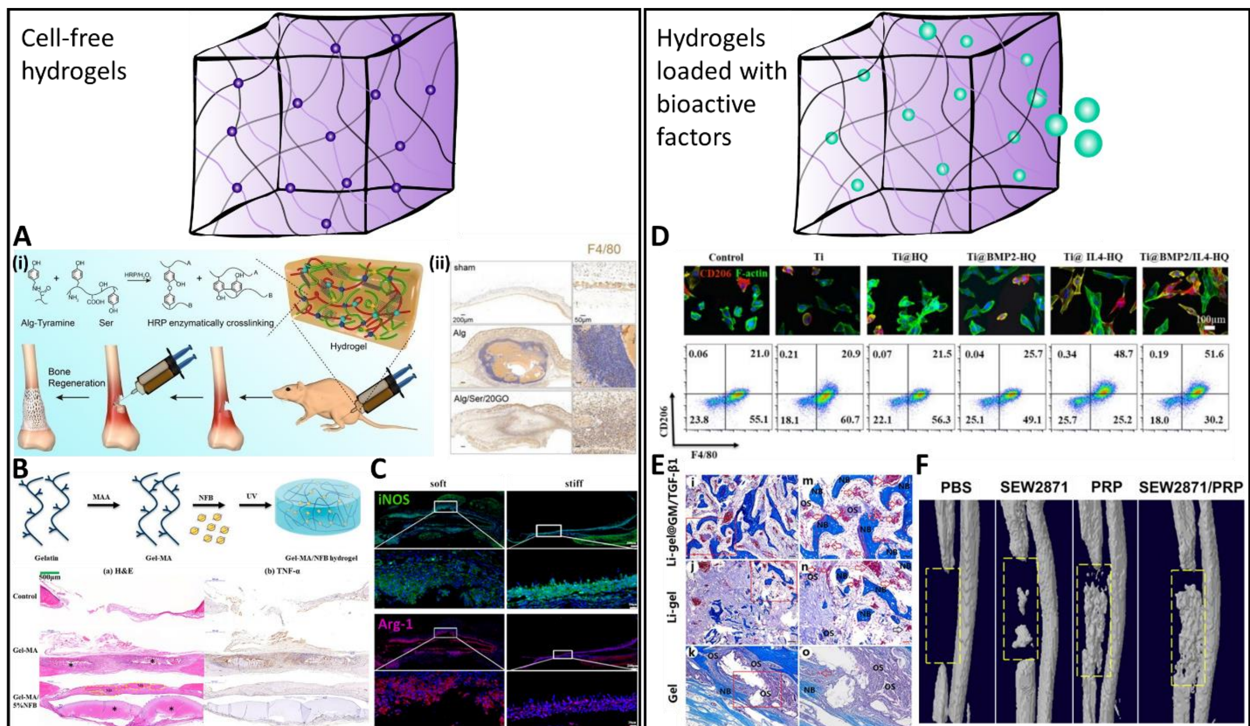
Injectable hyaluronic acid hydrogels loaded with endothelial cell-derived exosomes (EC-Exos), and an IRE-1α inhibitor (APY29) were fabricated to regulate the balance between osteoblasts and osteoclasts as well as M1 and M2 macrophages. MiR-26a-5p is well-known as an osteogenic differentiation regulator; however, due to miRNAs instability, EC-Exos were used as carriers (EC-Exos$^{miR\text{-}26a\text{-}5p}$), which in turn were also shown to have anti-osteoclast properties. Also, APY29 inhibits the phosphorylation of

IRE-1α, increasing the polarization of M2 macrophages. After injecting in a mouse femoral fracture, the *in situ* sustained release of this cocktail therapy improved bone formation, with increased levels of bone volume, total volume, and bone mineral density after 14 and 21 days. Additionally, the injectable hydrogel reduced the osteoclast differentiation, which was evaluated by tartrate-resistant acid phosphatase (TRAP) osteoclasts marker staining. At the same time, macrophages were shown to polarize into the M2 phenotype in the collected calluses.[159] In an alternative study, Mg^{2+}-releasing alginate hydrogels were investigated to evaluate the response of endogenous macrophages to bio-metal ions while regulating bone regeneration. After 7 days of implantation in a rat femoral defect model, macrophages were shown to infiltrate the injured site. TRAP-positive cells were suggested to migrate from the edge to the periphery of the defect, becoming multinucleated after 7 days of implantation. Interestingly, a gradual increase of M2 macrophages expressing CD206 was followed by a decrease in M1 macrophages over time. The defect was filled with mineralized bone after 56 days of implantation.[164]

A 3D-printed strategy was characterized by incorporating the anti-inflammatory phytomolecule honokiol in decellularized cartilage ECM/PEGDA hydrogels. The printed hydrogel, utilizing stereolithography technology, was co-cultured with LPS stimulated Raw 264.7 macrophages, differentiating macrophages to a M1 phenotype. The incorporation of honokiol significantly suppressed the release of TNF-α, IL-1β, and IL-6, known pro-inflammatory factors. After implantation in Sprague-Dawley rats with cylindrical osteochondral defects, micro-CT scans demonstrated more calcified tissue when implanted with ECM/PEGDA hydrogels with or without honokiol. The histological analysis revealed that at 4 and 8 weeks after surgery, the incorporation of honokiol significantly improved osteochondral regeneration.[137]

Resveratrol is also a known anti-inflammatory compound, which has been incorporated in collagen hydrogels to repair osteochondral defects. Due to its insolubility in water, resveratrol was grafted in polyacrylic acid. When transplanted to rabbit defects, the gene expression of anti-inflammatory IL-1β, MMP-13, and COX-2 markers in the osteochondral tissue defects was up-regulated; however, the expression decreased over time. Additionally, there was an up-regulation in the gene expression of osteochondral markers, including SOX-9, aggrecan, collagen II and I, compared to controls (only collagen or untreated).[165]

Figure 2. Cell-free and bioactive factors-loaded hydrogels with osteoimmunomodulatory properties. **A.** (i) Schematics on the fabrication of alginate/sericin/graphene oxide hydrogel. (ii) Recruitment of F4/80 macrophages detected by immunochemistry staining 4 days after implantation. Scale bars: 200 μm (left) and 50 μm (right). Reprinted with permission.[129] Copyright 2021, Elsevier. **B.** Illustration of the development of GelMA/nano fishbone hybrid hydrogels. Microscopic images of haematoxylin and eosin (H&E) and TNF-α staining 4 weeks after implantation in rat cranial bone defect models. NB: new bone. *: implants. Reprinted with permission.[134] Copyright 2020, American Chemical Society. **C.** Immunofluorescence microscopic images of macrophages positively stained for iNOS (green) and Arg-1 (pink) after soft and stiff GelMA subcutaneous implantation. Reprinted with permission.[142] Copyright 2020, American Chemical Society. **D**. Confocal microscopic images of macrophages primed



with IL-4 and BMP-2-loaded hydrogels (CD206 – red; F-actin – green; nuclei – blue). Scale bar: 100 µm. Respective cell surface expression of CD206 and F4/80 of macrophages in same conditions. Reprinted with permission.[161] Copyright 2022, Oxford University Press. **E.** Masson's trichrome staining of histological sections 12 weeks after TGF-β1-loaded hydrogel implantation in rabbit femoral defects. New blood vessels are indicated by red arrows. NB: new bone. OS: osteoid tissue. Reprinted with permission.[155] Copyright 2022, Elsevier. **F.** µCT images of bone defects 6 weeks after implantation of hydrogels containing SEW2871 and/or PRP in rat bone defects. Reprinted with permission.[163] Copyright 2014, Elsevier.

3.3. Co-encapsulation of immunomodulatory mesenchymal stem cells

MSCs are highly attractive for bone tissue engineering and cell therapy. These cells possess self-renewal capacity, multilineage differentiation potential, and exhibit powerful immunomodulatory properties.[166] Besides their ability to undergo osteogenic differentiation, MSCs can mediate the immune response under the influence of pro-inflammatory or anti-inflammatory environments.[167] Furthermore, MSCs were shown to interact with both innate and adaptive immune cells in the course of bone regeneration via paracrine and juxtacrine signalling. In fact, MSCs have the ability to polarize macrophages into a regulatory-like profile by releasing PGE2 via iNOS and a COX2 dependent pathway.[168,169] MSCs also have chemotaxis properties. The implantation of MSCs in a murine cranial defect model was shown to induce macrophage recruitment by secreting VEGF.[170] Under the influence of inflammatory signals, MSCs can also recruit and regulate T-lymphocyte activation and differentiation by expressing CXCL9, CXCL10, and CCL2.[171,172] To date, several MSC-based approaches have been suggested for bone regenerative applications, but the purpose has been to enhance the pool of osteoprogenitor cells instead of acting as immunomodulatory agents.[173,174]

To invert this trend, the potential of MSCs in bone tissue engineering strategies has been demonstrated by immunomodulating the host's implantation environment to support bone repair and osteointegration. For that, fibrin hydrogels encapsulating MSCs have been employed to promote bone healing by stimulating endogenous regeneration. Seebach and colleagues showed that implanting bone marrow MSC-laden fibrin hydrogels in rat femoral bone defects induced the rapid infiltration of pro-inflammatory M1 macrophages and endothelial cells (**Figure 3A**). On the other hand, cell-free fibrin hydrogels were not invaded. Authors attributed such enhanced infiltration of cell-laden

hydrogels to the release of bioactive factors, such as VEGF, by MSCs.[175] Human amniotic MSCs were also encapsulated in fibrin hydrogels to evaluate their ability to promote endogenous bone regeneration. Interestingly, *in vitro* studies demonstrated that the indirect co-culture of amniotic and bone-marrow MSCs could promote osteogenic differentiation of bone marrow MSCs. Amniotic MSCs can also stimulate the polarization of macrophages into alternatively activated M2 phenotype and induce capillary-tube formation of endothelial cells. When implanted in rabbit cranial defects, the amniotic MSC-laden hydrogels were shown to recruit mainly M2 macrophages. Additionally, M2 macrophages that infiltrated the hydrogel expressed a substantial amount of BMP-2 and VEGF, promoting cranial defect healing.[176] Ji *et al*. reported similar results regarding MSCs' osteoimmunomodulatory properties. In this study, MSCs encapsulated in hydroxypropyl chitin (HPCH) hydrogels were infused into a poly(ε-caprolactone)/nano-HAp (PCL/nHA) construct to create a 3D hybrid scaffold. MSC-laden hydrogel indirectly co-cultured with RAW 264.7 macrophages increased gene expression of VEGF, PDGF-BB, and MMP9 in macrophages, compared to individual HPCH and PCL/nHA scaffolds. MSCs could also trigger the polarization of macrophages from M1 to M2 phenotypes in an LPS-induced inflammatory environment *in vitro*. Moreover, the presence of MSCs shifted macrophages into a CD206-positive pro-regenerative profile when implanted subcutaneously in rats (**Figure 3B**) and increased the bone volume formation when implanted in a calvarial defect model.[177] Our group also proposed liquefied microcapsules for bone tissue engineering.[178] The concept relies on the subsequent *(i)* encapsulation of adipose-derived MSCs and poly(ε-caprolactone) microparticles within alginate beads; *(ii)* construction of a layer-by-layer membrane composed of poly(L-lysine), alginate, and chitosan as polyelectrolytes; and then *(iii)* liquefaction of the core by chelation with ethylenediaminetetraacetic acid (EDTA). The microparticles function as cell adhesion spots, allowing MSCs proliferation and ECM deposition. Besides excellent viability, *in vitro* results demonstrated that encapsulated MSCs within the liquefied microcapsules underwent osteogenic differentiation and deposition of mineralized matrix.[178–180] Interestingly, changing the outer layer of the liquid microcapsules can trigger different immunomodulatory responses. Expressly, when the microcapsules were cultured *in vitro* with macrophages, results indicated that chitosan-ending microcapsules, and the presence of MSCs, favoured the balance of macrophage polarization towards a more regenerative profile.[181]

Given the importance of polarizing macrophages to a pro-healing state in regenerative medicine strategies, Ueno and colleagues engineered a lentivirus-transduced IL-4 overexpressing MSCs (IL-4-MSCs). The purpose was to release IL-4 in a sustained fashion, aiming to shift macrophages towards a M2 phenotype. The IL-4-MSCs were incorporated within a gelatine microribbon hydrogel and implanted in mice critical-size femoral defects. After implantation, hydrogels encapsulating IL-4-MSCs were populated with significantly more macrophages compared to hydrogels encapsulating non-transduced MSCs (control). Importantly, the phenotype of such recruited macrophages was mainly related to the M2 state, with high expression of Arg-1 and CD206. Additionally, the IL-4-MSC-laden hydrogel promoted bone bridging and bone formation, whereas this was not observed in the controls.[182] Interestingly, such IL-4-MSC-laden hydrogel obtained better osteogenic differentiation and macrophage polarization when implanted in male mice. On the other hand, an increased number of TRAP-positive osteoclasts were observed in female mice.[183]

Glucomannan has been used to coat MSC-laden hydrogels in order to enhance their immunomodulatory potential since this polysaccharide shows an affinity for macrophage receptors. The coating of 3D cell-laden hydrogels revealed promising results, facilitating macrophage adhesion and activation while inducing the macrophage's gene expression of osteogenic and angiogenic markers. Upon subcutaneous implantation, it was observed the formation of a fibrous layer around the coated hydrogel (**Figure 3C**). However, the thickness of the layer did not increase over time, as observed in uncoated controls. Additionally, the osteogenic differentiation of MSCs embedded in the coated hydrogel was significantly higher than in uncoated hydrogels after implantation.[184]

Other strategies have also demonstrated the potential of MSCs-laden hydrogels in generating a favourable host's immune microenvironment for bone healing.[185,186] The encapsulation of MSCs with signalling factors or anti-inflammatory drugs has been successfully immunomodulating the response of macrophages and T-lymphocytes within the host's microenvironment in general tissue engineering applications.[187–189] Overall, these recent data highlight the potential of MSC-based bioengineered strategies for osteoimmunomodulation, and might be a starting point for translation into clinical practice.

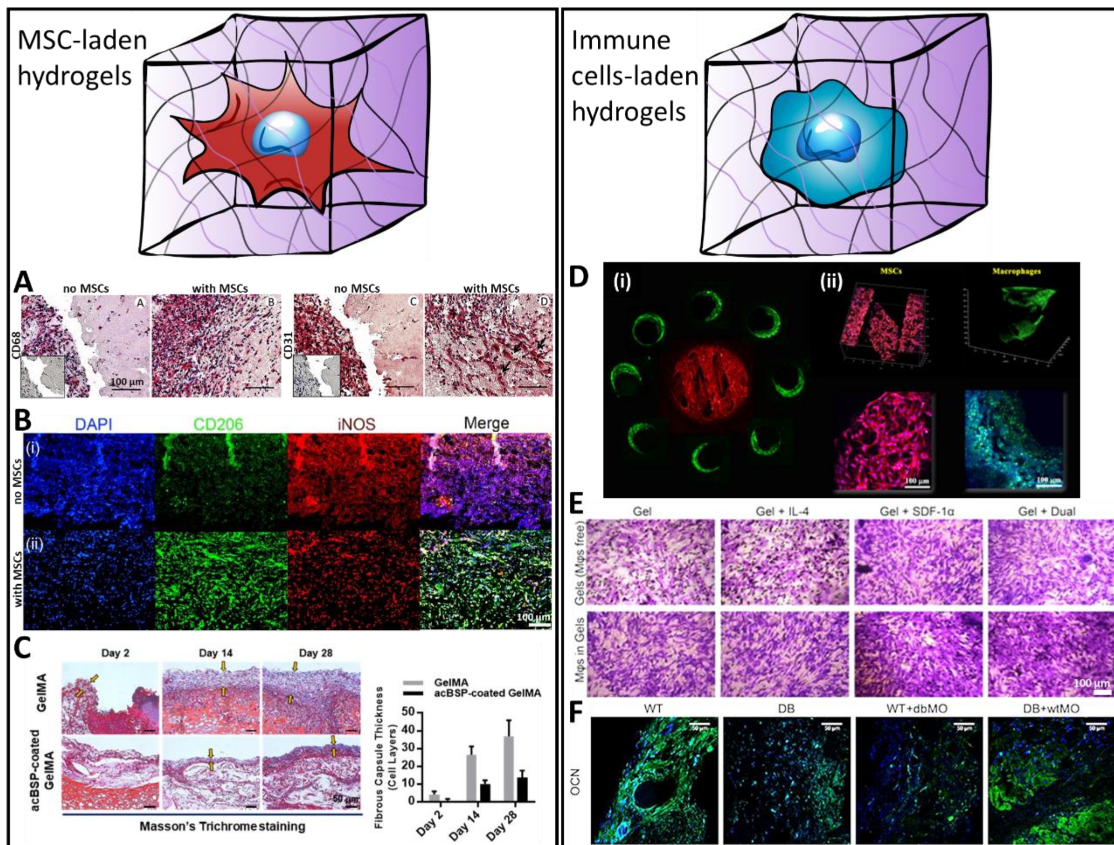


Figure 3. Encapsulation of immunomodulatory stem cells and immune cells for bone tissue engineering. **A.** Immunohistochemistry staining of infiltrated cells within the callus 6 days after implantation of MSC-laden fibrin hydrogel. Macrophages were identified by the expression of CD68 and endothelial cells by CD31. Arrows indicate primitive vessel formation. Scale bar: 100 µm. Reprinted with permission.[175] Copyright 2014, Elsevier. **B.** Immunofluorescence staining of hydrogels with or without MSCs 7 days after subcutaneous implantation (nuclei – blue; CD206 – green; iNOS – red). Scale bar: 100 µm. Reprinted with permission.[177] Copyright 2020, The authors. **C.** Masson's trichrome staining of MSC-laden hydrogels coated with glucomannan after subcutaneous implantation with the respective quantification of the fibrous capsule layer thickness. Scale bar: 50 µm. Reprinted with permission.[184] Copyright 2017, Elsevier. **D.** (i) Immunofluorescence microscopic images of the Haversian bone-mimicking

scaffold. Macrophages were distributed in the Haversian canals (green), and MSCs were allocated in the cancellous bone (red). (ii) Confocal microscopic images showing MSCs and macrophages within each compartment. Scale bar: 100 µm. Reprinted with permission.[190] Copyright 2021, Elsevier. **E.** Microscopic images revealing the migration of MSCs toward hydrogels with or without macrophages. Scale bar: 100 µm. Reprinted with permission.[156] Copyright 2019, Elsevier. **F.** Osteocalcin (green) immunofluorescence staining of the calvarial section after delivering wild-type (wtMO) and diabetes mellitus (dbMO) macrophages into healthy (WT) and unhealthy (DB) rats. Scale bar: 50 µm. Reprinted with permission.[191] Copyright 2021, The authors.

3.4. Incorporation of immune cells

Nowadays, it is well-established that immune cells play significant roles in the homeostasis and healing of multiple tissues. Consequently, the incorporation of immune cells within biomaterials has been investigated to understand their potential for cell-based therapies.[192,193] Indeed, exciting results have been gathered. For instance, macrophages encapsulated in gelatine hydrogels affect the incoming host's cell behaviour, facilitating the biomaterial integration upon implantation.[194] Also, macrophage-laden hydrogels were shown more effective in recruiting endothelial cells and fibroblasts than monocyte-laden hydrogels.[194] The incorporation of monocytes within a gelatine-hyaluronic acid hydrogel combined with a cocktail of anti-inflammatory cytokines was also designed to control the initial immune response, while the encapsulated monocytes differentiated into pro-healing macrophages.[195] Although the incorporation of bioactive factors in the hydrogel network could be a reliable strategy to immunomodulate the host's microenvironment, as discussed in Section 3.2., the encapsulation of immune cells would continuously provide an active resource of several cytokines and growth factors.

The presence of macrophages within hydrogels demonstrates as well potential advantages for tissue remodelling and vascularization. Barthes and colleagues found that macrophages co-cultured with endothelial cells within gelatine hydrogels enhanced the vessel-like structure organization, demonstrated by increased sprouting. When tri-cultured with endothelial cells and fibroblasts, macrophages enhanced the organization of the ECM structure with well-defined intercellular junctions.[196] Furthermore, the encapsulation of macrophages in alginate beads enhanced angiogenesis and arteriogenesis following implantation in a mouse hindlimb ischaemic model.[197] When

incorporated in poly(ethylene glycol)-based hydrogels, macrophages changed the morphology in an endothelial cell-dependent manner, and in the presence of endothelial cells, macrophages increased the vessel tubule volume.[198] Thus, incorporating these immune cells and controlling their phenotypes can develop superior physiological *in vitro* tissue models as well as improve the integration of biomaterials following implantation. Additionally, the immune microenvironment is also modulated by the direct cell-cell contact between macrophages and MSCs.[199] The juxtacrine interactions between macrophages and bone marrow-derived MSCs were shown to induce the release of IL-6 by MSCs through the gp130/STAT3 pathway, which consequently promoted the migration and proliferation of MSCs.[200] Thus, the encapsulation of macrophages can be particularly useful in co-cultured systems to recruit host's MSCs *in vivo*.

Few studies have applied macrophages within hydrogels aiming at specific tissues. However, the tissue engineering paradigm has been changing, and the role of macrophages in tissue healing has been recognized more than ever. Juhas *et al.* demonstrated that the integration of macrophages within 3D muscle constructs stimulated myogenesis of muscle satellite cells *in vitro* while enhancing the blood vessel ingrowth, muscle regeneration, and contractile function *in vivo*.[201] The delivery of monocytes and macrophages in pullulan-collagen composite dermal hydrogels enhanced the rate of wound healing in wild-type and diabetic mice, while transplanted macrophages displayed a mixture of phenotypes.[202] Some studies have also incorporated macrophages within 3D hydrogels for fracture healing in the bone tissue engineering context. **Table 1** summarizes the major researches that incorporated macrophages for bone tissue engineering. Designing bone tissue engineering strategies requires elucidating the interactions between macrophages, MSCs, and biomaterials. To better understand the influence of macrophages on the multipotency ability of MSCs, Cantu and colleagues cultured both cell phenotypes in gelatine/polyethylene glycol or collagen hydrogels. It was undeniable that macrophages positively affected MSC-osteogenic differentiation in both types of biomaterials, with higher Alizarin Red S and von Kossa staining. Contrarily, the role of macrophages on chondrogenic and adipogenic differentiation was attenuated or null, respectively.[203] The use of macrophages was also proposed to fabricate a Haversian bone-mimicking biomaterial. For that, a construct composed of Haversian canals, Volkmann canals, and cancellous bone structures was firstly printed and then cultured with THP-1 macrophages and

mouse-derived bone marrow MSCs. Considering the multiphenotypic cellular environment of bone, macrophages were distributed in the Haversian canals structural units, while MSCs were allocated in the cancellous bone (**Figure 3D**). A stronger expression of M2 macrophages was observed after only 3 days of co-culture when a ratio of MSCs-macrophages was 2:1. Moreover, the paracrine signalling mediated by macrophages, through the high expression of osteogenic and angiogenic markers, such as OSM, WNT10b, BMP-2, and PDGF-BB, up-regulated the MSC-osteogenic differentiation.[190]

The direct co-culture of human bone marrow-derived MSCs and different phenotypes of macrophages was proposed as a 3D model to recapitulate musculoskeletal tissues.[204] Following the polarization of macrophages through a pool of cytokines, M0, M1, and M2 macrophages were co-cultured with MSCs within GelMA hydrogels. The M1 macrophage phenotype decreased over time when co-cultured with MSCs, whereas M2 macrophages secreted more anti-inflammatory cytokines. Moreover, after 4 weeks of culture, the incorporation of macrophages enhanced MSC-osteogenic differentiation, with a higher amount of ALP activity, Alizarin Red S staining, and ECM mineralization. Interestingly, the co-culture with the M1 macrophage phenotype exhibited the highest levels of osteogenic differentiation.[204] In a similar strategy, He *et al.* loaded rat-derived MSCs, macrophages, IL-4, and SDF-1α within high-stiffness TG-gels to understand their efficacy in supporting periodontal tissue repair. Due to the inclusion of IL-4, macrophages were able to shift into an M2 profile, with higher gene expression of Arg-1 and CD206. After direct and indirect co-culture with MSCs, macrophages positively influenced MSC-osteogenic differentiation. Furthermore, by using a Transwell system, the encapsulation of macrophages was shown to enhance the MSCs migration independently of the delivery of IL-4 and SDF-1α (**Figure 3E**).[156] Within the same approach, encapsulated macrophages in TG-gels with different degrees of stiffness were indirectly co-cultured with MSCs. Remarkably, macrophages encapsulated in low-stiffness TG-gels enhanced the osteogenic differentiation of MSCs, with significantly more mineralized nodules and ALP activity. On the other hand, macrophages within mid-stiffness and high-stiffness TG-gels negatively influenced MSC-osteogenic differentiation, as they were more likely to polarize into a pro-inflammatory phenotype.[143] The immunomodulatory reaction of encapsulated macrophages as well as the osteogenic differentiation of MSCs, can also be modulated by other features of the biomaterials. For instance, needle-shaped HAp particles

embedded in collagen did not promote MSC-osteogenic differentiation compared to spherical large HAp particles. However, when macrophages were loaded into the collagen construct with needle-shaped particles, the osteogenic differentiation and calcium deposition of MSCs was significantly enhanced. The explanation for the results is most likely associated with the ability of needle-shaped particles to up-regulate the expression of BMP-2, OPN, and OSM in macrophages.[205]

Given the attractive potential of macrophages, their transplantation has been considered as an approach to ameliorate the bone fracture healing process. For that, macrophages were embedded in a combination of a blood clot and a commercially available mineral bone substitute and then implanted subcutaneously in BALB/c mice. Results revealed that the implantation of encapsulated macrophages enhanced the amount of collagen, osteocalcin, and osteopontin, compared to biomaterials without cells. Additionally, osteoblast-like cells and osteon-like structures could be observed 4 and 8 weeks after macrophage-laden biomaterials implantation. Macrophages also increased the number of new blood vessels.[206] Another personalized and functional bone substitute was proposed by Jeon *et al*. Herein, macrophages and MSCs were obtained by differentiation from human-induced pluripotent stem cells (hiPSC). Then, hiPSC-MSCs were cultured in HA-coated poly(lactic-co-glycolic acid)/poly(L-lactic acid) for 7 days under osteogenic differentiation factors, after which were added hiPSC-macrophages under osteoclastogenesis supplementation. The final construct induced bone-like tissue formation *in vitro* and *in vivo*. Although osteoclasts are shown as villains of bone healing, the authors demonstrated that they are essential for engineering mature bone tissues.[207] Although promising results have been reported, the delivery of macrophages still presents controversial outcomes. For instance, transplantation of allogeneic bone marrow-derived macrophages seeded onto HA-poly(lactic-co-glycolic acid) on large cranial defects in mice did not stimulate bone formation. The authors explained that the study conditions might impact these results and that the cranial defect microenvironment could be less inflammatory than expected. Thus, macrophages would not be stimulated to induce bone regeneration.[208] As observed in other approaches, a smart strategy could be based on delivering immune cells "educated" *ex vivo*. For instance, the previous exposure of macrophages to peripheral nerve segments or the pre-treatment of microglia (resident macrophages in the central nervous system) with a specific cytokine cocktail increased the potential of delivered macrophages to treat spinal cord injuries in animal models.[209,210] Also, it is necessary to consider other

factors, such as the presence of previous pathologies. Kang *et al*. studied the effect of type 2 diabetes mellitus on macrophages and bone healing. Although macrophages from wild-type and diabetes mellitus animals demonstrated similar surface antigen profiles, the unhealthy macrophages expressed a higher amount of pro-inflammatory factors. Then, the bone regeneration was negatively affected after delivering diabetes mellitus macrophages within collagen hydrogels in wild-type mouse critical-sized defects. On the other hand, the wild-type macrophages partially improved bone healing after transplantation into diabetic bone defects (**Figure 3F**).[191] The ability of wild-type macrophages to partially heal calvarial defects in mice with diabetes mellitus indicates that these immune cells can be advantageous or detrimental depending on the state profile

Table 1 – Examples of bone tissue engineering strategies using immune cells. The examples cover the following topics: the type of immune cells used to immunomodulate the microenvironment; other cells that were co-cultured with immune cells; the biomaterials used to incorporate the immune cells; the main results of the strategy; and the technique employed to study the cell-biomaterials constructs. Abbreviations: ALP – alkaline phosphatase; ECM – extracellular matrix; hiPSCs – human-induced pluripotent stem cells; MSCs – mesenchymal-derived stem cells.

Encapsulated immune cells	Co-culture systems	Biomaterial	Results	Technique	Ref.
Human blood macrophages	Human bone marrow-derived MSCs	Gelatine/polyethylene glycol or collagen hydrogels	Macrophages enhanced osteogenic differentiation, had an attenuated role on chondrogenic differentiation, and did not affect adipogenic differentiation of MSCs.	*In vitro*	[203]
RAW 264.7	Rat bone marrow-derived MSCs	Akermanite bioceramic scaffold	MSCs and macrophages cultured in a Haversian bone-mimicking biomaterial, up-regulated osteogenic differentiation, and polarized macrophages into a M2 profile.	*In vitro*	[190]
Human monocyte-derived macrophages	Human bone marrow-derived MSCs	Methacrylated gelatine (GelMA)	Direct co-culture of macrophages and MSCs in GelMA shifted macrophages to a M2 profile, while the calcium deposition and ECM mineralization were enhanced.	*In vitro*	[204]
Rat bone marrow-derived macrophages	Rat bone marrow-derived MSCs	High-stiffness transglutaminase crosslinked gelatines	Indirect and direct co-culture of cells within hydrogels enhanced osteogenic differentiation, with higher Alizarin Red S staining and ALP activity.	*In vitro*	[156]
RAW 264.7	Rat bone marrow-derived MSCs	Transglutaminase crosslinked gelatines with different degrees of stiffness	Macrophages loaded within low-stiffness hydrogels improved the osteogenic differentiation of MSCs, compared to macrophages within gels with harder degrees of stiffness.	*In vitro*	[143]
THP-1 differentiated macrophages	Human bone marrow-derived MSCs	Collagen-hydroxyapatite scaffolds	Macrophages significantly increased the MSC-osteogenic differentiation within collagen-hydroxyapatite scaffolds.	*In vitro*	[205]
Rat resident peritoneal macrophages	-	Blood clot and Bio-Oss®	Subcutaneous implantation of macrophage-laden biomaterials enhanced the amount of collagen and immunoexpression of osteocalcin and osteopontin.	*In vivo*	[206]

hiPSC-macrophages	hiPSC-MSCs	Hydroxyapatite-coated poly(lactic-co-glycolic acid)/poly(L-lactic acid) scaffolds	hiPSC-MSCs and hiPSC-macrophages were submitted to osteogenic and osteoclastogenic differentiation within the scaffold. The co-culture accelerated bone formation *in vitro* and *in vivo*.	*In vitro* and *in vivo*	[207]
Rat bone marrow-rived macrophages	-	Hydroxyapatite-poly(lactic-co-glycolic acid)	The transplantation of allogeneic macrophages did not influence the regeneration of critical-sized cranial defects.	*In vivo*	[208]
Diabetes mellitus and wild-type rat bone marrow-derived macrophages	-	Collagen hydrogels	The transplantation of diabetic macrophages in healthy rats impaired bone regeneration, while the transplantation of healthy macrophages in a diabetes mellitus model improved bone healing.	*In vivo*	[191]

4. Conclusions and future perspectives

The immune system plays a dual role in bone tissue engineering. Immune cells are essential for bone homeostasis and regeneration, and so far, macrophages are the most explored due to their multiple functions in bone healing. Additionally, the response of immune cells can dictate the success or failure after biomaterial's implantation. Thus, the inspiration from the multiphenotypic cellular environment of bone led to the development of more biomimetic tissue engineering strategies. Herein, we focused on hydrogels and cell encapsulation systems that harnessed the distinct power of immune cells, mainly macrophages, to enhance bone tissue engineering performance. Given the importance of macrophages polarization into an M2 profile for biomaterial's osseointegration and bone healing, we overviewed cell-free hydrogels and hydrogels loaded with cytokines and growth factors that appear to positively immunomodulate the bone environment following implantation. Cell therapy is also highly investigated as an alternative to bone grafting. The encapsulation of immunomodulatory MSCs, alone or co-cultured with immune cells, has been shown to have massive potential for bone tissue engineering. Although much work has to be done, incorporating immune cells within biomaterials has shown promising results. We believe that the presence of immune cells will reproduce the physiological development process of bone by continuously producing a cocktail of bioactive factors which can substitute the exogenous media factors usually added to scaffolds. Moreover, the encapsulation of immune cells enables the juxtacrine signalling with co-cultured MSCs, resulting in an immunopriviledge microenvironment due to the immunomodulatory properties of MSCs. Additionally, such strategy has the potential to influence through paracrine signalling the recruited immune cells and MSCs. The extent of automated immune cell isolation kits and methods and the ability to differentiate them into specific phenotypes allows the development of personalized engineered strategies using autologous cells.[211,212] Further research on macrophage-based therapies is needed. Additionally, it is necessary to consider the MSCs and macrophages ratios. For instance, increasing the macrophage:MSC seeding density to 5:1 has shown better osteogenic outcomes than 1:1.[91,213] However, one of the challenges in studying the direct co-culture of immune and stem cells is identifying the cell source of cytokine production. Other challenges that should be carefully evaluated are the translation into human clinics since successful regenerative solutions are still far from reality. From an immunological perspective, the

source of transplanted macrophages or other immune cells should be autologous to avoid the risk of chronic inflammation and graft-versus-host disease.[214] Although cord blood private banking has been highly advertised, the use of autologous cells in standard TE approaches seems unlikely due to the high costs, lack of availability, and possible presence of genetic diseases.[215] The transplantation of allogeneic cells requires the assessment of the human leukocyte antigens (HLA) match status to reduce the risk of post-transplant complications. Therefore, the banking of multi-ethnic HLA-homozygous iPSCs, as well as genome-editing strategies to engineer HLA matching in allogeneic settings via CRISPR-Cas9, have been envisioned to overcome the limitations of the use of autologous cells.[216–218] More high-throughput models are necessary to understand the osteoimmunomodulatory potential of different biomaterials while avoiding the FBR. Finally, further investigation is required to achieve advanced and multifunctional tissue engineering strategies that can regulate osteogenesis, osteoclastogenesis, and vascularization while modulating the response of the immune cells.

Acknowledgements

S. Nadine acknowledges the financial support given by the Portuguese Foundation for Science and Technology (FCT) with the doctoral grant of Sara Nadine (SFRH/BD/130194/2017). This work was financed by national funds (OE) through FCT/MCTES in the scope of the projects "TETRISSUE" (PTDC/BTM-MAT/3201/2020), and the European Research Council for project "ATLAS" (ERC-2014-AdG-669858). This work was developed within the scope of the project CICECO-Aveiro Institute of Materials, UIDB/50011/2020, UIDP/50011/2020 & LA/P/0006/2020, financed by national funds through the FCT/MEC (PIDDAC).

Declaration of competing interest

The authors declare no conflict of interest.

Bibliography

[1] S. J. Forbes, N. Rosenthal, *Nat. Med.* **2014**, *20*, 857.

[2] P. Hoff, T. Gaber, C. Strehl, M. Jakstadt, H. Hoff, K. Schmidt-Bleek, A. Lang, E. Röhner, D. Huscher, G. Matziolis, G.-R. Burmester, G. Schmidmaier, C. Perka, G. Duda, F. Buttgereit, *Int. J. Mol. Sci.* **2017**, *18*, 583.

[3] K. D. Hankenson, G. Zimmerman, R. Marcucio, *Injury* **2014**, *45 Suppl 2*, DOI

10.1016/J.INJURY.2014.04.003.

[4] D. Lopes, C. Martins-Cruz, M. B. Oliveira, J. F. Mano, *Biomaterials* **2018**, *185*, 240.

[5] L. Roseti, V. Parisi, M. Petretta, C. Cavallo, G. Desando, I. Bartolotti, B. Grigolo, *Mater. Sci. Eng. C* **2017**, *78*, 1246.

[6] C. Gao, S. Peng, P. Feng, C. Shuai, *Bone Res. 2017 51* **2017**, *5*, 1.

[7] M. M. Stevens, *Mater. Today* **2008**, *11*, 18.

[8] T. Ding, W. Kang, J. Li, L. Yu, S. Ge, *J. Nanobiotechnology* **2021**, *19*, 1.

[9] D. P. Vasconcelos, M. Costa, I. F. Amaral, M. A. Barbosa, A. P. Águas, J. N. Barbosa, *Biomaterials* **2015**, *37*, 116.

[10] T. Wang, J. Bai, M. Lu, C. Huang, D. Geng, G. Chen, L. Wang, J. Qi, W. Cui, L. Deng, *Nat. Commun.* **2022**, *13*, 160.

[11] W. Liu, J. Li, M. Cheng, Q. Wang, K. W. K Yeung, P. K. Chu, X. Zhang, W. Liu, M. Cheng, Q. Wang, X. Zhang, J. Li, K. W. K Yeung, P. K. Chu, *Adv. Sci.* **2018**, *5*, 1800749.

[12] R. M. Visalakshan, M. N. Macgregor, S. Sasidharan, A. Ghazaryan, A. M. Mierczynska-Vasilev, S. Morsbach, V. Mailänder, K. Landfester, J. D. Hayball, K. Vasilev, *ACS Appl. Mater. Interfaces* **2019**, *11*, 27615.

[13] L. Chen, D. Wang, F. Peng, J. Qiu, L. Ouyang, Y. Qiao, X. Liu, *Nano Lett.* **2019**, *19*, 3480.

[14] L. Lv, Y. Xie, K. Li, T. Hu, X. Lu, Y. Cao, X. Zheng, L. Lv, Y. Xie, K. Li, X. Lu, Y. Cao, X. Zheng, T. Hu, *Adv. Healthc. Mater.* **2018**, *7*, 1800675.

[15] Z. Chen, T. Klein, R. Z. Murray, R. Crawford, J. Chang, C. Wu, Y. Xiao, *Mater. Today* **2016**, *19*, 304.

[16] J. Lee, H. Byun, S. K. Madhurakkat Perikamana, S. Lee, H. Shin, *Adv. Healthc. Mater.* **2019**, *8*, 1801106.

[17] C. R. Correia, S. Nadine, J. F. Mano, *Adv. Funct. Mater.* **2020**, *30*, 1908061.

[18] J. O. Hollinger, J. C. Kleinschmidt, *J. Craniofac. Surg.* **1990**, *1*, 60.

[19] U. Kneser, D. J. Schaefer, E. Polykandriotis, R. E. Horch, *J. Cell. Mol. Med.* **2006**, *10*, 7.

[20] E. M. Bueno, J. Glowacki, *Nat. Rev. Rheumatol.* **2009**, *5*, 685.

[21] G. Fernandez de Grado, L. Keller, Y. Idoux-Gillet, Q. Wagner, A.-M. Musset, N. Benkirane-Jessel, F. Bornert, D. Offner, *J. Tissue Eng.* **2018**, *9*, 204173141877681.

[22] M. D. McKee, W. N. Addison, M. T. Kaartinen, *Cells Tissues Organs* **2005**, *181*, 176.

[23] B. Clarke, *Clin. J. Am. Soc. Nephrol.* **2008**, *3*, S131.

[24] L. F. Bonewald, *Ann. N. Y. Acad. Sci.* **2007**, *1116*, 281.

[25] F. Shapiro, *Eur. Cells Mater.* **2008**, *15*, 53.

[26] W. S. Jee, H. M. Frost, *Triangle.* **1992**, *31*, 77.

[27] A. G. Robling, A. B. Castillo, C. H. Turner, *Annu. Rev. Biomed. Eng.* **2006**, *8*, 455.

[28] J. T. Swarthout, R. C. D'Alonzo, N. Selvamurugan, N. C. Partridge, *Gene* **2002**, *282*, 1.

[29] X. Li, L. Qin, M. Bergenstock, L. M. Bevelock, D. V Novack, N. C. Partridge, *J. Biol. Chem.* **2007**, *282*, 33098.

[30] Y. L. Ma, R. L. Cain, D. L. Halladay, X. Yang, Q. Zeng, R. R. Miles, S. Chandrasekhar, T. J. Martin, J. E. Onyia, *Endocrinology* **2001**, *142*, 4047.

[31] K. P. McHugh, K. Hodivala-Dilke, M.-H. Zheng, N. Namba, J. Lam, D. Novack, X. Feng, F. P. Ross, R. O. Hynes, S. L. Teitelbaum, *J. Clin. Invest.* **2000**, *105*, 433.

[32] S. L. Teitelbaum, *Science (80-.).* **2000**, *289*, 1504.

[33] L. J. Raggatt, N. C. Partridge, *J. Biol. Chem.* **2010**, *285*, 25103.

[34] R. T. Franceschi, G. Xiao, D. Jiang, R. Gopalakrishnan, S. Yang, E. Reith, *Connect. Tissue Res.* **2003**, *44 Suppl 1*, 109.

[35] H. C. Anderson, *Curr. Rheumatol. Rep.* **2003**, *5*, 222.

[36] A. G. Robling, C. H. Turner, *Crit. Rev. Eukaryot. Gene Expr.* **2009**, *19*, 319.

[37] K. Schmidt-Bleek, A. Petersen, A. Dienelt, C. Schwarz, G. N. Duda, *Expert Opin. Biol. Ther.* **2014**, *14*, 247.

[38] T. A. Einhorn, L. C. Gerstenfeld, *Nat. Rev. Rheumatol.* **2015**, *11*, 45.

[39] A. Schindeler, M. M. McDonald, P. Bokko, D. G. Little, *Semin. Cell Dev. Biol.* **2008**, *19*, 459.

[40] G. C. Gurtner, S. Werner, Y. Barrandon, M. T. Longaker, *Nature* **2008**, *453*, 314.

[41] D. Nam, E. Mau, Y. Wang, D. Wright, D. Silkstone, H. Whetstone, C. Whyne, B. Alman, *PLoS One* **2012**, *7*, e40044.

[42] K. A. Alexander, M. K. Chang, E. R. Maylin, T. Kohler, R. Müller, A. C. Wu, N. Van Rooijen, M. J. Sweet, D. A. Hume, L. J. Raggatt, A. R. Pettit, *J. Bone Miner. Res.* **2011**, *26*, 1517.

[43] L. Gong, Y. Zhao, Y. Zhang, Z. Ruan, *Ann. Clin. Lab. Sci.* **2016**, *46*, 65.

[44] J. Richardson, A. M. Hill, C. J. C. Johnston, A. McGregor, A. R. Norrish, D. Eastwood, C. B. D. Lavy, *J. Bone Joint Surg. Br.* **2008**, *90-B*, 988.

[45] Z. Xing, C. Lu, D. Hu, T. Miclau, R. S. Marcucio, *J. Orthop. Res.* **2010**, *28*, 1000.

[46] N. A. Gasper, C. C. Petty, L. W. Schrum, I. Marriott, K. L. Bost, *Infect. Immun.* **2002**, *70*, 4075.

[47] D. Yang, A. R. Wijenayaka, L. B. Solomon, S. M. Pederson, D. M. Findlay, S. P. Kidd, G. J. Atkins, *MBio* **2018**, *9*, DOI 10.1128/MBIO.00415-18/FORMAT/EPUB.

[48] M. Timlin, D. Toomey, C. Condron, C. Power, J. Street, P. Murray, D. Bouchier-Hayes, *J. Trauma Inj. Infect. Crit. Care* **2005**, *58*, 1223.

[49] A. W. Segal, *Annu. Rev. Immunol.* **2004**, *23*, 197.

[50] Y. Y. Kong, U. Feige, I. Sarosi, B. Bolon, A. Tafuri, S. Morony, C. Capparelli, J. Li, R. Elliott, S. McCabe, T. Wong, G. Campagnuolo, E. Moran, E. R. Bogoch, G. Van, L. T. Nguyen, P. S. Ohashi, D. L. Lacey, E. Fish, W. J. Boyle, J. M.

Penninger, *Nature* **1999**, *402*, 304.

[51] K. Söderström, E. Stein, P. Colmenero, U. Purath, U. Müller-Ladner, C. Teixeira de Matos, I. H. Tarner, W. H. Robinson, E. G. Engleman, *Proc. Natl. Acad. Sci.* **2010**, *107*, 13028 LP.

[52] R. Bielby, E. Jones, D. McGonagle, *Injury* **2007**, *38*, S26.

[53] T. Kon, T.-J. Cho, T. Aizawa, M. Yamazaki, N. Nooh, D. Graves, L. C. Gerstenfeld, T. A. Einhorn, *J. Bone Miner. Res.* **2001**, *16*, 1004.

[54] A. Wu, Z. Zeng, *Commun. Nonlinear Sci. Numer. Simul.* **2013**, *18*, 373.

[55] F. Loi, L. A. Cordova, J. Pajarinen, T. Lin, Z. Yao, S. B. Goodman, *Bone* **2016**, *86*, 119.

[56] P. M. Mountziaris, A. G. Mikos, *Tissue Eng. Part B Rev.* **2008**, *14*, 179.

[57] J. A. Cameron, D. J. Milner, J. S. Lee, J. Cheng, N. X. Fang, I. M. Jasiuk, *Curr. Top. Microbiol. Immunol.* **2013**, *367*, 113.

[58] H. M. Kronenberg, *Nature* **2003**, *423*, 332.

[59] W. Lehmann, C. M. Edgar, K. Wang, T.-J. Cho, G. L. Barnes, S. Kakar, D. T. Graves, J. M. Rueger, L. C. Gerstenfeld, T. A. Einhorn, *Bone* **2005**, *36*, 300.

[60] M. Kondo, K. Yamaoka, K. Sonomoto, S. Fukuyo, K. Oshita, Y. Okada, Y. Tanaka, *PLoS One* **2013**, *8*, e79463.

[61] Z. S. AI-Aql, A. S. Alagl, D. T. Graves, L. C. Gerstenfeld, T. A. Einhorn, *J. Dent. Res.* **2008**, *87*, 107.

[62] M. M. McDonald, A. Morse, K. Mikulec, L. Peacock, P. A. Baldock, P. J. Kostenuik, D. G. Little, *J. Bone Miner. Res.* **2013**, *28*, 1550.

[63] M. Fajardo, C.-J. Liu, K. Ilalov, K. A. Egol, *J. Orthop. Trauma* **2010**, *24*, 557.

[64] R. Marsell, T. A. Einhorn, *Injury* **2011**, *42*, 551.

[65] J. E. Horton, L. G. Raisz, H. A. Simmons, J. J. Oppenheim, S. E. Mergenhagen, *Science (80-.).* **1972**, *177*, 793.

[66] M. Asagiri, H. Takayanagi, *Bone* **2007**, *40*, 251.

[67] L. E. Theill, W. J. Boyle, J. M. Penninger, *Annu. Rev. Immunol.* **2002**, *20*, 795.

[68] M. M. Zaiss, R. Axmann, J. Zwerina, K. Polzer, E. Gückel, A. Skapenko, H. Schulze-Koops, N. Horwood, A. Cope, G. Schett, *Arthritis Rheum.* **2007**, *56*, 4104.

[69] D. Toben, I. Schroeder, T. El Khassawna, M. Mehta, J.-E. Hoffmann, J.-T. Frisch, H. Schell, J. Lienau, A. Serra, A. Radbruch, G. N. Duda, *J. Bone Miner. Res.* **2011**, *26*, 113.

[70] S. Reinke, S. Geissler, W. R. Taylor, K. Schmidt-Bleek, K. Juelke, V. Schwachmeyer, M. Dahne, T. Hartwig, L. Akyüz, C. Meisel, N. Unterwalder, N. B. Singh, P. Reinke, N. P. Haas, H.-D. Volk, G. N. Duda, *Sci. Transl. Med.* **2013**, *5*, DOI 10.1126/scitranslmed.3004754.

[71] E. T. Khassawna, A. Serra, C. H. Bucher, A. Petersen, C. Schlundt, I. Könnecke, D. Malhan, S. Wendler, H. Schell, H. D. Volk, K. Schmidt-Bleek, G. N. Duda, T. El Khassawna, A. Serra, C. H. Bucher, A. Petersen, C. Schlundt, I. Könnecke, D. Malhan, S. Wendler, H. Schell, H. D. Volk, K. Schmidt-Bleek, G. N. Duda, *Front. Immunol.* **2017**, *8*, 562.

[72] T. Ono, K. Okamoto, T. Nakashima, T. Nitta, S. Hori, Y. Iwakura, H. Takayanagi, *Nat. Commun.* **2016**, *7*, 10928.

[73] H. Huang, H.-H. H.-M. H.-H. H.-M. J. Kim, E.-J. Chang, Z. H. Lee, S. J. Hwang, H.-H. H.-M. H.-H. H.-M. J. Kim, Y. Lee, H.-H. H.-M. H.-H. H.-M. J. Kim, *Cell Death Differ.* **2009**, *16*, 1332.

[74] I. Könnecke, A. Serra, T. El Khassawna, C. Schlundt, H. Schell, A. Hauser, A. Ellinghaus, H. D. Volk, A. Radbruch, G. N. Duda, K. Schmidt-Bleek, *Bone* **2014**, *64*, 155.

[75] Y. Li, G. Toraldo, A. Li, X. Yang, H. Zhang, W.-P. Qian, M. N. Weitzmann, *Blood* **2007**, *109*, 3839.

[76] C. Schlundt, S. Reinke, S. Geissler, C. H. Bucher, C. Giannini, S. Märdian, M. Dahne, C. Kleber, B. Samans, U. Baron, G. N. Duda, H.-D. Volk, K. Schmidt-Bleek, *Front. Immunol.* **2019**, *10*, 1954.

[77] C. H. Bucher, C. Schlundt, D. Wulsten, F. A. Sass, S. Wendler, A. Ellinghaus, T. Thiele, R. Seemann, B. M. Willie, H.-D. Volk, G. N. Duda, K. Schmidt-Bleek, *Front. Immunol.* **2019**, *10*, 797.

[78] C. R. Almeida, H. R. Caires, D. P. Vasconcelos, M. A. Barbosa, *Stem Cell Reports* **2016**, *6*, 466.

[79] A. Kovtun, S. Bergdolt, R. Wiegner, P. Radermacher, M. Huber-Lang, A. Ignatius, *Eur. Cells Mater.* **2016**, *32*, 152.

[80] L. Claes, S. Recknagel, A. Ignatius, *Nat. Rev. Rheumatol.* **2012**, *8*, 133.

[81] T. Wynn, L. Barron, *Semin. Liver Dis.* **2010**, *30*, 245.

[82] M. K. Chang, L.-J. Raggatt, K. A. Alexander, J. S. Kuliwaba, N. L. Fazzalari, K. Schroder, E. R. Maylin, V. M. Ripoll, D. A. Hume, A. R. Pettit, *J. Immunol.* **2008**, *181*, 1232.

[83] I. G. Winkler, N. A. Sims, A. R. Pettit, V. Barbier, B. Nowlan, F. Helwani, I. J. Poulton, N. Van Rooijen, K. A. Alexander, L. J. Raggatt, J. P. Lévesque, *Blood* **2010**, *116*, 4815.

[84] D. A. Hume, J. F. Loutit, S. Gordon, *J. Cell Sci.* **1984**, *66*, 189.

[85] P. J. Murray, T. A. Wynn, *Nat. Rev. Immunol.* **2011**, *11*, 723.

[86] A. Shapouri-Moghaddam, S. Mohammadian, H. Vazini, M. Taghadosi, S. A. Esmaeili, F. Mardani, B. Seifi, A. Mohammadi, J. T. Afshari, A. Sahebkar, *J. Cell. Physiol.* **2018**, *233*, 6425.

[87] P. L. Graney, S. Ben-Shaul, S. Landau, A. Bajpai, B. Singh, J. Eager, A. Cohen, S. Levenberg, K. L. Spiller, *Sci. Adv.* **2020**, *6*, eaay6391.

[88] L. J. Raggatt, M. E. Wullschleger, K. A. Alexander, A. C. K. Wu, S. M. Millard, S. Kaur, M. L. Maugham, L. S. Gregory, R. Steck, A. R. Pettit, *Am. J. Pathol.* **2014**, *184*, 3192.

[89] C. M. Champagne, J. Takebe, S. Offenbacher, L. F. Cooper, *Bone* **2002**, *30*, 26.

[90] R. P. Pirraco, R. L. Reis, A. P. Marques, *J. Tissue Eng. Regen. Med.* **2013**, *7*, 392.

[91] V. Nicolaidou, M. M. Wong, A. N. Redpath, A. Ersek, D. F. Baban, L. M. Williams, A. P. Cope, N. J. Horwood, *PLoS One* **2012**, *7*, DOI

10.1371/JOURNAL.PONE.0039871.

[92] P. Guihard, Y. Danger, B. Brounais, E. David, R. Brion, J. Delecrin, C. D. Richards, S. Chevalier, F. Rédini, D. Heymann, H. Gascan, F. Blanchard, *Stem Cells* **2012**, *30*, 762.

[93] R. Dresner-Pollak, N. Gelb, D. Rachmilewitz, F. Karmeli, M. Weinreb, *Gastroenterology* **2004**, *127*, 792.

[94] D. Liu, S. Yao, G. E. Wise, *Eur. J. Oral Sci.* **2006**, *114*, 42.

[95] C. Schlundt, T. El Khassawna, A. Serra, A. Dienelt, S. Wendler, H. Schell, N. van Rooijen, A. Radbruch, R. Lucius, S. Hartmann, G. N. Duda, K. Schmidt-Bleek, *Bone* **2018**, *106*, 78.

[96] B. N. Brown, S. F. Badylak, *Acta Biomater.* **2013**, *9*, 4948.

[97] C. Schlundt, H. Fischer, C. H. Bucher, C. Rendenbach, G. N. Duda, K. Schmidt-Bleek, *Acta Biomater.* **2021**, *133*, 46.

[98] K. L. Spiller, S. Nassiri, C. E. Witherel, R. R. Anfang, J. Ng, K. R. Nakazawa, T. Yu, G. Vunjak-Novakovic, *Biomaterials* **2015**, *37*, 194.

[99] S. K. Ramasamy, A. P. Kusumbe, M. Schiller, D. Zeuschner, M. G. Bixel, C. Milia, J. Gamrekelashvili, A. Limbourg, A. Medvinsky, M. M. Santoro, F. P. Limbourg, R. H. Adams, *Nat. Commun.* **2016**, *7*, 13601.

[100] N. Dirckx, M. Van Hul, C. Maes, *Birth Defects Res. Part C Embryo Today Rev.* **2013**, *99*, 170.

[101] J. M. Kanczler, R. O. C. Oreffo, *Eur. Cells Mater.* **2008**, *15*, 100.

[102] Z. Szekanecz, A. E. Koch, *Nat. Clin. Pract. Rheumatol.* **2007**, *3*, 635.

[103] H. Hong, X. Y. Tian, *Int. J. Mol. Sci. 2020, Vol. 21, Page 6328* **2020**, *21*, 6328.

[104] B. A. Corliss, M. S. Azimi, J. M. Munson, S. M. Peirce, W. L. Murfee, *Microcirculation* **2016**, *23*, 95.

[105] K. Schmidt-Bleek, H. Schell, J. Lienau, N. Schulz, P. Hoff, M. Pfaff, G. Schmidt, C. Martin, C. Perka, F. Buttgereit, H. D. Volk, G. Duda, *J. Tissue Eng. Regen. Med.* **2014**, *8*, 120.

[106] L. Arnold, A. Henry, F. Poron, Y. Baba-Amer, N. van Rooijen, A. Plonquet, R. K. Gherardi, B. Chazaud, *J. Exp. Med.* **2007**, *204*, 1057.

[107] D. T. Ploeger, N. A. Hosper, M. Schipper, J. A. Koerts, S. de Rond, R. A. Bank, *Cell Commun. Signal.* **2013**, *11*, 29.

[108] K. L. Spiller, R. R. Anfang, K. J. Spiller, J. Ng, K. R. Nakazawa, J. W. Daulton, G. Vunjak-Novakovic, *Biomaterials* **2014**, *35*, 4477.

[109] L. Jakobsson, C. A. Franco, K. Bentley, R. T. Collins, B. Ponsioen, I. M. Aspalter, I. Rosewell, M. Busse, G. Thurston, A. Medvinsky, S. Schulte-Merker, H. Gerhardt, *Nat. Cell Biol.* **2010**, *12*, 943.

[110] S. Levy, J. M. Feduska, A. Sawant, S. R. Gilbert, J. A. Hensel, S. Ponnazhagan, *Bone* **2016**, *93*, 113.

[111] B. Heissig, S. Rafii, H. Akiyama, Y. Ohki, Y. Sato, T. Rafael, Z. Zhu, D. J. Hicklin, K. Okumura, H. Ogawa, Z. Werb, K. Hattori, *J. Exp. Med.* **2005**, *202*, 739.

[112] O. M. Leung, J. Li, X. Li, V. W. Chan, K. Y. Yang, M. Ku, L. Ji, H. Sun, H.

Waldmann, X. Y. Tian, Y. Huang, J. Lau, B. Zhou, K. O. Lui, *Cell Rep.* **2018**, *24*, 1610.

[113] A. Facciabene, X. Peng, I. S. Hagemann, K. Balint, A. Barchetti, L. P. Wang, P. A. Gimotty, C. B. Gilks, P. Lal, L. Zhang, G. Coukos, *Nature* **2011**, *475*, 226.

[114] G. Christoffersson, E. Vågesjö, J. Vandooren, M. Lidén, S. Massena, R. B. Reinert, M. Brissova, A. C. Powers, G. Opdenakker, M. Phillipson, *Blood* **2012**, *120*, 4653.

[115] G. Christoffersson, J. Lomei, P. O'Callaghan, J. Kreuger, S. Engblom, M. Phillipson, *J. Leukoc. Biol.* **2017**, *102*, 741.

[116] R. Langer, J. P. Vacanti, *Science (80-.).* **1993**, *260*, 920.

[117] H. Petite, V. Viateau, W. Bensaïd, A. Meunier, C. de Pollak, M. Bourguignon, K. Oudina, L. Sedel, G. Guillemin, *Nat. Biotechnol.* **2000**, *18*, 959.

[118] R. Agarwal, A. J. García, *Adv. Drug Deliv. Rev.* **2015**, *94*, 53.

[119] F. J. O'Brien, *Mater. Today* **2011**, *14*, 88.

[120] M. Liu, X. Zeng, C. Ma, H. Yi, Z. Ali, X. Mou, S. Li, Y. Deng, N. He, *Bone Res.* **2017**, *5*, 17014.

[121] A. Gantar, L. P. Da Silva, J. M. Oliveira, A. P. Marques, V. M. Correlo, S. Novak, R. L. Reis, *Mater. Sci. Eng. C* **2014**, *43*, 27.

[122] M. T. I. Mredha, N. Kitamura, T. Nonoyama, S. Wada, K. Goto, X. Zhang, T. Nakajima, T. Kurokawa, Y. Takagi, K. Yasuda, J. P. Gong, *Biomaterials* **2017**, *132*, 85.

[123] R. Sridharan, A. R. Cameron, D. J. Kelly, C. J. Kearney, F. J. O'Brien, *Mater. Today* **2015**, *18*, 313.

[124] J. M. Anderson, A. Rodriguez, D. T. Chang, *Semin. Immunol.* **2008**, *20*, 86.

[125] J. M. Morais, F. Papadimitrakopoulos, D. J. Burgess, *AAPS J.* **2010**, *12*, 188.

[126] D. M. Mosser, J. P. Edwards, *Nat. Rev. Immunol.* **2008**, *8*, 958.

[127] G. Broughton, J. E. Janis, C. E. Attinger, *Plast. Reconstr. Surg.* **2006**, *117*, 12S.

[128] S. Gordon, *Cell* **2002**, *111*, 927.

[129] L.-B. Jiang, S.-L. Ding, W. Ding, D.-H. Su, F.-X. Zhang, T.-W. Zhang, X.-F. Yin, L. Xiao, Y.-L. Li, F.-L. Yuan, J. Dong, *Chem. Eng. J.* **2021**, *418*, 129323.

[130] X. Ding, J. Shi, J. Wei, Y. Li, X. Wu, Y. Zhang, X. Jiang, X. Zhang, H. Lai, *Sci. Adv.* **2021**, *7*, 7857.

[131] P. Qiu, M. Li, K. Chen, B. Fang, P. Chen, Z. Tang, X. Lin, S. Fan, *Biomaterials* **2020**, *227*, 119552.

[132] C. Yin, Q. Zhao, W. Li, Z. Zhao, J. Wang, T. Deng, P. Zhang, K. Shen, Z. Li, Y. Zhang, *Acta Biomater.* **2020**, *102*, 416.

[133] Q. Ou, K. Huang, C. Fu, C. Huang, Y. Fang, Z. Gu, J. Wu, Y. Wang, *Chem. Eng. J.* **2020**, *382*, 123019.

[134] L. Huang, J. Zhang, J. Hu, T. Zhao, Z. Gu, *ACS Biomater. Sci. Eng.* **2020**, *6*, 3270.

[135] W. Jiang, F. Hou, Y. Gu, Q. Saiding, P. Bao, J. Tang, L. Wu, C. Chen, C. Shen, C. L. Pereira, M. Sarmento, B. Sarmento, W. Cui, L. Chen, *Bioact. Mater.* **2022**,

12, 169.

[136] D. K. Patel, S. D. Dutta, J. Hexiu, K. Ganguly, K. T. Lim, *Carbohydr. Polym.* **2022**, *281*, 119077.

[137] S. Zhu, P. Chen, Y. Chen, M. Li, C. Chen, H. Lu, *Am. J. Sports Med.* **2020**, *48*, 2808.

[138] R. L. Duncan, C. H. Turner, *Calcif. Tissue Int.* **1995**, *57*, 344.

[139] L. Wang, X. You, L. Zhang, C. Zhang, W. Zou, *Bone Res.* **2022**, *10*, 16.

[140] S. Tan, J. Y. Fang, Z. Yang, M. E. Nimni, B. Han, *Biomaterials* **2014**, *35*, 5294.

[141] Y.-R. V Shih, K.-F. Tseng, H.-Y. Lai, C.-H. Lin, O. K. Lee, *J. Bone Miner. Res.* **2011**, *26*, 730.

[142] Z. Zhuang, Y. Zhang, S. Sun, Q. Li, K. Chen, C. An, L. Wang, J. J. J. P. Van Den Beucken, H. Wang, *ACS Biomater. Sci. Eng.* **2020**, *6*, 3091.

[143] X. T. He, R. X. Wu, X. Y. Xu, J. Wang, Y. Yin, F. M. Chen, *Acta Biomater.* **2018**, *71*, 132.

[144] M. Chen, Y. Zhang, P. Zhou, X. Liu, H. Zhao, X. Zhou, Q. Gu, B. Li, X. Zhu, Q. Shi, *Bioact. Mater.* **2020**, *5*, 880.

[145] E. M. Sussman, M. C. Halpin, J. Muster, R. T. Moon, B. D. Ratner, *Ann. Biomed. Eng.* **2014**, *42*, 1508.

[146] N. Jain, V. Vogel, *Nat. Mater.* **2018**, *17*, 1134.

[147] B. Cha, S. R. Shin, J. Leijten, Y. Li, S. Singh, J. C. Liu, N. Annabi, R. Abdi, M. R. Dokmeci, N. E. Vrana, A. M. Ghaemmaghami, A. Khademhosseini, *Adv. Healthc. Mater.* **2017**, *6*, 1700289.

[148] M. D. Swartzlander, C. A. Barnes, A. K. Blakney, J. L. Kaar, T. R. Kyriakides, S. J. Bryant, *Biomaterials* **2015**, *41*, 26.

[149] N. Jain, J. Moeller, V. Vogel, *Annu. Rev. Biomed. Eng.* **2019**, *21*, 267.

[150] K. M. Adlerz, H. Aranda-Espinoza, H. N. Hayenga, *Eur. Biophys. J.* **2016**, *45*, 301.

[151] C. Varol, A. Mildner, S. Jung, *Annu. Rev. Immunol.* **2015**, *33*, 643.

[152] H. Seeherman, J. M. Wozney, *Cytokine Growth Factor Rev.* **2005**, *16*, 329.

[153] W. J. King, P. H. Krebsbach, *Adv. Drug Deliv. Rev.* **2012**, *64*, 1239.

[154] D. Xue, E. Chen, H. Zhong, W. Zhang, S. Wang, M. U. Joomun, T. Yao, Y. Tan, S. Lin, Q. Zheng, Z. Pan, *Int. J. Nanomedicine* **2018**, *13*, 5799.

[155] D. Li, Z. Yang, X. Zhao, Y. Luo, W. Zhou, J. Xu, Z. Hou, P. Kang, M. Tian, *Chem. Eng. J.* **2022**, *435*, 134991.

[156] X.-T. He, X. Li, Y. Xia, Y. Yin, R.-X. Wu, H.-H. Sun, F.-M. Chen, *Acta Biomater.* **2019**, *88*, 162.

[157] S. Jain, T. H. Tran, M. Amiji, *Biomaterials* **2015**, *61*, 162.

[158] M. C. P. Sok, N. Baker, C. McClain, H. S. Lim, T. Turner, L. Hymel, M. Ogle, C. Olingy, J. I. Palacios, J. R. Garcia, K. Srithar, A. J. García, P. Qiu, E. A. Botchwey, *Biomaterials* **2021**, *268*, 120475.

[159] B. Mi, L. Chen, Y. Xiong, Y. Yang, A. C. Panayi, H. Xue, Y. Hu, C. Yan, L. Hu, X. Xie, Z. Lin, W. Zhou, F. Cao, X. Xiao, Q. Feng, G. Liu, *ACS Nano* **2022**, *16*,

771.

[160] Q. Fan, J. Bai, H. Shan, Z. Fei, H. Chen, J. Xu, Q. Ma, X. Zhou, C. Wang, *Bioact. Mater.* **2021**, *6*, 4014.

[161] Y. Wang, Z. Feng, X. Liu, C. Yang, R. Gao, W. Liu, W. Ou-Yang, A. Dong, C. Zhang, P. Huang, W. Wang, *Regen. Biomater.* **2022**, DOI 10.1093/RB/RBAC003.

[162] M. Zou, J. Sun, Z. Xiang, M. Zou, J. Sun, Z. Xiang, *Adv. Healthc. Mater.* **2021**, *10*, 2001502.

[163] Y.-H. Kim, H. Furuya, Y. Tabata, *Biomaterials* **2014**, *35*, 214.

[164] W. Qiao, H. Xie, J. Fang, J. Shen, W. Li, D. Shen, J. Wu, S. Wu, X. Liu, Y. Zheng, K. M. C. Cheung, K. W. K. Yeung, *Biomaterials* **2021**, *276*, 121038.

[165] W. Wang, L. Sun, P. Zhang, J. Song, W. Liu, *Acta Biomater.* **2014**, *10*, 4983.

[166] F. Gao, S. M. Chiu, D. A. L. Motan, Z. Zhang, L. Chen, H.-L. Ji, H.-F. Tse, Q.-L. Fu, Q. Lian, *Cell Death Dis.* **2016**, *7*, e2062.

[167] R. S. Waterman, S. L. Tomchuck, S. L. Henkle, A. M. Betancourt, *PLoS One* **2010**, *5*, e10088.

[168] K. Németh, A. Leelahavanichkul, P. S. T. Yuen, B. Mayer, A. Parmelee, K. Doi, P. G. Robey, K. Leelahavanichkul, B. H. Koller, J. M. Brown, X. Hu, I. Jelinek, R. A. Star, É. Mezey, *Nat. Med.* **2009**, *15*, 42.

[169] J. Maggini, G. Mirkin, I. Bognanni, J. Holmberg, I. M. Piazzón, I. Nepomnaschy, H. Costa, C. Cañones, S. Raiden, M. Vermeulen, J. R. Geffner, *PLoS One* **2010**, *5*, e9252.

[170] Y. Zhou, R. Huang, W. Fan, I. Prasadam, R. Crawford, Y. Xiao, *J. Tissue Eng. Regen. Med.* **2018**, *12*, e566.

[171] K. Akiyama, C. Chen, D. Wang, X. Xu, C. Qu, T. Yamaza, T. Cai, W. Chen, L. Sun, S. Shi, *Cell Stem Cell* **2012**, *10*, 544.

[172] G. Ren, L. Zhang, X. Zhao, G. Xu, Y. Zhang, A. I. Roberts, R. C. Zhao, Y. Shi, *Cell Stem Cell* **2008**, *2*, 141.

[173] G. J. Meijer, J. D. de Bruijn, R. Koole, C. A. van Blitterswijk, *PLoS Med.* **2007**, *4*, e9.

[174] T. K. Kovach, A. S. Dighe, P. I. Lobo, Q. Cui, *J. Immunol. Res.* **2015**, *2015*, DOI 10.1155/2015/752510.

[175] E. Seebach, H. Freischmidt, J. Holschbach, J. Fellenberg, W. Richter, *Acta Biomater.* **2014**, *10*, 4730.

[176] F. Jiang, W. Zhang, M. Zhou, Z. Zhou, M. Shen, N. Chen, X. Jiang, *Theranostics* **2020**, *10*, 6216.

[177] X. Ji, X. Yuan, L. Ma, B. Bi, H. Zhu, Z. Lei, W. Liu, H. X. Pu, J. Jiang, X. Jiang, Y. Zhang, J. Xiao, *Theranostics* **2020**, *10*, 725.

[178] S. Nadine, S. G. Patrício, C. R. Correia, J. F. Mano, *Biofabrication* **2019**, *12*, 15005.

[179] C. R. Correia, R. P. Pirraco, M. T. Cerqueira, A. P. Marques, R. L. Reis, J. F. Mano, *Sci. Rep.* **2016**, *6*, 21883.

[180] C. R. Correia, T. C. Santos, R. P. Pirraco, M. T. Cerqueira, A. P. Marques, R. L.

Reis, J. F. Mano, *Acta Biomater.* **2017**, *53*, 483.

[181] S. Nadine, C. R. Correia, J. F. Mano, *Adv. Healthc. Mater.* **2021**, *10*, 2001993.

[182] M. Ueno, C. Lo, D. Barati, B. Conrad, T. Lin, Y. Kohno, T. Utsunomiya, N. Zhang, M. Maruyama, C. Rhee, E. Huang, M. Romero-Lopez, X. Tong, Z. Yao, S. Zwingenberger, F. Yang, S. B. Goodman, *J. Biomed. Mater. Res. Part A* **2020**, *108*, 2240.

[183] M. Ueno, N. Zhang, H. Hirata, D. Barati, T. Utsunomiya, H. Shen, T. Lin, M. Maruyama, E. Huang, Z. Yao, J. Y. Wu, S. Zwingenberger, F. Yang, S. B. Goodman, *Front. Bioeng. Biotechnol.* **2021**, *9*, 972.

[184] Y. Niu, Q. Li, R. Xie, S. Liu, R. Wang, P. Xing, Y. Shi, Y. Wang, L. Dong, C. Wang, *Biomaterials* **2017**, *139*, 39.

[185] H. L. Xu, W. G. Yang, P. P. Chen, R. Chen, P. P. Xue, L. F. Wang, J. D. Yuan, Q. Yao, B. Chen, Y. Z. Zhao, *Biotechnol. J.* **2020**, *15*, 2000004.

[186] A. H. Lourenço, A. L. Torres, D. P. Vasconcelos, C. Ribeiro-Machado, J. N. Barbosa, M. A. Barbosa, C. C. Barrias, C. C. Ribeiro, *Mater. Sci. Eng. C* **2019**, *99*, 1289.

[187] A. Gonzalez-Pujana, K. H. Vining, D. K. Y. Zhang, E. Santos-Vizcaino, M. Igartua, R. M. Hernandez, D. J. Mooney, *Biomaterials* **2020**, *257*, 120266.

[188] J. R. García, M. Quirós, W. M. Han, M. N. O'Leary, G. N. Cox, A. Nusrat, A. J. García, *Biomaterials* **2019**, *220*, 119403.

[189] A. Moshaverinia, C. Chen, X. Xu, S. Ansari, H. H. Zadeh, S. R. Schricker, M. L. Paine, J. Moradian-Oldak, A. Khademhosseini, M. L. Snead, S. Shi, *Adv. Funct. Mater.* **2015**, *25*, 2296.

[190] B. Zhang, M. Zhang, Y. Sun, M. Li, F. Han, C. Wu, *Prog. Nat. Sci. Mater. Int.* **2021**, *31*, 883.

[191] M. Kang, G. Thalji, C.-C. Huang, S. Shirazi, Y. Lu, S. Ravindran, L. F. Cooper, *Front. cell Dev. Biol.* **2020**, *8*, 596622.

[192] S. E. Hanson, S. N. King, J. Kim, X. Chen, S. L. Thibeault, P. Hematti, *Tissue Eng. - Part A* **2011**, *17*, 2463.

[193] S. N. King, S. E. Hanson, X. Chen, J. Kim, P. Hematti, S. L. Thibeault, *J. Biomed. Mater. Res. Part A* **2014**, *102*, 890.

[194] C. Dollinger, S. Ciftci, H. Knopf-Marques, R. Guner, A. M. Ghaemmaghami, C. Debry, J. Barthes, N. E. Vrana, *J. Tissue Eng. Regen. Med.* **2018**, *12*, 330.

[195] I. Ščigalková, J. Bystroňová, L. Kovářová, M. Pravda, V. Velebný, V. Riabov, H. Klüter, J. Kzhyshkowska, N. E. Vrana, *RSC Adv.* **2019**, *9*, 21396.

[196] J. Barthes, C. Dollinger, C. B. Muller, U. Liivas, A. Dupret-Bories, H. Knopf-Marques, N. E. Vrana, *Front. Bioeng. Biotechnol.* **2018**, *6*, DOI 10.3389/fbioe.2018.00108.

[197] F. E. Ludwinski, A. S. Patel, G. Damodaran, J. Cho, J. Furmston, Q. Xu, S. N. Jayasinghe, A. Smith, B. Modarai, *npj Regen. Med.* **2019**, *4*, 6.

[198] E. M. Moore, G. Ying, J. L. West, *Adv. Biosyst.* **2017**, *1*, 1600021.

[199] J. Kim, P. Hematti, *Exp. Hematol.* **2009**, *37*, 1445.

[200] J. Chang, A. J. Koh, H. Roca, L. K. McCauley, *Bone Res. 2015 31* **2015**, *3*, 1.

[201] M. Juhas, N. Abutaleb, J. T. Wang, J. Ye, Z. Shaikh, C. Sriworarat, Y. Qian, N. Bursac, *Nat. Biomed. Eng.* **2018**, *2*, 942.

[202] M. S. Hu, G. G. Walmsley, L. A. Barnes, K. Weiskopf, R. C. Rennert, D. Duscher, M. Januszyk, Z. N. Maan, W. X. Hong, A. T. M. Cheung, T. Leavitt, C. D. Marshall, R. C. Ransom, S. Malhotra, A. L. Moore, J. Rajadas, H. P. Lorenz, I. L. Weissman, G. C. Gurtner, M. T. Longaker, *JCI Insight* **2017**, *2*, DOI 10.1172/jci.insight.96260.

[203] D. A. Cantu, P. Hematti, W. J. Kao, *Stem Cells Transl. Med.* **2012**, *1*, 740.

[204] M. Romero-López, Z. Li, C. Rhee, M. Maruyama, J. Pajarinen, B. O'Donnell, T. H. Lin, C. W. Lo, J. Hanlon, R. Dubowitz, Z. Yao, B. A. Bunnell, H. Lin, R. S. Tuan, S. B. Goodman, *Tissue Eng. - Part A* **2020**, *26*, 1099.

[205] R. Sridharan, K. J. Genoud, D. J. Kelly, F. J. O'Brien, *ACS Appl. Bio Mater.* **2020**, *3*, 7562.

[206] J. M. Živković, S. T. Stojanović, M. Đ. Vukelić-Nikolić, M. B. Radenković, J. G. Najdanović, M. Ćirić, S. J. Najman, *Int. Orthop.* **2021**, *45*, 1087.

[207] O. H. Jeon, L. M. Panicker, Q. Lu, J. J. Chae, R. A. Feldman, J. H. Elisseeff, *Sci. Rep.* **2016**, *6*, 26761.

[208] M. R. Borrelli, M. S. Hu, W. X. Hong, J. D. Oliver, D. Duscher, M. T. Longaker, H. P. Lorenz, *J. Craniofac. Surg.* **2019**, *30*, 2640.

[209] O. Rapalino, O. Lazarov-Spiegler, E. Agranov, G. J. Velan, E. Yoles, M. Fraidakis, A. Soloman, R. Gepstein, A. Katz, M. Belkin, M. Hadani, M. Schwartz, *Nat. Med.* **1998**, *4*, 814.

[210] S. Kobashi, T. Terashima, M. Katagi, Y. Nakae, J. Okano, Y. Suzuki, M. Urushitani, H. Kojima, *Mol. Ther.* **2020**, *28*, 254.

[211] I. J. Fuss, M. E. Kanof, P. D. Smith, H. Zola, *Curr. Protoc. Immunol.* **2009**, *85*, 7.1.1.

[212] B. S. Sheridan, L. Lefrançois, *Curr. Protoc. Immunol.* **2012**, *99*, 3.19.1.

[213] L. Y. Lu, F. Loi, K. Nathan, T. H. Lin, J. Pajarinen, E. Gibon, A. Nabeshima, L. Cordova, E. Jämsen, Z. Yao, S. B. Goodman, *J. Orthop. Res.* **2017**, *35*, 2378.

[214] L. Jardine, U. Cytlak, M. Gunawan, G. Reynolds, K. Green, X. N. Wang, S. Pagan, M. Paramitha, C. A. Lamb, A. K. Long, E. Hurst, S. Nair, G. H. Jackson, A. Publicover, V. Bigley, M. Haniffa, A. J. Simpson, M. Collin, *J. Clin. Invest.* **2020**, *130*, 4574.

[215] K. Ballen, *Stem Cells Transl. Med.* **2017**, *6*, 1312.

[216] P. A. Gourraud, L. Gilson, M. Girard, M. Peschanski, *Stem Cells* **2012**, *30*, 180.

[217] A. Morizane, T. Kikuchi, T. Hayashi, H. Mizuma, S. Takara, H. Doi, A. Mawatari, M. F. Glasser, T. Shiina, H. Ishigaki, Y. Itoh, K. Okita, E. Yamasaki, D. Doi, H. Onoe, K. Ogasawara, S. Yamanaka, J. Takahashi, *Nat. Commun. 2017 81* **2017**, *8*, 1.

[218] H. Xu, B. Wang, M. Ono, A. Kagita, K. Fujii, N. Sasakawa, T. Ueda, P. Gee, M. Nishikawa, M. Nomura, F. Kitaoka, T. Takahashi, K. Okita, Y. Yoshida, S. Kaneko, A. Hotta, *Cell Stem Cell* **2019**, *24*, 566.