Osteoimmunomodulation for the development of advanced bone biomaterials

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Z. Murray 1 Ross Crawford 13, Jiang Chang 23, Chengtie Wu 23, Yin Xiao 13 As direct <u>crector cells</u> for osteogenesis, osteoblastic cells are commonly used for evaluating the *in vitro* osteogenic capacity of bone biomaterials, and the traditional biological principle for developing bone biomaterials is to directly stimulate osteogenic differentiation. With this principle, most efforts are currently spent on optimizing the bio-mechanical and physicochemical properties to induce osteogenic differentiation of <u>mesenchymal stem cells</u>. This strategy has achieved certain success in the development of bone biomaterials; however, inconsistencies between *in vitro* and *in vivo* studies are not uncommon, implying the mechanisms that govern the material's capacity to mediate osteogenesis is not well-

understood steoimmunology has revealed the vital role of <u>immune cells</u> in regulating bone <u>dynamics</u>. Neglecting the importance of the immune response is a major shortcoming of the traditional strategy, and may explain inconsistencies between *in vitro* and *in vivo* conditions. Here, we proposed osteoimmunomodulation (OIM) in recognition of the importance of the immune response during biomaterial-mediated osteogenesis. Accordingly, we proposed the **paradigm** shift of bone biomaterials to an osteoimmunomodulatory material and discussed the evaluation strategy for the osteoimmunomodulation property of bone biomaterials. It is the ambition of authors that this review will change traditional methods for bone biomaterials with the osteoimmunomodulatory property for orthopedic and dental applications.

Introduction

<u>Bone defects</u> caused by tumor resection, traumatic fracture, <u>aseptic</u> <u>necrosis</u>, <u>osteolysis</u>, <u>osteomyelitis</u>, <u>periodontitis</u>, and spinal fusion, to name but a few, typically require surgical <u>remediation</u> using bone biomaterials [1], [2], [3], [4]. Due to the direct relationship between osteoblastic <u>lineage</u> and bone formation, the major principle for developing bone biomaterials is to manipulate the *in vitro* osteogenic differentiation of the osteoblastic lineage and then investigating the potential osteogenic biomaterials in an *in vivo* model. With this principle, many strategies have been developed to fabricate an ideal bone biomaterial that can gain desired <u>osseointegration</u> and osteogenesis. The fabrication techniques are quite advanced that materials scientists can somehow prepare bone biomaterials with the desired physicochemical and mechanical properties (from nano to particle size, from 2D to customized 3D structure, from hydrophilic to hydrophobic, etc.).

However, this principle often does not lead to clinically useful bone implant materials, with many candidates failing to make it beyond the confines of the laboratory. When analyzing the possible reasons, we focus on optimizing the compositions, the bio-physicochemical and mechanical properties of the candidate materials, but we rarely think that perhaps the basic biological principle also needs optimization. Bone biology has made great progress and the mechanisms underlying osteogenesis have been much better understood. We now know that osteogenesis is not simply accomplished by bone cells from skeleton system, but a collaboration of multiple systems. This indicates that the traditional biological principle is outdated and insufficient, which could be a leading reason for the failure of trials. To fabricate an ideal bone biomaterial, we need to keep the pace with the development of bone biology and keep on modifying the basic biological principle. Among all the achievements made in the area of bone biology, the development of osteoimmunology is one of the greatest. The immune and skeletal systems are found to be closely related, sharing a number of cytokines, receptors, signaling molecules and transcription factors [5], [6]. <u>Immune cells</u> play a key role in bone <u>homeostasis</u>. Being a foreign body, an implant is recognized by the immune system and triggers a significant immune reaction that affects the biological behavior of bone cells. Such an event may eventually determine the *in vivo* fate of bone biomaterials [7], [8]. The immune response may, therefore, be a key factor that is neglected when evaluating the osteogenic capacity of bone biomaterials. Accordingly, the design paradigm for advanced bone biomaterials should be shifted from being relatively inert to having immunomodulatory properties, emphasizing the important role of immune responses [7]. A new generation of bone biomaterials should be able to modulate the local immune environment such that it favors osteogenesis and the <u>osseointegration</u> of the implant.

Developing such biomaterials would require an in-depth understanding of a number of important issues. Firstly, it is important to understand the relationship between immune cells and bone cells and what effect the immune environment induced by implanted biomaterials has on osteogenesis. Secondly, the mechanisms underlying the material-mediated immune response must be understood in order to aid the design and preparation of biomaterials to induce an immune environment that provides conditions that balance osteogenesis and steoclastogenesis for optimal osseointegration. Finally, determining whether or not a biomaterial can induce a favorable immune response should be a routine screening process and part of a standard evaluation protocol when developing advanced immunomodulatory bone biomaterials. In this review, we define such a capacity as osteoimmunomodulation (OIM) – a novel property of bone biomaterials. Favorable OIM properties are of great importance when attempting to produce advanced bone biomaterials for clinical application with optimal osteogenesis and osseointegration.

Overview of the integration between bone tissue and implants The mechanism underlying bone biomaterial-mediated osteogenesis involves at least three interactive components: the host immune cells, the host bone cells and the materials. Following implantation, the host body will first undergo a universal response to the materials, which is an extension of the mammalian response following tissue injury [9]. Proteins from blood and interstitial fluids, such as fibrinogen, vitronectin, complement, and fibronection [7], will adsorb to the material's surfaces within seconds and then form a transient surface matrix. In response, the coagulation cascade and complement systems are activated, leading to thrombus formation and activation of other cell populations.

After the initial blood/material interaction, an <u>acute inflammation</u> is initiated, which features the recruitment and activation of <u>neutrophils</u>,

or <u>polymorphonuclear leukocytes</u> (PMNs). PMNs, in an effort to degrade the materials, release proteolytic enzymes and reactive oxygen species (ROS) [10], which will corrode the surface of the material. The PMNs rapidly become exhausted, undergoing apoptosis and disappearing from the implantation sites within the first two days [11]. Mast cells are also active participants in the acute inflammatory reaction and <u>degranulation</u> of these cells leads to the release of inflammation-enhancing cytokines and histamine, which amplify the immune reaction [12].

emoattractants and activation cytokines released in the previous phase result in <u>monocyte</u> recruitment to the implant, where the cells differentiate

into macrophages. Macrophages are able to engulf particles of up to $5 \mu m$ [7], [13]; if the particle size is larger, the macrophages will coalesce to form foreign body giant cells (FBGCs), driven by stimulation with IL-4 and IL-13 [14]. The foreign components and molecules released during the host-body/implant interaction can positively regulate osteogenic differentiation to form new bone on the surface of the implant and entrap it. In the bone remodeling phase newly formed bone undergoes functional remodeling and some entrapped implant materials, such as Ca–P based bioceramics, can be further degraded. Functional loading and the mechanical strain is the main cause for the remodeling. Osteocytes are known to translate signals related to mechanical strain into biochemical signals and regulate osteoblasts and osteoclasts and, therefore, may play a regulatory role in this late stage [15], [16], [17] (Fig. 1a)



(a) Macrophage phenotype switch pattern during the formation of new bone

(b) Macrophage phenotype switch pattern during the formation of fibrous capsule



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 - Figure 1. Schematic illustration of bone biomaterials mediated *de novo* bone formation (a) and the failure of bone biomaterials that lead to the fibrous encapsulation (b). (a) This process can be divided into

three phases: early, bone formation and remodeling phase, with the involvement of multiple systems: coagulation system, immune system, skeleton system. The most likely macrophage phenotype switch pattern is also presented. The early stage of the repair response is dominated by the inflammatory phase, when the majority of macrophages would be of the inflammatory M1 phenotype. An efficient and timely switch from M1 to M2 macrophage phenotype results in an osteogenic cytokine release and with it the formation of new bone tissue. (b) It is also divided into three phases: acute, chronic inflammation and fibrous encapsulation. The likely macrophage phenotype switch pattern is shown. The early stage is dominated by the inflammatory phase, during which the majority of macrophages would be of the inflammatory M1 phenotype. However, a prolonged M1 polarization phase leads to an increase in fibrosis-enhancing cytokine release pattern by the M2 macrophages, which results in the formation of a fibrocapsule. Adapted from [182].

- 4. The close relationship that exists between the immune and skeletal systems makes the proposition that stimulated <u>immune cells</u> may contribute to both the success and failure of an implant seem feasible (Fig. 1). The immune cells would exert this effect by releasing cytokines that regulate osteogenesis, in addition to their well-known effects on inflammation, thus inducing or inhibiting bone formation. The effects of the immune response to bone biomaterials in regulating osteogenesis are, therefore, 'a double-edged sword'. A favorable immune reaction creates an osteogenic microenvironment that can improve osteogenesis, whereas an inappropriate immune reaction may lead to the <u>chronic inflammation</u> and the formation of a fibrous capsule around the implant.
- 5. The capsule formed by the <u>foreign body reaction</u> (FBR) effectively separates the implant from the surrounding environment, such that it can remain safely in the host body throughout its lifetime [18]. However, this renders the implant an 'inert' mechanical support, a scenario which fails to meet the demands of a bone substitute material since it is intended to induce new bone formation and fill the defect space with fully functional bone. A fibrous encapsulation prevents direct interaction between bone marrow and the implants such that bone cells cannot attach to the surface of implants to form new bone. Instead, the defect will be filled by a fibrous tissue, resulting in the

failure of bone reconstruction. This illustrates the importance of creating a local immune environment that favors <u>bone</u> <u>regeneration</u> and <u>osseointegration</u>; manipulating the immune response by targeted modifications of the bone biomaterials is, therefore, a good strategy to swing the balance toward this direction.

Immune cells regulation of osteoclastogenesis and osteogenesis The field of osteoimmunology seeks to understand the interaction between the immune and skeletal systems [6]. Immune cells participate actively in bone physiology and pathology by releasing regulatory molecules which elicit significant effects on osteoclastogenesis and osteogenesis (Fig. 2). Abnormal functioning of immune cells can lead to an imbalance between osteoclasts and osteoblasts and result in conditions such as osteolysis, osteoporosis, osteoarthritis and rheumatoid arthritis. In this part, we will review the role of immune cells in osteoclastogenesis and osteogenesis and possible molecular mechanisms at play.



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Figure 2. Schematic illustration of the role of immune cells in bone dynamics. Immune cells participate actively in osteoclastogenesis and osteogenesis by releasing regulatory molecules.

Immune cells and osteoclastogenesis

Immune cells regulate osteoclastogenesis by three major cytokines: macrophagecolony stimulating factor (M-CSF), receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG). M-CSF binds to its cognate receptor c-FMS on osteoclast precursors and signals through the Akt and MAP kinase pathway [19]. RANKL binds to RANK, a receptor on the surface of osteoclast precursors, thereby transducing via TNF receptor associated factor 6 (TRAF6), NF-κB, activator protein 1 (AP-1) and nuclear factor of activated T cells 2 (NFAT2) to upregulate expression of genes for the survival and differentiation of osteoclasts [5], [20]. RANKL is expressed, not only by osteoblastic cells that maintain normal osteoclastogenesis in bone tissue, but also by activated T cells and neutrophils, indicating the involvement of these immune cells during osteoclastogenesis [21], [22]. Macrophages are the precursor of osteoclasts, which under the stimulation of M-CSF and RANKL can differentiate into osteoclasts during bone remodeling.

IL-6 and oncostatin M (OSM) are important mediators of osteoclast formation and function. IL-6 is known to induce the expression of RANKL, and utilize the RANKL/RANK-OPG system to elicit indirect effects on promoting osteoclastogenesis and osteoclast activation [23], [24]. IL-6 is also found to participate in the TNF- α and IL-I induced osteoclast formation [25]. OSM uses gp130, the same receptor subunit as does IL-6, for signaling and these two cytokines often have similar and overlapping functions [26]. OSM can also stimulate the production of RANKL by osteoblasts and enhance the formation of osteoclasts in a dose dependent manner, which might be related to its synergistic effects with IL-6 [27], [28]. By contrast, interferon- γ (IFN γ) promotes the degradation of TRAF6, a key intermediate in RANKL/RANK pathway, thereby preventing massive bone destruction during inflammation [5]. OPG, a decoy receptor for RANKL, interrupts the interaction of RANKL/RANK, thereby inhibiting both differentiation and the function of osteoclasts [29], [30]. B cells have been shown to be a major source of bone marrow-derived OPG [31], [32], which implies that B cells are one of the main inhibitor of osteoclastogenesis in normal physiology. Depletion of CD4 and CD8 T lymphocyte subsets in mice results in a vitamin D3-stimulated osteoclast formation through a mechanism involving upregulated prostaglandin E production [32], [33]. T-cells are found to work cooperatively with B-cells and increase OPG production by CD40/CD40L costimulation, based on data showing that T-cell-deficient CD40 and CD40L-knock-out mice are osteoporotic [31].

Mast cells also participate actively in osteoclastogenesis [34]. A reduction in the number of mast cells reduces bone remodeling, whereas the enhancement of systemic mastocytosis leads to an increase of bone loss [35], [36]. Histamine, rather than pro-inflammatory cytokines, may be the major mediator during this interaction; a study in mice showed that a targeted disruption of the histidine decarboxylase gene, which leads to a deficiency of histamine, had the effect of reducing bone loss, even in response to ovariectomy [37], [38].

The interaction between immune cells and osteoclast cells plays a key role in the pathology of many bone diseases, such as osteoarthritis, rheumatoid arthritis. Persistent excessive inflammation is a feature of the continuous release of pro-inflammatory cytokines (TNF- α , IL-1 α/β and IL-6) and usually accompanies an increased RANKL/OPG ratio and elevated osteoclast activity [39]. The result is a shift of bone remodeling toward osteoclast-mediated progressive bone erosions, characterized by derangement of mineral and organic components, which results in excessive bone loss and functional disability.

Immune cells and osteogenesis

Immune cells play an indispensable regulatory role in osteogenesis. They can act positively in the progress of bone regeneration. Resident macrophages (osteomacs) are crucial for efficient osteoblast mineralization, as depletion of macrophages leads to the complete loss of osteoblast-mediating bone formation in vivo [40]. Bone fracture healing is significantly enhanced in knockout mice that lack T and B cells, indicating they may also have a detrimental function during this process [41], [42]. These data together suggest the dual roles of immune cells in osteogenesis, through their expression and secretion of a wide range of regulatory molecules [43], such as inflammatory cytokines, bone morphogenetic protein 2 (BMP2), transforming growth factor β (TGF- β), and vascular endothelial growth factor (VEGF) [44], [45]. A full understanding on how immune cells and their secreted cytokines act on osteogenesis will help to develop immunomodulatory intervention strategies that maximize the regenerative and minimize the destructive effects of immune response, leading to the desired bone regeneration.

A combination of four major inflammatory cytokines, TNF- α , TGF- β , IFN- γ , and IL-17 at physiological concentrations could induce the production of mineralized matrix as effectively as dexamethasone, a commonly used osteogenic medium supplement [46]. TNF- α is found to increase alkaline phosphatase (ALP) activity and mineralization by mesenchymal stromal cells (MSCs) in a dose-dependent manner through activation of the NF- κ B signaling pathway [47], [48]. The stimulatory effect of the conditioned medium from the lipopolysaccharide (LPS)-activated inflammatory M1 macrophages on ALP activity is attenuated when the conditioned medium is pretreated with TNF- α neutralizing antibody [46]. It is also found that the knockout of IL-6 delays callus maturity, mineralization, and remodeling indicating the essential role of IL-6 in the early stages of fracture healing [49], while the knockout of OSM in early stage leads to the reduced amount of new bone [50].

However, the inhibitory effects of TNF- α have also been observed on the differentiation of osteoblastic cells, by suppressing the release of BMP2 and eliciting pro-apoptotic effects on osteoblasts [51], [52]. The upregulation of IFN- γ and TNF- α by T lymphocytes is also thought to be responsible for the failure of MSCs based bone tissue regeneration and its inhibitory effects can be eliminated by the application of aspirin (an anti-inflammatory drug) [53]. The underlying mechanism may be related to the stimulation of NF- κ B in MSCs, which promotes degradation of β -catenin, thereby inhibiting osteogenic differentiation [54]. This reasoning leads to the hypothesis that the effects of inflammatory cytokines on osteogenesis may be dose and time dependent and that an adequate concentration and appropriate timing of these cytokines could induce osteogenesis. The flip side to this is that inadequate concentrations and/or stimulation time of the inflammatory cytokines may lead to bone resorption.

The observation that immune cells have an important role in bone dynamics is a strong argument for considering the immune responses in bone tissue engineering. The traditional strategy of focusing on the reactions of the bone cells is clearly insufficient, as they do not reflect the in vivo condition, which involves immune reaction during the process of bone repair. If the importance of the immune system is neglected, the conclusions will invariably be drawn when interpreting the results of bone tissue engineering experiments.

Multiple roles of macrophages in the bone healing process Macrophages, among all the immune cells, tend to receive the most attention due to their multiple roles in the bone healing process and their high plasticity. Macrophages play a central role in inflammation and host defense, especially in the innate immune response. Based on distinct functional properties, surface markers, and inducers, macrophages have been broadly characterized into M1 and M2 phenotypes, mirroring the Th1/Th2 nomenclature described for T helper cells [55]. M2 macrophages include three sub-populations: M2a, M2b, and M2c. The inducers, surface markers and functions of each phenotype are summarized in Fig. 3. It should be noted that this classification represents only a simplification of the in vivo scenario. Most likely the macrophage phenotype occupies a continuum between M1 and M2 designations, with many shades of activation yet to be identified [56]. This makes distinguishing M1 from M2 macrophages more difficult since transient macrophages may possess some characteristics of both phenotypes, resulting in unreliable surface markers. Thus, reliance on markers to detect a macrophage population would be problematic and multiple criteria are required.



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Figure 3. Inducers, surface markers and functions of macrophage phenotypes. Under the influences of different inducers (next to the arrows), macrophages can switch into different phenotypes [M1, M2 (M2a, M2b, M2c)]. Each phenotype has special surface markers and different functions.

There is still no consensus as to which macrophage phenotype is the most beneficial for osteogenesis. Classically activated inflammatory macrophages (M1) are well known to secrete many pro-inflammatory cytokines (TNF α , IL-6, IL-1 β), which are traditionally recognized to induce the osteoclastogenesis, and enhance osteoclastic activities, leading to bone resorption. However, some recent studies have found that osteogenesis is enhanced in the response of M1 macrophages, rather than M2. Guihard et al., reported that the classically activated inflammatory M1, but not M2 macrophages, induced osteogenesis in MSCs via OSM [57]. Similar results can be found in LPS activated M1 T lymphocyte, which were found to secrete high levels of BMP2, enhancing osteogenic differentiation of MSCs [46].

Alternative activated M2 macrophages tend to be more closely associated with late stage of the tissue repair, resulting in either a fibrocapsule (Fig. 1b) or the formation of new bone (Fig. 1a), by the secretion of relevant cytokines. They not only contribute osteoinductive and osteogenic cytokines, such as BMP2 and VEGF, to the process of osteogenesis, but also elaborate inflammatory and fibrous agents (TNF α , TGF- β 1, TGF- β 3) to promote pathological fibrosis [58], [59]. In response to excessive inflammation, fibrosis-enhancing M2 phenotypes would be induced to regulate the formation of a fibrous capsule, thereby separating the inflammatory reaction center from normal bone tissue. This confines the inflammation and preserves the normal bone tissue, which would otherwise lead to failure of bone regeneration (Fig. 1b). An excessive switch to the M2 phenotype, on the other hand, leads to scar tissue or a delayed wound healing [9], [60]. It is, therefore, most probable that both macrophage phenotypes play indispensable roles during the bone healing process and that it is the macrophage phenotype (Fig. 1).

There are only a limited number of studies that have examined the specific effects of macrophage polarization patterns following bone injury. Some have investigated the effects of macrophages in the healing of other tissues such as skin and muscle: one study used macrophage depletion to determine the effects of immune response during the sequential stages of skin wound healing [61]. It was found that depletion of macrophages in the early stage resulted in less scar formation, whereas depletion of macrophages in the mid-stage resulted in severe hemorrhage. No effect was noted in the depletion of macrophages in the late stage. These results indicate diverse roles of macrophages during various phases of skin repair.

The healing process of bone defects share some commonalities with wound healing in general. The early stage of the repair response is dominated by the inflammatory phase, when the majority of macrophages would be of the pro-inflammatory M1 phenotype. This stage plays an important role in determining the long-term success of bone repair, either by promoting a fibrotic foreign body reaction or by a bone wound healing response with bone matrix production and vascularization [62]. The M1 macrophages may determine subsequent immune cell behaviors, either by restraining the inflammation and initiating the tissue repair, or amplifying the inflammatory response with subsequent destruction of normal bone tissue. More efforts need to go into determining the appropriate early immune environment for activating osteogenesis-enhancing M2 macrophages, thereby enhancing the new bone regeneration.

The M2 macrophages play a more prominent role during mid- to late stages of the repair response compared with M1 macrophages. The cytokine release pattern of

M2 macrophages is decided by the M1 polarization during the early phase of bone wound healing. Prolonged M1 polarization can lead to an increase in fibrosisenhancing cytokine pattern released by the M2 macrophages, which results in the formation of a fibrocapsule (Fig. 1b). By contrast, an effective and timely switch in M1 macrophage phenotype can result in an osteogenesis-enhancing cytokine release pattern from M2 macrophages and with it the formation of new bone tissue (Fig. 1a). Macrophage phenotypes are dynamic and plastic and respond to environmental cues, allowing these cells to alter their phenotype and physiology and this accounts for their multiple roles in the bone healing process [63]. The phenotype switch relates to the type, concentration and duration of the polarizing signals. When treated with IL-4, macrophages change into a reparative M2 phenotype [64]; however, with the addition of LPS and immune complexes, macrophages can take on a hybrid phenotype possessing both characteristics of wound-healing and regulatory macrophages [65]. It is still unclear whether this phenotypic alteration is derived from the in situ macrophages or a new population of macrophages migrated from circulating immune cells into the tissue spot to replace the local cells. Macrophages can be considered as a model cell type for the evaluation of immune response. In addition, the heterogeneity and plasticity of macrophages also makes them a prime target for modulation of immune response. Therefore, more studies are required to better understand the macrophage switch pattern and how it affects the bone healing process.

Bone biomaterials modulating the immune response

Bone biomaterials are recognized by the host's immune system as a foreign body, arousing multiple directional immune responses. The biomaterials are not simply passive targets for attacking immune cells, but elicit significant effects that determine the type and extent of implant-mediated immune responses. Surface properties, particle size, porosity, and the released ions from the biomaterials are all factors involved in these responses (Fig. 4, Table 1).



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Figure 4. Possible properties of bone biomaterials, such as topography, surface charge, wettability particle size, ion release and porosity and pore size, can modulate the immune cells (macrophages, lymphocytes, etc.) and the subsequent major immune responses (innate, humoral immune response, etc.).

Table 1. Surface and bulk properties of biomaterials affecting the immune cells.

Properties of biomaterials Effects on immune cells

Surface properties

Surface wettability Hydrophobic materials enhance monocyte adhesion [68], [69]; hydrophilic/neutral surfaces inhibit macrophages adhesion, but enhance the release of pro-inflammatory cytokines and chemokines [66], [67].

Surface chargeCationic particles are more likely to induce inflammation, compared with anionic and neutral species [70], [71].

Surface topography, roughness Affect immune cells adhesion [75]; sandblasting and acid etching surfaces elicit significant immune reaction [74].

Particle size Immune cells internalize materials in a size dependent manner [70], [102], [103]; size affects the immune reaction [70], [107], [108], [109], [110], but no consensus on what range of size is more effective.

Porosity and pore size Larger pore size enhance angiogenesis while inhibit inflammation [112], [113], [116].

Released bioactive ions Some bioactive ions have immunoregulatory effects. Upregulation of inflammation: Ca, Co, Si [124], [140], [158], [159]. Downregulation effects: Ca, Zn, Mg, Sr [125], [145], [172], [178]. Could be in a dose dependent manner.

Surface properties of bone biomaterials

Biological behaviors of immune cells on the surfaces of the bone biomaterials are largely determined by the surface properties, such as surface microstructure and wettability [66], [67], [68]. Generally, hydrophobic materials tend to improve monocyte adhesion in comparison to hydrophilic materials resulting in a local immune reaction in situ [68], [69]. It was found that the hydrophilic/neutral copolymer surfaces inhibited macrophage adhesion and fusion into FBGCs. However, the adherent cells produced larger amounts of cytokines (IL-6 and IL-1 β) and chemokines [IL-8, RANTES (regulated on activation, normal T cell expressed and secreted; also known as CCL5), ENA-78 (epithelial-derived neutrophil-activating peptide 78; also known as CXCL5), and MCP-1 (monocyte chemotactic protein 1; also known as CCL2)] than the hydrophobic and hydrophilic ones [66], [67]. The surface charge also elicits significant effects on the immune response. It is generally accepted that cationic (positively charged) particles are more able to boost inflammatory response than anionic (negatively charged) and neutral species [70], [71]. Most mammalian cells, including immune cells, have an overall negative surface charge [72]. The loss of the negative surface charge of the cell membrane by the positively charged particles may influence protein localization and confirmation, which, under normal circumstances, induce signal transduction into the cytoplasm resulting in significant biological responses, including inflammatory reaction. The surface topography of biomaterials is another important property that affects the interaction of immune cells [73], [74], [75], [76]. The roughness of titanium, for example, affects the attachment and spread of immune cells: macrophage adhesion increases with time on all surfaces (polished, machined, and grit-blasted commercially pure titanium), whereas cell spreading increases with increased surface roughness [75]. In addition to the effects on cell attachment, the roughness of titanium could also modulate the production of inflammatory cytokines and chemokines by macrophages, with the sandblasting and acid etching surface eliciting significant stimulatory effects [74].

It is estimated that the surface roughness of bone is around 32 nm, making nanomaterials potentially most biomimic [77]. Nanoscale microstructures have been found to stimulate human MSCs into producing bone minerals in vitro, even in the absence of osteogenic supplements. This has generated the considerable interest in the applications of nanomaterials in bone tissue engineering [78]. It has also been found that micro-structured, rather than nanostructured topography, induced macrophages to an activated state that have both M1 and M2 characteristics [79],

and titanium surfaces, modified by titania nanotube arrays, can reduce in vitro immune response compared to the raw titanium surface [80]. Nanoscale topographic structures may, therefore, prove to be a good strategy to modulate the immune response of biomaterials.

The underlying mechanisms of biomaterial surface properties regulating the immune response may be related to how they affect protein adsorption, such as complement components, fibrinogen, fibronectin and vitronectin. Upon adsorption, the protein structures may experience some changes, leading to the exposure of some masked domains or epitopes, which can then be recognized by host cells (including immune cells). Binding to these epitopes via specific receptors allow host cells to attach to the surfaces of the materials [81]. The initial adsorption of proteins forms a temporal matrix on the surface, which then becomes an important link between the material and host response. For this reason, it is important to know not only what proteins are adsorbed, but also the manner of how they interact with the surface when determining subsequent behaviors of host cells [82]. Both surface chemistry and wettability influences the conformational changes of adsorbed proteins and modulate adsorption kinetics, binding strengths, and protein activities [83]. Most proteins in blood are hydrophilic on their outside while their hydrophobic domains are turned inwards, thus serum proteins tend to bind on the hydrophobic surfaces [83], [84].

Complement components, fibrinogen, fibronectin and vitronectin have all been found to attach to the implant surfaces and elicit significant effects on the immune response [85], [86]. The complement system participates in the degradation of implants, mainly by enhancing phagocytosis of implants and attracting macrophages and neutrophils [86]. The main event in the activation of the complement system is the enzymatic cleavage of C3 into C3b and C3a, where all three complement pathways (the classical pathway, the mannose-binding lectin pathway and the alternative pathway) converge [87]. C3 can be adsorbed to the material surface, and the adsorption causes conformational changes that make C3 into a C3b-like molecule, which can bind to Bb and become C3 convertase, initiating the alternative pathway [88]. C3b molecules can themselves bind to the plasma proteins coating the material surface, triggering the alternative pathway amplification loop, which produces the majority of the C3b molecules for the normal functioning of complement system in the implant-mediating immune response [86]. Attachment of plasma fibrinogen exposes the pro-inflammatory sequence fragment D30, which can bind to the integrin Mac-1 (CD11b/CD18) on the surface of phagocytes, participating actively in the accumulation of phagocytes [85]. Fibrinogen may also convert to a fibrin-like conformation on material surfaces, facilitating the

binding and activation of inflammatory cells [85]. Plasma fibronectin is also found to participate actively in the fusion of FBGCs, thereby modulating the fibrous encapsulation of implanted materials [89]. Vitronectin can adsorb to a surface in the face of competition from other plasma proteins [90] and has been found to be a vital protein adhesion substrate for IL-4-induced FBGCs formation [91], [92]. Adsorption of these proteins from plasma onto biomaterial surfaces can bind to the integrin receptors on the surface of immune cells, activating the signaling pathways [85], [93], [94], [95]. Integrins have been characterized extensively as adhesion receptors with the capacity of transducing external signals inside cells [96]. It has been suggested that β 1 integrin is highly expressed on the surface of undifferentiated monocytes, while β 3 integrin expression upregulates upon the differentiation of macrophage [96]. During the formation of FBGCs, both β 1 and β 2 integrin-mediated adhesion are present, while β3 integrin-mediated adhesion are not detected, a process that is known to be important in mediating the adhesion of osteoclasts onto bone surfaces [97]. The depletion of β3 integrin affects the polarization of macrophages, switching the phenotype to M2 extreme [98]. Anti-β2 integrin antibodies can partly block the adhesion of macrophages to the implants, reducing IL-1 β production to basal levels, while anti- β 1 and anti- $\alpha\nu\beta$ 3 antibodies have no effect [99]. Macrophage-associated integrins also participate in regulation of BMP2 expression: anti- β 1 integrin antiserum had a relatively greater effect on macrophage BMP2 mRNA expression than did anti- β 3 integrin antiserum [75]. Therefore, integrins are likely to play an important role in transducing the signal from the matrix on implant surfaces to the immune cells, leading to the cell attachment, spread, division, and differentiation.

In response to matrix signals integrins can also transmit signals inside the cell, affecting the cytoskeleton (especially microfilaments), thereby altering cell morphology. The correlation of macrophage morphology and inflammatory cytokine production deriving from the material contact has also been investigated and macrophages with an amoeboid shape produce more TNF α , compared to macrophages with hemispherical and spherical shapes [100]. Previous studies have also associated spread morphology with the level of activation where a decrease in cell spreading indicates a reduction in the level of activation [76]. The conclusion one can draw from these findings is that surface properties affect the biological behaviors of immune cells by affecting the cytoskeleton. Cytochalasins can bind to actin filaments and block actin polymerization and elongation, thereby suppressing cytoskeleton-dependent cell shape remodeling [75], [101]. Cytochalasin D was applied to block polymerization of the actin cytoskeleton and results showed that an intact cytoskeleton was necessary for the generation of pro-inflammatory cytokine

IL-1 β [99]. Macrophages did not spread but showed a round shape in response to high concentrations (50 μ m) of cytochalasin B, which inhibited the expression of the osteogenesis-enhancing gene BMP2 [75].

Biomaterials particle size

Immune cells degrade and process particles from implants in a size dependent manner. Particles less than 0.5 μ m in size are internalized by macropinocytosis, clathrin-mediated, caveolin-mediated, and clathrin/caveolin-independent endocytosis [70], [102], whereas particles larger than 0.5 μ m are ingested by phagocytosis [70], [103]. Macrophages could phagocytose particles of up to 5 μ m [7], [13], but with larger particle size macrophages will coalesce to form FBGCs. The phagocytosis of microbial pathogens usually results in the generation of inflammatory cytokines and subsequent pathogen digestion with lysosomal enzymes [70]. However, it is still unclear whether the endocytosis of bone biomaterials can activate the inflammation response, which may vary depending on particle size and the corresponding endocytosis pathway. Poly(lactic-glycolic) acid (PLGA) does not elicit any significant immune response, although PLGA particles are readily phagocytosed by macrophages. By comparison, polystyrene latex elicits a robust release of inflammatory cytokines (TNF α , TGF- β and nitric oxide) when ingested by macrophages [104], [105].

For same amount of particles, decrease in the particle size increases the surface area and enhances chemical reactivity, thereby strengthening the effects on target cells, or even elicit a different effect altogether [102], [106]. Hydroxyapatite particles with the smallest size (1–30 μ m) stimulate immune cells to produce the greatest amount of pro-inflammatory cytokines (TNF α , IL-1 β , IL-6) [107]. Bulk gold samples are practically inert, whereas gold nanoparticles have been reported to be highly reactive for several immune responses, including production of reactive oxidative species (ROS) [108]. However, it does not follow that smaller sized particles necessarily mediate a more severe immune reaction. It has been found that large (>1 μ m) particles can induce a Th1 response, whereas particles smaller than 0.5 μ m) are associated with Th2 [70], [109]. An in vivo study has shown that a decrease in the size of irregularly shaped hydroxyapatite particles inhibits the inflammatory response [110]. A systematic examination of a range of particle sizes within each class of bone biomaterials is therefore necessary to quantify how these parameters affect the inflammatory reaction.

Porosity and pore size of biomaterials

Porosity and pore size are two key parameters for the fabrication of bone tissue engineering scaffolds, which are important in determining the ingrowth tissue types (inflammatory granuloma tissue, vascular tissue, bone tissue) [111]. Small pores may severely hamper the diffusion of nutrients and oxygen supplied from blood and interstitial fluid, especially in the center of the implant, resulting in a local hypoxic microenvironment [112]. Hypoxia could enhance local inflammation, ending up with the formation of granuloma. This would completely block the small pores, creating a barrier between the implant and the surrounding bone cells that prevents bone tissue ingrowth from taking place [113]. In addition, a hypoxic environment also favors angiogenesis and vascularization by stabilization of hypoxia-inducible factors (HIFs), which is beneficial for bone regeneration. Proper pore size should be able to induce a moderate hypoxia environment which can avoid significant inflammatory reaction but reserve the angiogenic effects. It has been found that pores ranging in the size of 90–120 μ m hamper vascularization and leads to chondrogenesis, whereas larger pores (350 μ m) enhance vascularization and results in higher oxygen tension and supply of nutrients and enhanced osteogenesis [114].

Higher porosity (80–88%) and macroporosity (pore size > 50 μ m) are thought to be more beneficial for the ingrowth of bone tissue [111]. Apart from the relevance for the behaviors of bone cells, the importance of porosity and pore size is demonstrated in the interaction of implant and host immune system [115]. It seems that with an increase in pore size, the activity of the foreign body reaction decreases [113], [116]. The underlying mechanism may be related to macrophage polarization [117], [118], [119], since there appears to be a correlation between increasing fiber/pore size and upregulated expression of the M2 markers, along with downregulated expression of the M1 markers [117].

Released ions from bone biomaterials

Bioactive bone biomaterials normally undergo degradation to different extents following implantation, either by physicochemical dissolution, cell-medicated dissolution, hydrolysis, enzymatic decomposition, or corrosion [120]. Ions released from the biomaterials during degradation can elicit significant effects by altering the local biological environment [121], [122], [123].

Calcium (Ca) is one of the major components of calcium phosphate bone biomaterials and is well documented to be involved in certain inflammatory signaling pathways [124], [125]. The noncanonical Wnt5A/Ca2+ signaling pathway is found to enhance inflammation [124]. When Wnt5A binds to Fz5 it can activate the Wnt/Ca2+ signaling pathway via Ca2+/Calmodulin (CaM)-dependent protein kinase II (CaMKII) and protein kinase C, which culminates in the upregulation of downstream inflammatory cytokine genes through the transcription factor NF-κB [126]. CaMKII, in particular, acts with the cyclic AMP-response element binding protein (CREB) in macrophages and activates cyclooxygenase-2 (COX-2) to produce the proinflammatory hormone prostaglandin E2 (PGE2) [127]. High concentration of extracellular Ca2+ has also been found to be able to activate the calcium sensing receptor (CaSR) signaling cascade leading to the production of Wnt5A, which can reduce the expression of TNF α via the inhibition of NF- κ B and downregulate the TNFR1 via the Wht5a/Ror2 signaling pathway, thereby reducing inflammation [125]. Silicon (Si) is an essential trace element for bone development [128], [129] and is found in active calcification sites during the early mineralization phase of bone regeneration [130]. Lack of dietary Si intake leads to deformities in bones [131], whereas dietary Si supplementation could suppress the bone resorption in ovariectomized animals [132]. Aqueous Si has been reported to improve the proliferation, differentiation and collagen production of osteoblasts [133], [134], [135]. Si-containing ionic products released from bioactive glass, bioceramics and coatings have similar stimulating effects on regulating the proliferation and differentiation of osteoblastic cells [136], [137], [138], [139]. A more complex response could be found in osteoclasts, with Si levels below 30 ppm enhancing the development of osteoclasts, while higher levels of Si inhibit development of osteoclasts and their bone resorption activities [134]. Si ions may also elicit an immune reaction, for example, the inhalation of silica particle is the major cause of pneumosilicosis. Nanometer sized silica has a milder fibrogenic effects than does micrometer sized silica, potentially because nanoparticles diffuse and translocate more readily compared to microparticles [140]. In addition, it is also thought that long-term exposure to components from silicone gel-filled breast implants could be related to autoimmune or inflammatory diseases, since Si is found at higher concentrations in the lesions and blood in this patient cohort [141]. On the other hand, it has been reported that the immunogenicity and biocompatibility of flat, nano-channeled, and nano-porous Si toward human monocytes are almost equivalent to tissue culture polystyrene, indicating the inertness of Si [142]. Magnesium (Mg) is a biodegradable and biocompatible metal that is mechanically similar to bone and, therefore, eliminates the effects of stress shielding and improves in vivo degradation properties [143]. Mg has been proposed as biodegradable metallic bone biomaterials for applications in orthopedics [143], [144]. Mg2+ ions can suppress inflammatory cytokine production by inhibiting toll-like receptor (TLR) pathway [145]. Macrophages recognize foreign bodies through the TLR pathway, which induce an innate immune reaction in order to degrade or reject the implants [7]. Most of the activated TLRs are bound by the adaptor protein MyD88, which then activate a downstream cascade [146]. However, TLR3 can only conduct through a MyD88-independent pathway, using the adaptor protein toll-like receptor adaptor molecule (Ticam), also known as TIR domain containing adapter inducing IFN β (TRIF), whereas TLR4 can signal through both pathways [147], [148]. Although they

produce signals via different adapter proteins, both MyD88-dependent and TRIFdependent pathways recruit NF-κB eventually, which then proceed to express inflammatory cytokines [149].

Cobalt (Co) can be used to facilitate angiogenesis by stabilizing the HIFs and subsequently activating HIF target genes such as VEGF [150], [151]. Accordingly, a number of studies have been carried in which bone substitute materials were modified with Co, resulting in the incorporation of Co into tricalcium phosphate, 45S5 bioglass[®] and mesoporous bioactive glass [152], [153], [154], [155]. These bone biomaterials showed significant enhancements of in vitro angiogenesis; however, in addition to the effects on angiogenesis, HIF was also found to have some pro-inflammatory effects. Stabilization of HIF-1 α was found to be essential for the infiltration and activation of myeloid cells in vivo through a mechanism independent of VEGF [156]. HIF-1 α is also required for the functional maturation of macrophages [157] and pro-inflammatory cytokines, such as TNF α and IL-1, could stabilize HIF-1, thereby amplifying the inflammatory response [158], [159]. It is well established that Co ions are toxic and have been implicated in the failure of joint prostheses [160], [161], [162]; its use in biomaterials is, therefore, controversial.

Zinc (Zn) has been found to stimulate bone formation and mineralization [163], [164] and dietary Zn deficiency can result in retardation of bone growth [165], [166]. Zn has therefore been incorporated into CaP biomaterials to enhance their osteogenic capacity. However, in addition to its positive effects on osteogenesis, it also regulates the immune response [167]. Zn-substituted ceramics can increase the release of anti-inflammatory cytokine IL-10, while reducing the expression of TNF α and IL-1 β , which may be due to the regulation of TLR-4 pathway [168], [169], [170], [171]. Patients with inflammatory diseases such as rheumatoid arthritis have been found to present with low blood Zn levels and a corresponding increased TNF α production [172]; the pathological process can be reversed by the supplementation of Zn [173]. Zn regulates the immune cell responses in a concentration-dependent manner: the addition of Zn salt to peripheral blood mononuclear cells grown in complete medium led to a concentration-dependent stimulation of TNF α (peaking at 250 µmol/L) [174].

Strontium (Sr) is a physiological trace element that enhances osteogenesis while inhibiting osteoclastogenesis and has been applied as a treatment for osteoporosis [175], [176], [177]. The underlying mechanism appears to be related to antagonizing the inflammatory role of NF- κ B, which suggests that Sr is an anti-inflammatory agent [178]. Studies in which Sr was introduced into Ca/P materials found that it could inhibit the release of pro-inflammatory cytokine TNF α in human primary monocytes, at both high (500 µmol/L) and low (10 µmol/L) ion concentrations [179], [180]. Sr has also been shown to promote cell proliferation and suppress the expression of the pro-inflammatory cytokines IL-6 in periodontal ligament cells [180], [181]. Bioactive ions elicit a range of effects from the immune system, which differ in composition and concentration. The strategy to manipulate the immune reaction by controlled release of defined combinations of bioactive elements is, therefore, one worthy of careful consideration. Some preliminary studies have been performed to investigate the mechanisms of how the bioactive elements affect the immune response, but much work remains to fully understand the molecular mechanisms that would provide the basic biological knowledge for the development of bioactive bone substitute materials.

Definition and evaluation strategy of the osteoimmunomodulation property of bone biomaterials.

Defining the osteoimmunomodulation property

Bone biomaterials have the capacity to modulate the local immune environment and elicit a significant effect on the functioning of bone cells, thereby determining the final outcome of bone regeneration and osseointegration. Most of the efforts related to material-mediated immune responses have focused on whether the foreign body reaction elicited by the materials would lead to excessive inflammation and rejection or encapsulation by fibrous tissue in a concept referred to as 'biocompatibility'. Immune cells also release cytokines that regulate osteogenesis, thus inducing or inhibiting bone formation. Given the importance of immune cells in bone dynamics, a novel property involving biomaterials, bone cells, and immune cells together must be defined in an effort to optimize the development of bone biomaterials. In a recent study by our laboratory, we investigated how macrophages, in response to cobalt incorporated β-TCP (CCP) stimulation, affected osteogenic differentiation of bone marrow stromal cells (BMSCs) [182]. CCP on its own could enhance the osteogenic differentiation of BMSCs, indicating from the upregulation of osteogenesis markers (ALP, OPN, OCN, and COL1). However, when macrophages were involved, the osteogenic effect was attenuated. We then carried out an in vivo study to test the accuracy of these testing methods (Fig. 5). Interestingly, results from biomaterials/bone cells/immune cells are consistent with in vivo findings and are indicative of the important role of immune cells and macrophages, in particular, in biomaterial-induced osteogenesis. It further highlights the necessity of evaluating the role of the immune response when considering the in vitro osteogenesis capacity of bone substitute biomaterials.



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Figure 5. H&E staining of rat femoral condyle defects four weeks after implantation of β -tricalcium phosphate (β -TCP) group (a, c, e) and cobalt incorporated β -tricalcium phosphate (CCP) group (b, d, f) four weeks after implantation. (a) Overview of the defect filled with β -TCP; new bone formation was observed in the defect. (b) Overview of the defect treated with CCP; no new bone formation was observed and the defect was mainly filled by fibrous inflammatory tissue. The defect size was much larger than β -TCP group. (c) New bone formation on the surface of the remaining β -TCP particle. (d) Inflammatory tissue surrounded the remaining CCP particle. (e) The defect boundary became blurred with osteoblasts forming the new bone (arrows). (f) The defect boundary was clear and surround by fibrous inflammatory tissue (arrows).

The weight of evidence from both the literature and our own studies, makes it clear that a novel property involving biomaterials, bone cells, and immune cells together must be defined and added to the system of evaluating bone biomaterials in an effort to optimize the development of such materials. Thus, we propose to name it osteoimmunomodulation (OIM) in recognition of the importance of the immune response during biomaterial-mediated osteogenesis. In contrast to biocompatibility, OIM does not simply describe the immune response in relation to the implants, but focuses more on the effects of the immune environment resulting from the interaction with biomaterials on the behavior of bone cells. OIM is a specific property that describes the ability of biomaterials to alter the local immune environment, affecting the balance of osteogenesis over osteoclastogenesis, thereby determining the in vivo fate of bone biomaterials in terms of new bone formation or fibrous encapsulation (Fig. 6). To be specific, advanced bone biomaterials with favorable OIM are those that can induce both an adequate and appropriate inflammatory response by local immune cells, which also release factors that enhance the recruitment and osteogenic differentiation of BMSCs, resulting in new bone formation. These materials must further be able to induce proper osteoclastogenesis, which is important for bone remodeling and cell-mediating materials degradation, while also avoiding excessive bone resorption. Materials with poor OIM properties will cause excessive inflammation, an imbalance of osteoclastogenesis over osteogenesis and lead to the destruction of normal bone tissue and the formation of a fibrous capsule. Such materials would, therefore, be excluded from further preclinical or clinical testing.



		Mater	rial stimu	lating immune cells	(macrophag	es)	
Phenoty (Surface	pe change markers)	Inflam cytoł	matory cines	Osteogenic factors	Osteoclas	togenic ors	Fibrosis enhancing factors
Ml	M2	Pro-	Anti-				
CCR7 CD11c	CD163 CD206	TNF-α IL-6 IL-1β IFN-γ	IL-10 IL-1ra	BMP2, BMP6, WNT10b, VEGF, OSM, TGF-β	RANKL, OPG, M-CSF, OSM, IL-6, IL-1β, TGF-β, TNF-α VEGF		TGF-β, TNF-α, VEGF
		Bone cell	s stimulat	ed by both material	and immun	e cells	
		Osteogen	esis			osteoclast	ogenesis
Signalling pathway Markers for osteogenesis		Osteoclastogenic factors	Signalling pathway Osteoclast activities		Osteoclastic activities		
BMP (BMPR2, BMPR1A, SMAD 1/4/5/8/9)	OSM (OSMR, gp130, STAT3)	ALP, O OCN, O IBSP, R	OPN, COL1, UNX2	RANKL, OPG, M-CSF,	M-CSF (c-FMS, AKT, MAPK)	RANKL (RANK NF-kB, AP-1, NFAT2)	MMP9, CA2, CTSK, TRAP, RANK, CT

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Figure 6. Contents of the osteoimmunomodulation. Different types of immune cells elicit different effects in the bone dynamics. This figure used macrophages as an example, due to its multiple roles in bone dynamics and pivotal role in the degradation of bone biomaterials. Osteoimmunomodulation refers to the immune environment that is created by the interaction of biomaterials with immune cells and bone cells. Such an immune environment contains inflammatory cytokines, osteogenic and osteoclastogenic factors, and fibriosis enhancing factors, which determines the outcome of bone biomaterial's effect on bone repair.

Evaluation of OIM will be challenging, because it involves the interaction between biomaterials, bone cells, and immune cells but can be achieved by applying a coculture system that includes all three factors. The co-culture systems are outlined below and would include indirect co-culture using conditioned medium, indirect coculture using Boyden chambers, and direct co-culture (Fig. 7).



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Figure 7. Suggested evaluation methods for the osteoimmunomodulation. All three factors (bone biomaterials, immune cells, and bone cells) should be involved in the system of evaluation. (a) Immune cells first interact with biomaterial, and then the effects of the created immune environment (conditioned medium) on osteogenesis/osteoclastogenesis are tested in bone cells. (b) Bone cells are indirectly contact with immune cells/biomaterials; the Boyden chambers allows cell migration

or molecular penetration depends on the Boyden chambers pore size. (c) Bone cells and immune cells are co-cultured on the biomaterials directly.

Indirect co-culture using conditioned medium

Immune cells are first cultured on bone biomaterials to stimulate an immune response. The conditioned medium will subsequently be applied to osteoblastic and osteoclastic cells, to determine its effects on osteogenesis and osteoclastogenesis, respectively. This model has the advantage of being a short and simple procedure [183]. Conditioned media can be frozen down so that the same batch of medium can be used for several replicates. Not only that, it can also be applied when immune cells and bone cells are from different species, thereby extending the range of applications (direct co-culture system requires the use of cells from the same species, since immune cells will react to xenogeneous cells and trigger an unwanted immune reaction). It should be noted that one potential complication, when comparing to a non-conditioned medium, is the variation in levels of other aspects of the medium (such as glucose and serum components).

Instead of playing a passive role during the interaction with immune cells, bone cells actively regulate the immune response. For example, parathyroid hormone stimulated osteoblasts can express CXCL12 and IL-7, which are known to regulate B cell development [184]. Osteoclasts have recently been found to have innate immune cell properties and participate in the systemic immune responses [185] by secreting TNF and other cytokines, such as IL-1, IL-6 and VEGF-C [186], [187], thereby auto-amplifying osteoclastogenesis and enhancing inflammation. MSCs are also recognized as having immunomodulatory properties that protects against excessive inflammation [188]. Activated MSCs can induce the alternative macrophage phenotype M2 [189], which reduces inflammation and speeds up the healing process. This mechanism involves the expression of prostaglandin E2 (PGE2) that acts on macrophages via the prostaglandin EP2 and EP4 receptors [190], [191]. They can also express TNF- α stimulated gene/protein 6 (TSG-6) and IL-1ra to decrease the amplifying effects of pro-inflammatory cytokines (IL-1, IL-6, and TNF- α) [192], [193]. Therefore, indirect co-culture using Boyden chambers or direct co-culture can better mimic the in vivo environment, in relation to the interaction between bone cells and immune cells.

Indirect co-culture using Boyden chambers

The initial phase of the biomaterials/host body reaction is typically an acute inflammation that results in immune cells attaching to the surface of the materials before bone cells, which therefore elicit a greater effect during the early stages of biomaterials mediated osteogenesis. For this reason, in an indirect co-culture system,

the immune cells can be cultured in indirect contact with the materials and the bone cells in a Boyden chamber insert. The small pore size (such as 0.4 μ m) of the insert keeps the bone cells within the insert but allows secreted molecules to flow freely. Such an approach allows for the study of the molecular communications that results from the interaction between bone cells and immune cells in relation to the bone biomaterials. Changes to bone cells and immune cells, including gene and protein expression, can be investigated separately, which allows for greater resolution in unraveling the underlying mechanisms. By using larger pore sizes (3.0 μ m or larger depending on the cell size), the migration of bone cells can also be investigated, to study the effect of chemoattractant that results from stimulated immune cells. Direct co-culture

Direct co-cultures enable both immune and bone cells to be in direct contact with the materials at the same time, which would be the case in an in vivo environment. It can be performed by layering one cell type on top of another, although this method is fraught with technical difficulties of which reproducibility of results is the most prevalent [194]. It requires the involvement of cells sourced from one individual patient, especially when immune cells are involved. Primary culture of whole bone marrow tissue derived from surgery or biopsy may meet the requirement of providing various types of cells from different tissues, including skeleton and immune systems. Inter-patient variability and limited source of suitable donor tissues makes it difficult to establish this technique as the standard of in vitro evaluation. Another limitation is separating the effects of the different cell types. The cells could be sorted by fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) and then evaluated by gene expression, but this is fraught with difficulties. Also, the proteins in the media cannot be ascribed to one particular cell type. Indirect co-culture using Boyden chambers may, therefore, be the most suitable and reproducible evaluation system for the assessment of OIM. Possible evaluation process of osteoimmunomodulation

As has been pointed out throughout this review, immune cells play a number of roles during the various phases of bone wound healing. This implies that the evaluation of OIM must be phase-oriented. During the early phase, acute inflammation reaction is predominant and the induced immune environment will decide the recruitment of MSCs and initiation of osteogenesis. There are four major events that takes place during the early phase: (1) the activation of putative inflammation related signaling pathways in response to the implants; (2) release of inflammatory cytokines responsible for the acute inflammation; (3) release of chemokines and their effect on the recruitments of MSCs; and (4) release of factors that regulate osteogenesis and fibrosis, which ultimately will determine the outcome of new bone regeneration.

The mid phase of the repair response is characterized by three major events: (1) activation of osteogenic signaling pathways in response to the factors released during the early stage; (2) osteogenic differentiation of MSCs that determines the osteogenesis; and (3) release of osteoclastogenic factors from both immune and osteoblast cells that accelerates bone remodeling and degradation of the remaining biomaterials in the late phase.

During the late phase of the repair response the induced immune environment enhances osteoclastogenesis. There are two major events taking place during the late phase: (1) activation of osteoclastogenic signaling pathways in response to factors released during the mid-phase; (2) differentiation of osteoclasts and enhanced activity shift the overall balance from osteogenesis toward osteoclastogenesis. Should there be a failure to activate osteoclastogenesis during this phase this may prevent the timely degradation of biomaterials and impede a proper integration between bone and materials.

Possible strategies to endow advanced bone biomaterials with favorable osteoimmunomodulation

It has emerged from both our own and other studies that bone biomaterials elicit significant effects on the immune system. The immune response determines subsequent osteogenesis and osseointegration and is a reflection of the importance of the immune system to both normal and pathological bone physiology. Immune cells, such as macrophages, have a high degree of plasticity, which makes it possible to modulate the immune response by manipulating the materials' composition or structure. Potential strategies for such manipulations are (Table 2): (1) modifying the surface topology of materials (size and roughness) – material surfaces that provide biomimetic cues, such as nanoscale structures, have been found to regulate cell/biomaterial interactions [80], [195]; (2) modifying the surface chemistry of materials (hydrophility and electric potential) – macrophages cultured on surfaces with different hydrophilicity results in different protein expression profiles and cytokine responses [66]; (3) changing the particles size – the immune response varies with the change of material sizes, even though the composition remains unchanged; (4) optimizing pore size and porosity – a uniform $30-40 \mu m$ pore size appears to promote the polarization toward the M2 phenotype [118], whereas non-porous or random-porous-sized materials appear to favor the M1 phenotype [196]; (5) incorporating nutrient elements [148], [197] – the immune response can be manipulated by a combination of bioactive elements (e.g. Ca, Mg, Sr, Co, Zn) in a controlled-release manner; (6) incorporating bioactive molecules [198] such as macrophage inducers (e.g. IL-4, LPS) or inflammatory cytokines (e.g. IL-10, TNF α , IFNγ); and (7) coupling with immunomodulatory drugs [199], [200], for example

inflammatory modulation drug (steroid and non-steroidal anti-inflammatory drugs). These strategies should all be considered when designing biomaterials, since their combination can have synergistic effects. Needless to say, a better understanding of the mechanisms underlying the immune response and their effects on osteoclastogenesis and osteogenesis is essential for developing advanced bone biomaterials with favorable OIM properties (Table 3).

Table 2. Possible strategies to endow bone biomaterials with favorableosteoimmunomodulation.

Strategies	Methods	Examples
	Incorporate nutrient elements	Different combinations of nutrient elements (e.g. Mg, Sr, Si) have been applied to manipulate OIM[148],[202].
Modify the composition	Incorporate bioactive molecules, such as macrophage inducer (e.g. IL- 4, LPS) or inflammatory cytokines (e.g. IL-10, TNF α , IFN γ , OSM)	OSM has received great attention in osteoimmunology area, due to its dual effects on regulating both osteogenesis and osteoclastogenesis[28]. Incorporating proper dose of OSM can be a valuable strategy to direct bone regeneration.
Optimize the	Immune reaction can be manipulated by changing the particle size: from bulk, particle, powder, microscale, to nanoscale.	Inert materials such as gold can become immunogenic when it is fabricated into nano particles[108].
fabrication form	For those fabricated into 3D structured scaffolds, pore size and porosity should be optimized.	A uniform $30 - 40 \mu$ m pore size appears to promote the polarization toward the M2 phenotype[118], whereas non-porous or random-porous- sized materials appear to favor the M1 phenotype[196].
Change the surface properties	Modify the surface topology of materials (size and roughness)	Material surfaces that provide biomimetic cues, such as nanoscale structures, have been found to regulate cell/biomaterial interactions[80],[195].

Strategies	Methods	Examples
	Modify the surface chemistry of materials (hydrophility and electric potential)	Macrophages cultured on surfaces with different hydrophilicity results in different protein expression profiles and cytokine responses[66].
Others	Couple with immunomodulatory drugs	The local administration of anti- inflammatory drug (aspirin) enhances the bone tissue regeneration outcome via regulating the immune response toward implanted stem cells and bone substitute materials[53].

Abbreviation	Name	Explanation
TNF α	Tumor necrosis factor	
IFN γ	Interferon γ	Pro-inflammatory cytokines.
IL-1 β ,6	Interleukin 1 β , 6	
IL-1ra, 10	Interleukin-1 receptor antagonist, 10	Anti-inflammatory cytokines.
ROS	Reactive oxygen species	Natural byproducts from normal metabolism of oxygen
NF- κ B	Nuclear factor kappa- light-chain-enhancer of activated B cells	NF- κ B is a major transcription factor that regulates genes responsible for the enhancement of inflammation.
ΙκΒ-α	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	The major function is to inhibit the NF- κ B transcription factor.
RANTES	Regulated on activation, normal T cell expressed and secreted; also known as CCL5	Chemokines, participate in cell migrations.

Table 3. Glossary of proteins or cytokines appeared in this review.

Abbreviation	Name	Explanation
	Epithelial-derived	
ENA-78	neutrophil-activating	
	peptide 78; also known as	
	CXCL5	
MTNF α Tumor		
necrosis factor	Monocyte chemotactic	
Pro-	protein 1; also known as	
inflammatory	CCL2)	
cytokines.CP-1		
TT / / A	Wingless-related MMTV	
Wnt5A	integration site 5A	
Fz5	Frizzled-5	Wnt5A/Ca ²⁺ signaling pathway
	Calmodulin (CaM)-	components.
CaMKII	dependent protein kinase	
	II	
		CaSR signaling cascade leading to
CaSR	Calcium sensing receptor	reduce inflammation.
	Receptor activator of	
KAINKL	ligend	RANKL, an osteoclastogenic
	ligaliu	factor, binding to RANK, signaling
AP-1	Activator protein 1	through AP-1 and NFAT2 to
	Nuclear factor of activated	enhance osteoclastogenesis.
NFAT2	T cells 2	
	Myeloid differentiation	
MyD88	primary response gene 88	Toll life appendix (TI D) and
		1 OII-IIKE RECEPTOR (1LK) pathway
Ticam	Toll-like receptor adaptor	components.
	molecule	
HIFs	Hypoxia-inducible factors	Transcription factors that respond
	http://a madelole lactors	to hypoxia microenvironment.
MORE	Macrophage colony-	MCSF, an osteoclastogenic factor.
IVICOL	stimulating factor	MCST binds to c-FMS, signaling

Abbreviation	Name	Explanation
c-FMS	Colony stimulating factor 1 receptor	through AKT and MAPK to enhance osteoclastogenesis.
AKT	Protein Kinase B	
МАРК	Mitogen-activated protein kinases	
OPG	Osteoprotegerin	A decoy receptor for RANKL, inhibiting osteoclastogenesis.
TRAP	Tartrate-resistant acid phosphatase	TRAP is largely expressed in osteoclasts and activated macrophages.
CTSK	Cathepsin K	It is a lysosomal cysteine protease involved in bone remodeling and resorption, which is expressed mainly in osteoclasts.
CA 2	Carbonic Anhydrase II	CA 2 plays an important role in osteoclast differentiation and bone resorption by regulating the homeostasis of intracellular pH and Ca ²⁺ .
CT	Calcitonin receptor	CT binds the peptide hormone calcitonin, involving in bone formation and metabolism.
MMP9	Matrix metalloproteinase- 9	MMP9 is a protease involving in breaking down bone matrix.
TGF β 1/3	Transforming growth factor	Enhance the formation of fibrocapsule.
VEGF	Vascular endothelial growth factor	An angiogenic factor.
WNT10b	Wingless-related MMTV integration site 10b	A member of WNT family. Can activate canonical WNT pathway and enhance osteogenesis.

Abbreviation	Name	Explanation	
BMP2/6	Bone morphogenetic protein 2/6	An osteogenic factor.	
BMPR2	Bone morphogenetic protein receptor type II		
BMPR1A	Bone morphogenetic protein receptor, type IA	BMP2 signaling pathway	
SMAD 1/4/5	Mothers against decapentaplegic homolog 1/4/5	components	
OSM	Oncostatin M	An inflammatory cytokine; also a regulator of osteoclastogenesis and osteogenesis.	
OSMR	Oncostatin M receptor		
gp130	Glycoprotein 130	OSMR, A receptor of OSM. Signaling through gp130 and STAT3 to enhance osteogenesis.	
STAT3	Signal transducer and activator of transcription		
ALP	Alkaline phosphatase	ALP is a byproduct of osteoblast activity. ALP increases when active bone formation is occurring.	
OPN	Osteopontin	An early indicator of osteogenic differentiation.	
OCN	Osteocalcin	OCN is supposed to be expressed by more mature osteoblastic phenotypes. Therefore, it is a late indicator of osteogenic differentiation.	
COL1	Collagen type 1	COL1 is another marker of osteogenic differentiation, which will be increased when the osteogenesis is enhanced	

Abbreviation	Name	Explanation
		IBSP is a significant component of
IBSP	Bone sialoprotein 2	the bone extracellular matrix.

Of these strategies, modifying the biomaterials with nutrient elements may have the greatest potential, not least because nutrient elements are fundamental for human physiology. The integration of nutrient elements into biomaterials has already been widely applied to modify materials in an effort to improve their bioactivity. For example, incorporating strontium into bioactive glasses inhibits osteoclast activity and enhances the osteogenesis and has, therefore, expanded the application of hydroxyapatite to patients with osteoporosis [177], [201]. The scientific literature clearly reveals that nutrient elements can elicit significant effects on immune response regulation depending on the concentration and combination of the various elements. The implication of this is the possibility of finding an optimal combination of nutrient elements such that one can obtain an advanced bone biomaterial with multiple functions, one of which is OIM. Based on this strategy, we recently combined the elements Sr, Mg, and Si, to fabricate two novel bioceramic coating materials (Sr2MgSi2O7, MgSiO3). Both materials were found to endow the inert titanium substrate (Ti-6Al-4V) with favorable OIM and reduce inflammation and osteoclastogenesis, while maintaining or enhancing osteogenic capacity compared with hydroxyapatite coated materials [148], [202]. These are promising results that highlight the feasibility of this strategy and also that the design and preparation of bone biomaterials must be done with OIM properties in mind. Conclusions

Bone biomaterials can determine an immune response. The type of response is related to the properties of the biomaterials, such as surface topography, particle size, porosity and pore size, and ion release. Components from the degraded materials and released molecular signals from the interaction between immune system and implants, significantly affect the biological behaviors of bone cells, thereby determining the bone regeneration outcome. Accordingly, OIM is proposed to define this process, which involves the interaction between biomaterials, immune cells and bone cells, thus emphasizing the importance of immune cells during the biomaterial-mediated osteogenesis. Biomaterials with the appropriate OIM can create an immune environment that enhances osteogenesis, and regulates proper osteoclastogenesis that can participate in the bone remodeling and cell-mediated materials degradation. Such a property is important for the development of advanced immunomodulatory bone biomaterials. Biomaterials with poor OIM may

cause excessive inflammation and lead to an imbalance of osteoclastogenesis over osteogenesis; such materials would, therefore, be excluded from further tests. Future studies should focus on determining the kind of immune environment that is conducive for osteogenesis and osseointegration, thus providing a set of design aims for the modification of bone biomaterials. Understanding how the biomaterials modulate the immune environment can guide the development of modification strategies, and integral to this enterprise is a systematic evaluation of modification strategies to explore an optimized modification strategy to endow advanced bone biomaterials with favorable OIM.

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