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A practical guide for evaluating the osteoimmunomodulatory properties of biomaterials

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ABSTRACT

Biomaterials offer a promising approach to repair bone defects. Whereas traditional studies predominantly focused on optimizing the osteogenic capacity of biomaterials, less focus has been on the immune response elicited by them. However, the immune and skeletal systems extensively interact, a concept which is referred to as 'osteoimmunology'. This realization has fuelled the development of biomaterials with favourable osteoimmunomodulatory (OIM) properties, aiming to modulate the immune response and to support bone regeneration, thereby affecting the success of an implant.

Given the plethora of *in vitro* assays used to evaluate the OIM properties of biomaterials, it may be challenging to select the right methods to produce conclusive results. In this review, we aim to provide a comprehensive and practical guide for researchers interested in studying the OIM properties of biomaterials *in vitro*. After a concise overview of the concept of osteoimmunology, emphasis is put on the methodologies that are regularly used to evaluate the OIM properties of biomaterials. First, a description of the most commonly used cell types and cell culture media is provided. Second, typical experimental set-ups and their relevant characteristics are discussed. Third, a detailed overview of the generally used methodologies and readouts, including cell type-specific markers and time points of analysis, is given. Finally, we highlight the promise of advanced approaches, namely microarrays, bioreactors and microfluidic-based systems, and the potential that these may offer to the osteoimmunology field.

Statement of Significance

Osteoimmunology focuses on the connection and communication between the skeletal and immune systems. This interaction has been recognized to play an important role in the clinical success of biomaterials, which has resulted in an increasing amount of research on the osteoimmunomodulatory (OIM) properties of biomaterials. However, the amount of literature makes it challenging to extract the information needed to design experiments from beginning to end, and to compare obtained results to existing work. This article intends to serve as a guide for those aiming to learn more about the commonly used experimental approaches in the field. We cover early-stage choices, such as cell types and experimental set-ups, but also discuss specific assays, including cell markers and time points of analysis.

1. Introduction

1.1. The concept of 'Osteoimmunology'

The interplay between the skeletal and immune systems was early identified by the role of immune cells in the regulation of osteoclastic activity [1–4], and later coined as osteoimmunology

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by Arron and Choi when demonstrating that T lymphocytes regulate osteoclast activation [5]. Since then, osteoimmunology has evolved into an active field of research, addressing the cross-talk between immune and skeletal systems, aiming to improve strategies for bone healing.

From a physiological perspective, bone provides the microenvironment for the development of hematopoietic stem cells (HSC), which give rise to cells of the immune system [6]. Despite the ability of bone to remodel or repair, both processes can be impaired by severe trauma or diseases. In case of severe bone defects that cannot heal intrinsically, so called 'critical size defects', biomaterials may be used. Biomaterials can replace the bone defects, but can also be used in the form of resorbable implants that enhance bone regeneration.

Many biomaterials that performed promisingly in the setting of reductionist *in vitro*-models have never made it into clinically applicable implants [7]. This is mostly due to their disappointing *in vivo*-behaviour, which, quite often, is related either to insufficient stimulation of osteogenic cells or to triggering of inflammatory reactions and excessive bone resorption (*i.e.* osteolysis) [8]. Optimally, biomaterials should synergistically stimulate both immune and skeletal cells towards successful bone healing and avoid stimulation of detrimental inflammatory pathways that may lead to bone resorption.

1.2. Motivation and scope of the review

Knowledge of the complex interactions between the immune and skeletal systems upon biomaterial implantation is still only rudimentary. In addition, the methodologies to evaluate such complex biological events are heterogeneous and difficult to standardize. Proof of that is the available literature on the osteoimmunomodulatory (OIM) potential of specific biomaterials [6,9–12]. The aim of this review is to provide practical guidelines for the *in vitro* evaluation of biomaterials in terms of their OIM properties. The different approaches used to culture immune and skeletal cells are discussed, after which special attention is given to the specific experimental settings used to evaluate the OIM properties of biomaterials. This includes suggestions for potential advanced methods that could be further explored in the future to deepen the understanding of the complex triad: biomaterial, immune system, and skeletal system.

The methodologies described in this review are a fair representation of those performed to evaluate the OIM properties of biomaterials. The included articles were selected based on a structured online search in three scientific databases (Web of Sciences, Scopus, and PubMed). The search was restricted to articles written in English and covering the *in vitro* evaluation of the OIM properties of biomaterials, using both immune and skeletal cells. Articles in which the *in vitro* studies did not comply with the strict definition of osteoimmunology were excluded. Moreover, articles investigating particles instead of bulk materials and articles investigating drug-loaded biomaterials were also excluded, since the methodologies may differ substantially from bulk biomaterials. *In vivo* studies were beyond the scope of this review and were therefore not included.

2. Background knowledge

2.1. Bone remodelling and fracture healing

Bone turnover is a constant and lifelong process that is maintained by the balance between mainly osteoblasts (bone depositing cells), and osteoclasts (bone resorbing cells). However, the cross-talk between these cell types is strongly orchestrated by immune cells. For instance, fracture healing is initiated by the recruitment of immune cells such as granulocytes, lymphocytes and macrophages, within the fracture hematoma, orchestrating the subsequent chondro- and osteogenic responses [13]. Physiological bone turnover can be hampered by several diseases which mainly involve an altered activity of immune or skeletal cells such as osteoporosis, hyperparathyroidism, and immune diseases [14–18]. These diseases often lead to a reduced bone mineral density that increases the risk of fractures. Furthermore, non-union after long bone fractures can occur in up to 10% of clinical cases [19], and in open tibial fractures they can be as common as 40% [20]. In such cases, the restoration of bone homeostasis and bone function is sometimes overcome by the implantation of synthetic bone grafts. When this happens, bone remodelling has a new actor in play, the biomaterial. Hence, the importance of understanding how biomaterials can modulate bone remodelling by favourably interacting with both immune and skeletal systems.

When a biomaterial is implanted in bone, a hematoma will appear and the immune system will be activated, leading to overlapping stages of inflammation, bone formation and later remodelling [21]. The first event, inflammation, occurs by the formation of a blood clot around the biomaterial, and the adsorption of proteins at the biomaterial surface. These first events are highly driven by the immune system.

2.2. Immune cells in the remodelling process

Several leukocytes (T lymphocytes, B lymphocytes, dendritic cells (DC), natural killer (NK) cells, macrophages, monocytes, and neutrophils) hosted in bone [22] are involved in the inflammation process as well as in the subsequent bone repair and remodelling stages after biomaterial implantation. Upon biomaterial implantation, lymphocytes, monocytes and neutrophils arrive at the wound site from circulating blood. These cells are stimulated by pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), and interleukins (IL-1, and IL-6) that are secreted by the platelets aggregated around the hematoma. Neutrophils clear damaged cells and debris [23] and become apoptotic within hours to days, while monocytes become adherent and turn into macrophages. Macrophage differentiation from mononuclear cells is highly dependent on colony stimulating factor 1 (CSF-1) [24] released by osteoblasts. In addition, the chemical balance of factors such as reactive oxygen and nitrogen species (ROS, and NOS, respectively) dictate the first response to inflammation, and the modulation towards repair and remodelling is signalled by the phenotype changes of macrophages.

Macrophages are key modulators of both inflammation and bone remodelling. Enormous attention has been paid to their role in healing, especially their plasticity to adapt to exogenous stimuli that can influence the healing cascade [10,25–28]. In addition, macrophages are responsible for guiding the soft-to-hard callus formation through the release of matrix metalloproteinases (MMPs) [29]. Upon biomaterial implantation and eventual degradation and debris, macrophages are able to engulf particles below 5 µm [30] or fuse together into foreign body giant cells (FBGC) to engulf particles up to 100 µm [31]. In addition, macrophages can adopt different phenotypes upon activation through toll-like receptors (TLRs) and nuclear factor-κB (NF-κB) [32], known as dynamic polarization. The acquired phenotypes can broadly be categorized into pro-inflammatory or anti-inflammatory, often referred to as M1 and M2, respectively. Both phenotypes are needed for successful healing; key being the timing of the switch between them, which depends on the composition and kinetics of the total biochemical milieu they are exposed to. M1 macrophages are predominant during the early stages of acute inflammation, they perform cleaning duties, release oxidative metabolites [28], and pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, and ROS

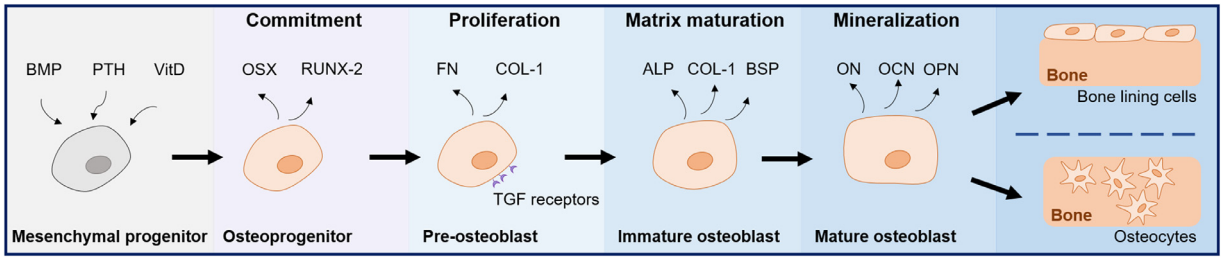

Fig. 1. MSCs formation and differentiation into osteogenic lineage and the different stages of osteoblasts maturation and differentiation [62,67].

[33]. In opposition, M2 macrophages, which are predominant during later stages of fracture healing and remodelling, express important anti-inflammatory cytokines such as IL-4 or IL-10, or enzymes such as arginase-1. Nowadays, M2 macrophages are divided in 4 subsets depending on their role during repair (Table 1). Generally, they are classified as M2a, stimulating fibroblast and extracellular matrix formation (ECM) [34,35]; M2b, responsible for balancing the inflammatory process [36]; M2c, responsible for matrix renovation and vascularization [37,38]; and M2d, important pro-angiogenic modulators [39].

Macrophage polarization towards M1 or M2 phenotypes is influenced by biomaterials [40,41]. More specifically, porosity [42,43], surface nanotopography [44] and roughness [45–48], surface chemical cues [49–51], mechanical cues [52], and biomaterial dimensions/geometry [53] modulate inflammation and bone healing. For instance, neutrophils, as early colonizers of fracture sites, have been suggested to be relevant modulators of the anti-inflammatory switch in macrophages [54]. The time switch from M1 to M2, driven by IL-4 stimulation, has been shown to be crucial for enhanced osteogenesis at later periods [55]. Prolonged presence of M1 macrophages and their characteristic cytokines may result in chronic inflammation and lead to fibrous encapsulation. However, under physiological conditions, M1 macrophages have been found to promote bone formation, especially in trabecular bone sites stimulated by receptor activation of NF-κB ligand (RANKL) [56]. Several reviews on the OIM potential of biomaterials are available for more detailed information on this topic [40,57,58].

2.3. Bone cells in the remodelling process

Four main cell types are naturally residing in bone tissue: bone lining cells, osteocytes, osteoblasts and osteoclasts. The most studied cells in terms of osteoimmunology with regards to interactions with biomaterials are osteoblasts, and their precursors, mesenchymal stem cells (MSCs). The relevance of MSCs lies in their active cross-talk with the immune system and their ability to further modulate inflammation and repair. Through the release of chemo- and cytokines and their receptors, immune cells enable the migration and activation of MSCs to the injured site [59]. Interferon gamma (IFN-γ), TNF-α, IL-1, IL-8, IL-17, as well as TLRs [60] are the main responsible proteins for MSC migration and activation. At later stages of inflammation, MSCs are responsible for controlling the influx of regulatory T cells, a subpopulation of T lymphocytes known for their immune regulatory potential, to the injured site. MSC induce their apoptosis, downregulate B cells, NK cells and DC, and finally, modulate macrophage response [61].

Successful bone repair and remodelling entails the differentiation of MSCs into the osteogenic lineage at later stages. This differentiation into the osteogenic lineage can be systemically stimulated via parathyroid hormone (PTH) [62], or vitamin D3 [63] (Fig. 1). Secondly, factors such as bone morphogenetic proteins (BMPs), insulin growth factor (IGF) or transforming growth factor beta (TGF-β) can also stimulate the differentiation of MSCs into the osteoblastic lineage [64,65]. The common mechanism for osteoblast differentiation is through autocrine pathways via Wnt-catenin, Runt-related transcription factor 2 (RUNX-2) and osterix (OSX) [66]. The expression of RUNX-2 and OSX transcriptional factors in MSCs represents their commitment towards the osteogenic lineage. Subsequently, committed pre-osteoblasts proliferate and secrete fibronectin (FN), collagen proteins (*e.g.* type I, COL-1) and express TGF-β receptors. In a second stage, pre-osteoblasts differentiate into immature osteoblasts and continue secreting COL-1, together with alkaline phosphatase (ALP) and bone sialoprotein (BSP) to trigger bone deposition [67]. Finally, mature osteoblasts release osteonectin (ON), osteocalcin (OCN) and osteopontin (OPN) proteins, which regulate calcium binding and mineralization allowing the formation of calcium deposits. Although not implicitly studied when evaluating the OIM potential of biomaterials, bone lining cells and osteocytes (both derived from mature osteoblasts, Fig. 1) are natural 'switches' to initiate and mediate bone remodelling process [68–70]. Osteocytes, which are osteoblasts engulfed by unmineralized osteoid, can sense and respond to mechanical loads, identify microdamages in bone tissue and secrete cytokines to activate the healing cascade [71]. Bone lining cells are also relevant during the coupling of bone resorption and bone deposition, enabling new bone formation by the deposition of fibrillar collagen, which is later mineralized by osteoblasts [72].

Osteoclasts, which are the cells responsible for the catabolic activity in bone tissue, play a crucial role in osteoimmunology, although they are until now mostly disregarded in studies on this topic. Osteoclastogenesis occurs upon the stimulation of mononuclear precursors from the immune system by paracrine factors secreted by leukocytes, osteoblasts or MSCs [73,74]. Osteoclast formation is highly dependent on macrophage colony stimulating factor (M-CSF) and receptor activation of NF-κB ligand (RANKL), together with osteoprotegerin (OPG) antagonist, factors secreted by osteoblasts and MSCs. Usually, bulk biomaterials that do not elicit prolonged inflammation, lead to the formation of osteoclasts, after which extracellular degradation will take place [57]. Osteoclast-mediated degradation balanced with new bone growth is the desired scenario for resorbable biomaterials. However, exacerbated osteoclast activity surrounding biomaterials may lead to reduced contact between bone and implant and eventually osteolysis [75]. Although beyond the scope of this review, several studies have investigated biomaterial-modulated osteoclastogenesis [76–80].

More recently, bone residing macrophages, known as osteomacs, have gained interest. These cells are particularly interesting due to their versatile functions, including phagocytosis, detection of bacteria, activation of bone remodelling and modulation of osteoblast activity [27,81].

3. The basics of cell culture studies

3.1. Cell types

In research, cell models are used to evaluate cellular interactions with a biomaterial. The type of cells and their origin are the first decisions that have to be made when planning an experi-

Table 1
Summary of macrophage characteristics, functions, inducers, typical surface markers and the most important released cytokines [34,37,39].

Type	Function	Stimulation/Inducers	Surface markers	Secreted cytokines
M1	Pro-inflammatory, microbial and tumoral activity, tissue damage	IFN-γ, TNF-α, LPS	CD80, CD86, TLR-2, TLR-4, iNOS, MHC-II	TNF-α, IL-1α, IL-1β, IL-6, IL-12, IL-23
M2a	Endocytic activity, cell growth, tissue repair	IL-4, IL-13	CD206, FIZZ1, Arg-1, Ym1/2, IL1R	TNF-α, IL-1α, IL-1β, IL-6, IL-12, IL-23
M2b	Immunoregulatory (Th2 differentiation)	IL-1β, TLR ligands, immune complex	CD86, MHC-II	TNF-α, IL-1α, IL-1β, IL-6, IL-10
M2c	Tissue repair (phagocytosis of apoptotic cells)	TGF-β, IL-10, glucocorticoids	CD163, CD206, CD86, Arg-1, TLR-1, TLR-8	IL-10, TGF-β
M2d	Pro-angiogenic	TLR antagonists, IL-6	CD163, IL-10R, IL-12R, CD14, CD16	IL-10, IL-12 TGF-β

ment. While the selection of a cell type depends on the application planned for the biomaterial, choosing their origin may be less straightforward. Generally, two main groups can be defined: primary cells and cell lines. Primary cells are isolated from human or animal tissue and therefore represent more realistic and complex physiological behaviour. However, these cells are more sensitive and can only be used for a relatively short number of passages, since they undergo senescence processes and have limited potential for self-renewal and differentiation. In contrast, cell lines are derived from genetically modified tissue or spontaneous tumours, which grants them immortality and offers unlimited amounts of cells, while bypassing ethical concerns associated with the use of animal and human tissue. Other advantages related to cell lines are their low cost, easy manipulation and possibility to keep them in culture for longer periods. However, the main problem associated with cell lines is that their phenotype is modified, meaning native functions and cell response to stimuli may also be altered. For this reason, cell lines rarely replicate the behaviour of primary cells, which causes controversy regarding the relevance of the results obtained although cell lines are widely used [82]. The general recommendation is to use cell lines as a tool to set up experiments and afterwards validate the results with primary cells.

Multiple cell types within the family of immune and skeletal cells, both primary and cell lines, have been used to study the OIM properties of biomaterials. Among the immune cells, the commercially available monocyte/macrophage cell line RAW 264.7 has been used in the majority of studies in the field. The main reasons explaining this choice are that macrophages are one of the primary immune cells involved in osteoimmunology and that this particular cell line, is considered an appropriate model of macrophages [83]. As alternative cell lines, a porcine macrophage 3D4/21, a murine monocyte/macrophage J774A.1, human monocyte such as U937 and THP-1, and a murine-derived dendritic cell line DC2.4 have been used. Apart from these cell lines, a variety of primary immune cells can be isolated from peripheral blood mononuclear cells (PBMCs), the most common source being buffy coats. These include monocytes (used either directly after isolation or after further differentiation towards macrophages), NK cells and T lymphocytes. In the case of monocyte-derived macrophages, different purification methods have been used, such as plastic adhesion and magnetic bead-based immunoisolation kits to for instance isolate CD14+ cells. Noteworthy, the isolation methods can influence the cells' phenotype, subsequently altering the results of the experiments performed [84]. A summary of the immune cells that are typically used to assess the OIM properties of biomaterials is displayed in Table 2.

Regarding the skeletal cells, two main cell types have been used: primary MSCs and bone cells, which are either directly isolated from human or animal tissue (primary cells), or cell lines that are commercially available, as previously explained. The most commonly used skeletal cells are bone marrow MSCs isolated from humans. Among the bone cells, osteoblasts clearly dominate the studies over osteoclasts and osteocytes, which are both in fact almost absent in the field. The osteoblast-like cell lines used are from both human and animal origin, mouse cells being the most common source for animal cells. Similar to immune cells, MSCs' potential for differentiation into an osteogenic lineage and subsequent mineralization relevant to study OIM is dependent on several factors, such as patient-related factors, harvest site (femur or iliac crest) and isolation methods (*e.g.* enzymatic digestion or explant outgrowth) [85]. A compilation of the different skeletal cells used in studies testing the OIM properties of biomaterials is presented in Table 3. Noteworthy, angiogenesis, another crucial process during bone regeneration, is also triggered by immune and skeletal cells. The role of endothelial cells in osteoimmunology has been mainly studied using a human umbilical vein endothelial cell (HUVEC cell

Table 2
Overview of the immune cell types that are typically used to evaluate the osteoimmunomodulatory properties of biomaterials, including their origin and typical cell culture medium supplements.

			Fetal bovine serum (FBS)							Human plasma
			Regular FBS			Heat-inactivated FBS			N.I.	Regular plasma
	Cells' origin	Cells type (name)	10%	20%	N.I.	5%	10%	N.I.		10%
Primary cells	Human buffy coat	Monocytes	[186]				[100]			[50]
		Macrophages derived from monocytes					[100]			
		Neutrophils								[50]
		Peripheral blood mononuclear cell (PBMC)					[100]			
		Natural killer (NK) cells					[100]			[122]
		T cells								[122]
		B cells	[122]							
	Mouse	Pan T lymphocytes							[187]	
Cell lines	Human	Monocytes (U937)	[109]		[112]					
		Monocytes (THP-1)	[113,114,116]							
	Mouse	Macrophages (RAW 264.7)	[87–90,113,118,121,134–137,139]	[126]		[86,98,123,133]	[48]		[110]	
		Monocytes/macrophages (J774A.1)	[107]							
		Dendritic cell line (DC2.4)	[124]							
	Porcine	Macrophages (3D4/21)	[115]							

N.I. not indicated.

Table 3
Overview of the skeletal cell types that are typically used to evaluate the osteoimmunomodulatory properties of biomaterials, including their origin and typical cell culture medium supplements.

			Fetal bovine serum (FBS)				
			FBS			Heat-inactivated FBS	
	Cells' origin	Cells type (name)	10%	20%	N.I.	10%	N.I.
Primary cells (MSCs)	Human bone marrow	MSCs	[86,87,90,100,110,113,114,116,121,123,125,133,139,186]	[126]			
	Cord blood	MSCs	[122]				
	Human teeth	MSCs (SHED)			[187]		
	Rat bone marrow	MSCs	[50,88,98,136]		[134]		
	Mouse bone marrow	MSCs	[137]				
	Pigs (tissue source N.I.)	MSCs	[115]				
	N.I.	Osteoblasts	[107]				
Cell lines (bone cells)	Human	Bone osteosarcoma (MG-63)	[109]		[112]		
		Osteoblast (Saos-2)				[48]	[110]
	Calvaria murine	Pre-osteoblast (MC3T3-E1)	[89,99,118,124,135]				

SHED: stem cells from human exfoliated deciduous teeth; N.I. not indicated.

lines) [86–90]. However, the detailed evaluation of methods using endothelial cells was beyond the scope of this review.

3.2. Cell culture medium

Cell culture medium supports cell survival, proliferation and a variety of cellular functions. Since each cell type has specific growth requirements, it is crucial to choose the right cell culture medium, as this will directly affect the cells' performance and therefore the results of a study. Today, in the majority of scientific studies, commercially available culture media is used. It is generally accepted that most adhesive cells can be cultured with a single basal medium of the family of Eagle media or Ham media, whereas RPMI-1640 medium is often used in suspension cultures [91]. For this reason, choosing the right culture media for osteoimmunology-related co-cultures using both suspension and adherent cells may be an extra challenge. This may require optimizing the combination of media to ensure the best cell growth for both cell types [91,92], as is further elaborated on in section 4.1.2, 'Indirect co-culture'.

Equally important as choosing the correct basal medium is selecting the right supplemental components to maintain the cells. This mainly includes, but it is not restricted to, an antibiotic solution (*e.g.* penicillin/streptomycin) and a serum. Although the use of antibiotics is common in research, especially when working with cell lines, concerns regarding their potential to alter the gene expression and regulation of cells have been raised. This should be especially taken into account when performing genetic, genomic or other biological assays involving cell cycle regulation, differentiation, and growth [93]. Regarding the serum, the most popular one nowadays is foetal bovine serum (FBS), which, among other compounds, consists of amino acids, proteins, vitamins, carbohydrates, lipids, hormones and growth factors. FBS promotes the proliferation of cells and is commonly added to cell cultures in studies that evaluate the OIM properties of biomaterials. However, the reported studies differ in the FBS concentration (often between 5 and 20%) and whether the FBS was heat-inactivated before use, as is reflected in Table 2 and 3 for immune and skeletal cells, respectively.

Heat-inactivating FBS is a simple process in which the FBS is heated at 56°C for 30 minutes before adding it to the basal medium [94]. The purpose of heating FBS is mainly to inactivate both the complement system, a number of small proteins present in the serum that are part of the immune system, and lipopolysaccharide (LPS)-binding proteins (LBP), which could activate immune cells [95]. For studies on osteoimmunology with biomaterials, most of them use heat-inactivated FBS for primary immune cells, whereas both regular and heat-inactivated FBS has been used for studies with cell lines, showing the lack of consensus in the field (Table 2). In contrast, the large majority of studies using skeletal cells do not use heat-inactivated FBS (Table 3). However, previous studies have indicated that some of the proteins present in the serum can affect the behaviour of osteoblasts and MSCs, meaning that the results can be indirectly influenced by the type of FBS used [96,97]. While most of the studies use the same concentration of FBS for cell maintenance and the subsequent experiments, there are a few experiments that use a serum-free culture medium when immune cells are extrinsically activated with lipopolysaccharide (LPS) [90,98,99] or during a particular study such as migration [100].

Noteworthy, since a couple of decades there is a strong push to use synthetic serum instead of FBS. There are several reasons behind this, such as the presence of many unidentified substances, batch-to-batch variation including different growth factors and growth inhibitors, and the potential to transfer viral and bacterial contaminations. On top of that, the procedure to obtain FBS is ethically debatable [101].

In addition to the serum and antibiotics, supplementing the medium with other components may be crucial to provide a specific environment to the cells during an experiment. In particular, chemical substances can be added into the immune cells to trigger their pro-inflammatory response, while growth factors or chemical compounds may be added to promote the differentiation of MSCs towards a skeletal cell type. The most common chemical compounds to extrinsically induce the activation of immune cells are LPS and phorbol 12-myristate 13-acetate (PMA). LPS is the main component of the outer membrane of gram-negative bacteria and triggers the activation of macrophages and other cells of the immune system [102,103]. PMA is a synthetic chemical compound that strongly activates protein kinase C, resulting in the activation of several immune cells. Upon activation, certain immune cells can respond by expressing pro-inflammatory cytokines and releasing ROS [103,104].

Based on the relevant articles selected for this review, a trend in the use of LPS and PMA was observed. The addition of LPS into a cell culture often aims to create an inflammatory environment that activates the immune cells, skewing them towards a M1 phenotype [105–107]. LPS can therefore be used to produce a set of activated inflammatory cells that can serve as a positive control [108,109] or to evaluate the anti-inflammatory properties of biomaterials [110]. LPS has occasionally been used to stimulate skeletal cells such as MSCs [107] or pre-osteoblasts [111]. In contrast, PMA is usually added to non-adherent cells such as THP-1 and U937 to differentiate them into adherent macrophages [109,112–114]. The range of concentrations in which these compounds are applied varies significantly between studies. The most common concentration for LPS is 1 µg/ml [99,105,107,110,115], although there are studies using it at both lower (1 ng/ml [50], 100 ng/ml [116], 200 ng/ml [106]), and higher (2 µg/ml [109], 1000 µg/ml [90,98]) concentrations. Cells are usually incubated with LPS for a short period between 6-24 hours. For PMA, more disperse concentrations have been used, with values ranging from 80 nM to 1000 nM [50,109,112–114]. Cells are usually incubated with PMA for a period of 24-72 hours. Other activators such as 20 ng/ml M-CSF (20 ng/ml) [117], IL-4 (10-20 ng/ml) [106,118], and a combination of activators such as IL-4 combined with IL-13 (20 ng/ml) [116] or LPS and IFN-γ [115,116] are also reported.

When skeletal cells (*e.g.* MSCs or pre-osteoblast-like cells) are cultured with/in/on biomaterials, supplements are often added into the medium to enhance their differentiation towards bone cells. The combined addition of ascorbic acid, β-glycerophosphate and dexamethasone is a particularly effective combination to enhance the differentiation of MSCs or pre-osteoblasts towards mature osteoblasts, which increase the number of bone nodules formed as a sign of mineralization in a time-dependent manner [119]. The potential mechanisms involved have been already discussed elsewhere [120]. The most common concentrations used experimentally are the following: between 8 and 50 µg/ml of L-ascorbic acid [48,50,88,108,110,113,121–126], but values of one order of magnitude higher have also been used [89,98]; mainly 10 mM β-glycerophosphate [48,50,89,108,110,113,121,125,126] but values between 1 and 5 mM are also reported [88,98,122–124]; and 10 and 100 nM of dexamethasone [48,50,89,98,122]. The classical protocol to induce osteogenic differentiation involves culturing a monolayer of cells in combination with the mentioned compounds over several weeks. However, the majority of studies are evaluated over a two-week period, although one and three-week intervals are also used. Importantly, the timing for the addition of osteogenic supplements in monolayer formation is often omitted, and only few studies indicate osteogenic supplements were added after 24h of cell seeding [88,110].

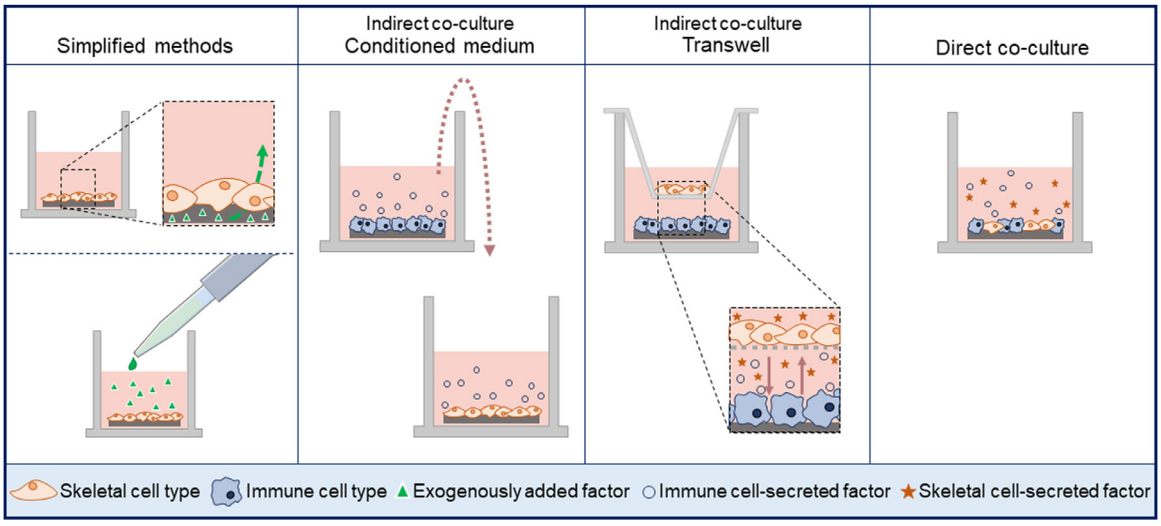

Fig. 2. Schematic of the main experimental configurations to study the osteoimmunomodulatory properties of biomaterials: simplified methods, indirect co-culture using conditioned medium or transwells, and direct co-culture.

Beyond the addition of exogenous biochemical signals into the cell culture media, more recent strategies have explored the stimulation of MSC differentiation through intrinsic properties of biomaterials. For example, biomaterials with tailored mechanical properties, mainly stiffness, induce cell differentiation through extracellular matrix mechano-stimulation, mimicking the native tissue environment of the cells [127,128]. In addition, skeletal cells can also be extrinsically stimulated by the addition of exogenous factors in the media or their release by a biomaterial [129], as is further elaborated on in section 4.1.1. 'Simplified methods to study osteoimmunology'.

4. *In vitro* assessment of the OIM properties of biomaterials

4.1. Experimental approaches

Multiple strategies have been explored to assess the OIM potential of biomaterials *in vitro*. These range from more simplified approaches, in which one of the key cell types (*i.e.* immune cells or skeletal cells) is exposed to regulatory factors expected to affect this cell type, to more complex approaches in which both cell types are involved. Such co-culture studies can be subdivided into indirect co-culture studies, typically using either conditioned medium (CM) or transwells, and direct co-culture studies, where two cell types are grown simultaneously with/in/on the same biomaterial (Fig. 2). Based on the literature selected for this review, the CM approach has shown to be the most common experimental set-up used to assess osteoimmunology in the context of biomaterials, followed by the direct contact studies and indirect contact studies employing transwells (Table 4).

4.1.1. Simplified methods to study osteoimmunology

In this approach, the cells are extrinsically stimulated by supplementation of commercially available regulatory factors, which are either added directly to the cell culture medium or incorporated in/on a biomaterial (Fig. 2). For example, in a study performed by Mountziaris *et al.*, the dose-effect of the pro-inflammatory cytokine TNF-α on the osteogenic differentiation of MSCs was investigated [130]. Rat MSCs were pre-treated with dexamethasone to induce osteogenic differentiation and subsequently grown on biodegradable polycaprolactone scaffolds. Afterwards, the cells were exposed to a continuous delivery of 0.1, 5 or 50 ng/ml TNF-α and assessed for osteogenic differentiation. Whereas the lowest dose of TNF-α inhibited osteogenic differentiation, exposure to the intermediate and highest concentrations resulted in a stimulation of differentiation and significant mineralized matrix deposition, respectively. This study was elaborated on in later work, in which it was shown that temporal variations in TNF-α affect osteogenic differentiation of rat mesenchymal stem cells [131]. An example of adding relevant factors to biomaterials, rather than supplementing these to the medium, is given by Lv *et al.* [129]. In their work, high mobility group box 1, a chemoattractant that directs the migration of among others inflammatory cells and MSCs, was immobilized on the surface of poly-L-lactide/polycaprolactone scaffolds. One key finding in this work was that such scaffolds accelerated the adhesion and osteogenic differentiation of MSCs *in vitro*. Although offering a relatively simple method to mimic and evaluate the OIM properties of biomaterials, these simplified approaches lack the real-time interplay between biomaterials and the two families of cells.

4.1.2. Indirect co-culture

Conditioned medium (CM). In this approach, one cell type, typically an immune cell type, is grown with/in/on a biomaterial, modifying the surrounding cell culture medium with cell-secreted factors. After incubation, this modified medium (*i.e.* CM) is collected and added to skeletal cells, which either grow on standard tissue culture plastic or on the biomaterial (Fig. 2). The CM may also be supplemented with extrinsic osteogenic compounds (*e.g.* ascorbic acid, β-glycerophosphate and dexamethasone) to further enhance the differentiation, as mentioned before in section 3.2 'Cell culture medium'. Multiple studies have used CM as the approach to study the interplay between macrophages and skeletal cells (Table 4). For example, Sadowska *et al.* studied the influence of the inflammatory environment generated by the physicochemical features of calcium phosphate substrates and its effect on the osteogenic differentiation of human MSCs and Saos-2 cells [110]. In their work, CM was prepared by culturing RAW 264.7 cells on different calcium phosphate substrates. This CM was subsequently fed to MSCs and Saos-2 cells, which had been growing either or both on the calcium phosphate substrates. The results showed that calcium phosphates with different physicochemical properties modulate the immune cell response differently, which on its turn also affected osteogenic differentiation. Interestingly, the substrate that caused the most prominent decrease in pro-inflammatory cytokines, was not the

Table 4
Overview of the experimental approaches and characterisation/analysis methods used to evaluate the osteoimmunomodulatory properties of biomaterials. In case that both monoculture (M) and a co-culture (C) were performed, only the latter is indicated.

			Experimental approach			Characterisation and analysis methods																					
	Materials	Properties evaluated	Direct	Transwells	CM	Morphology		Cell viability & proliferation		Gene expression		ELISA		Western Blot		ALP activity	Mineralization	Immunofluorescence		Flow cytometry	Oxidative comp.		Migration	Microarray		References
						IM	SK	IM	SK	IM	SK	IM	SK	IM	SK	SK	SK	IM	SK	IM	IM	SK	SK	IM	SK	
Ceramics	β-TCP	_			X			M	C	M	C			M		C	C									[124]
	β-TCP + heparine	Chem			X	M		M	M		C	M				M					M					[50]
	Mg/β-TCP	Chem			X	M				M	C			M	C	C	C			M						[123]
	β-TCP and CDHA	Cry, Rou			X	M	C			M	C	M			C		C		C							[110]
	HA/TCP	_	X					C	C	M				M		M	M			C						[122]
	HA	Rou, Chem			X	M				M		M			C	C	C					M				[121]
	HA	Rou			X	M				M	C									M						[87]
	Sr-HA + phospho-serine	Chem			X	M		M	C	M	C	M														[135]
	CDHA	Rou			X	M		M		M	C				C		C		C							[48]
	CPC, MCPC, MPC	Chem			X					M	C	M				C	C			M						[88]
	BCP	_		X	X					M	C	M		M									C	M		[139]
	Bioactive glass	_			X		M		M	M	C					C							C			[118]
	Cu-bioactive glass	Chem			X				M	M					M								C			[86]
	Alumina	Por			X	M		M		M	C	M		M			C				M					[98]
Polymers	PGA/PLA	_		X						C		C						C								[115]
	PLA; chitosan	Chem			X	M		M				M											C	M		[100]
	Chitosan	Chem	X			C	C					C	C						C							[107]
	Hyaluronic acid	_		X						C	C									C					C	[186]
	Zn-PEEK	Chem			X	C	C	M	M	M		M				C	C	M	C	M				M		[136]
	SBS-PEEK	Chem			X					M	C	M				C	C	M	C	M						[134]
	PEEK	Che, Rou, Wet			X	M	M	M	M	M	M	M				C	M				M			M		[113]
	Silk fibroin PCL	Rou	X			C	C		M			C				M	C									[112]
Composites	Mineralized collagen	_			X	M				M	C	M														[114]
	Coll-HA	Por, Mech	X					M	M	M	C	M				C	C									[116]
	Bioactive glass coated collagen	Rou			X	M				M	C				C		C									[133]
	Nanoparticle fibroin	Chem	X			C	C		M			C				M	M									[109]
	Transglutaminase gelatin	Mech		X	X	M	M		M	M	M	M				C	C	M								[126]
Metals	Titanium	Rou			X	M				M	C			M		C	C									[137]
	Titanium	Chem, Rou, Crys, Wet			X	M			M	M	C								M							[99]
	316L stainless steel	Rou			X	M	C	M	C	M	C								C							[90]
	Magnesium alloy	Chem			X			M	M	M	C					M	M		C							[89]

Abbreviations material: β-TCP: beta-tricalcium phosphate; BCP: bicalcium phosphate; CDHA: calcium deficient hydroxyapatite; CPC: calcium phosphate cements; HA: hydroxyapatite; HMW: High molecular weight; MCPC: magnesium-calcium phosphate cements; MPC: magnesium phosphate cements; PEEK: polyetheretherketone; PCL: polycaprolactone; PLA: polylactic acid; SBS: sodium buryrate-sulfonated; **Abbreviations types of culture:** M: monoculture; C: co-culture; **Abbreviations cell types:** IM: immune cells; SK: skeletal cells, **Abbreviations properties evaluated:** Chem: chemistry; Cry: crystalline phases; Mech: mechanical; Por: porosity; Rou: roughness; Wet: wettability.

one resulting in the most favourable environment for osteogenic differentiation.

Despite offering an accessible way to study the interactions between biomaterial, immune and bone cells, it should be noted that CM may not only be altered in terms of the desired cell-secreted factors, but may also show variation in other medium components, such as glucose and serum proteins. These factors can impact the biological outcome and can easily be overlooked. In addition, for all co-culture configurations, medium optimization may be required to select a common medium that sustains all the cell populations to a desirable degree [132]. This can be a challenging and lengthy process, especially when considering that cells that would make sense to culture together from a physiological point of view, do not necessarily share the same basal medium and supplements *in vitro*. A way to tackle this is to dilute CM with the complete medium of skeletal cells at different ratios, such as 1:1 [86,88,90], 1:2 [48,50,118,133–137], or 1:3 [123]. The observed variety in ratios seems to point at prior optimization experiments, in which the ratios between the different cell media were evaluated before performing the final studies for publication. However, data supporting the reasoning behind choosing a certain medium or media combination is unfortunately omitted.

Lastly, it should be mentioned that although CM has been widely used in studies on osteoimmunology, this set-up does not allow real-time cross-talk between the cells, but instead, cell-secreted factors accumulate in the medium over time. To circumvent this, transwells may be used.

Transwells. Transwells are inserts that contain a porous membrane at the bottom and can be positioned inside the wells of a cell culture plate. They have been used to provide physical isolation of different cell types, while allowing bidirectional exchange of secreted molecules within each well. In the context of osteoimmunology, the biomaterial is usually placed at the bottom of the traditional well plate, together with one of the cell types of interest. The other cell type is seeded on the porous membrane in the transwell insert, often without the biomaterial, enabling the diffusion of cell-secreted factors (Fig. 2). Apart from real-time exposure to secreted factors, using a transwell has the advantage of capturing the two-way dynamic interaction between immune and bone cells, thereby more closely mimicking the *in vivo* environment. An example of such a set-up is provided by Wang *et al.*, who studied how mouse bone marrow-derived MSCs respond to chemokines secreted by macrophages that had been in contact with a biphasic calcium phosphate biomaterial [138]. The biomaterial was placed in a well and subsequently seeded with RAW 264.7 cells. The transwell insert was placed into the well and seeded with bone marrow-derived MSCs. When membranes with sufficiently large pore sizes are used, cell migration could also be studied using this same set-up [86,100,118,139].

Noteworthy, to adequately cover the cells in a transwell insert with medium, larger volumes than in traditional well plates may be needed. This may dilute factors of interest and should therefore be considered when designing experiments. In addition, the selected cell culture medium itself could affect the experimental results.

4.1.3. Direct co-culture

In the direct co-culture approach, the two cells types are grown simultaneously with/in/on the same biomaterial, allowing direct cell-to-cell contact (Fig. 2). This strategy was chosen in a study aiming to determine the OIM properties of chitosan-based scaffolds. Co-cultures of osteoblasts and J774A.1 macrophages with and without LPS stimulation were used as a model for an inflamed bone environment and a regular environment, respectively [107]. The results showed that the chitosan-based scaffolds had the ability to inhibit pro-inflammatory cytokine (IL-1 and IL-6) production, indicating the potential of such materials to promote bone regeneration under favourable inflammatory conditions.

Although offering an environment even closer to the *in vivo* situation, having two cell types in close vicinity on the same biomaterial has its challenges. Not only is there a need for compatibility of the two cell types, optimizing cell densities and corresponding culture conditions (*e.g.* basal medium and supplements), but working with direct co-cultures also increases the complexity of analysis. This is particularly relevant when aiming to separate the effects from either of the cell types on the other, and to simultaneously ascribe the effects to the biomaterial, based on using common cell-secreted factors or other non-cell type specific methods. Moreover, similarly to other approaches, the general choice of cell culture medium is also important for the outcome of the study.

4.2. Methodologies used to assess osteoimmunology

4.2.1. General overview

The OIM properties of many different types of biomaterials have been tested, using either novel biomaterials or well-known biomaterials with modified physicochemical properties (*e.g.* chemistry, roughness and stiffness). The materials, classified by their chemical nature (ceramics, polymers, composites and metals) and their physicochemical property under study, are listed in Table 4, accompanied with the methods that have been used to test the OIM properties of the material.

Generally, the *in vitro* evaluation of biomaterials' OIM properties includes an assessment of cell viability and/or cellular morphology. Regardless of the experimental setup, the more specific evaluation often focuses first on determining the immune cell response upon biomaterial contact, and subsequently examines how this response affects the differentiation of osteoblasts/MSCs. The immune response is assessed by identifying the inflammatory profile of macrophages as either M1 (pro-inflammatory) or M2 (anti-inflammatory) through the study of expressed genes, released cytokines or surface markers. The evaluation of osteoblast or MSC differentiation comprises the detection of osteogenic genes, ECM proteins and calcium deposition. Moreover, migration of MSCs triggered by cytokines and chemoattractants has been evaluated with some migration tests.

The mentioned cellular responses are evaluated using a plethora of biochemical methods and techniques (Table 4). A more detailed overview of the different methodologies, including factors and time points assessed, are included in Tables 5 and 6 for inflammatory and skeletal cells, respectively. Noteworthy, for those co-culture studies using indirect contact approaches (CM or transwells), the characterisation could be directly performed and focused on the cell family of interest, meaning that the time points and factors are only indicated in one of the tables (Table 5 or 6). In the case of direct approaches, since both cell types were cultured together, the output from the characterisation, could in principle originate from either of the two cell types. Therefore, information regarding morphology may be indicated in both tables. On the contrary, specific markers (inflammatory or osteogenic) were added to the corresponding table based on the current knowledge of the factors and which cell type they originate from. Last but not least, while the general characterisation methods employed in each article are indicated in Table 4, due to the high number of specific factors evaluated, Tables 5 and 6 gather those factors tested in more than one work (using either the same or different techniques). Factors that have only been tested once have been included in Table S.1 and S.2, since these may serve as inspiration for future studies.

Table 5
Methodologies used to evaluate immune cells, including the factors and the time points at which they are commonly evaluated.

Method	Factor/assay	Monoculture	Co-culture
Morphology	Fluorescence/confocal microscopy	1 day [90,110,114,126] 2 days [100] 3 days [110, 126] 7 days [126] 9 days [100]	1 day [112] 3 days [109] 4 days [107]
	SEM (Scanning electron microscopy)	6 hours [50,110] 1 day [50,87,90,98,99,113,114,121,123,133,137] 2 days [50] 3 days [50,110] 4 days [136]	1 day [136] 4 days [136] 7 days [112]
Cellular viability & proliferation	Resazurin-based assays, live-dead imaging, etc.	1 day [48] 3 days [48,89] 5 days [48] 7 days [88,89,109,112] 14 days [109,112] 21 days [112]	6 days [122]
Gene expression (PCR)	Arg-1 (Arginase 1)	1 day [114] 2 days [88] 3 days [87,114,137] 7 days [126] [114]	1 day [115]
	BMP-2 (Bone morphogenetic protein 2)	6 hours [98] 1 day [113,123,124] [99] 36 hours [123] 2 days [88] 3 days [113,137] 4 days [134,136] 5 days [113]	
	CCL2 (C-C Motif Chemokine Ligand 2) / MCP-1	1 day [139] 3 days [89,135,139] 5 days [139]	
	CCL3 (C-C Motif Chemokine Ligand 3) / MIP-1a	1 day [139] 3 days [135,139] 5 days [139]	
	CCL5 (C-C Motif Chemokine Ligand 5) / RANTES	1 day [139] 3 days [135,139] 5 days [139]	
	CCR-7 (C-C Motif Chemokine Receptor 7)	6 hours [98] 3 days [87,137] 4 days [136]	
	CD11c (Cluster of differentiation 11c)	6 hours [98] 2 days [121] 3 days [137]	
	CD80 (Cluster of differentiation 80)	1 day [124]	
	CD86 (Cluster of differentiation 86)	6 hours [90,98] 1 day [124] [99] 2 days [121] 4 days [134]	
	CD163 (Cluster of differentiation)	1 day [99] 3 days [89,137] 4 days [134]	
	CD206 (Cluster of differentiation 206)	6 hours [90,98] 12 hours [48] 1 day [99,113,126] 2 days [121] 3 days [87,113,137] 4 days [136] 5 days [113] 7 days [126]	
	CTSK (Cathepsin K)	1 day [99,110] 12 hours [48] 3 days [110]	
	IFN-γ (Interferon gamma)	1 day [123,124] 36 hours [123] 3 days [137]	
	IL-4 (Interleukin 4)	1 day [124] 3 days [137]	
	IL-6 (Interleukin 6)	6 hours [90,98,110] 12 hours [48] 1 day [99,113,114,123,124] 36 hours [123] 2 days [88,121] 3 days [113,114,118,135,137] 5 days [113] 7 days [114]	24 hours [115]

(*continued on next page*)

Table 5 (*continued*)

Method	Factor/assay	Monoculture	Co-culture
	IL-10 (Interleukin 10)	6 hours [90] 1 day [99,114,123,124] 36 hours [123] 2 days [88] 3 days [87,89,114,118,137] 7 days [114,126]	24 hours [115]
	IL-18 (Interleukin 18)	6 hours [98] 1 day [124] [99]	
	IL-1β (Interleukin 1 beta)	6 hours [90,98,110] 12 hours [48] 1 day [99,113,123,124] 36 hours [123] 2 days [88,121] 3 days [113,118,135,137] 5 days [113] 7 days [126] N.I. [86]	24 hours [115]
	IL-1ra (Interleukin 1 receptor antagonist)	1 day [123,124] 36 hours [123] 2 days [88] 3 days [118,137] N.I. [86]	
	iNOS (Inducible nitric oxide synthase)	6 hours [98,110] 12 hours [48] 1 day [99,114] 2 days [88,121] 3 days [87,114,118,137] 7 days [114,126]	24 hours [115]
	MMP-9 (Matrix metallopeptidase 9)	6 hours [98,110] 12 hours [48]	
	OSM (Oncostatin M)	6 hours [98] 12 hours [48]	7 days [116]
	TGF-β1 (Transforming growth factor beta 1)	6 hours [98] 12 hours [48] 1 day [99,113,123,124] 36 hours [123] 2 days [88] 3 days [113,137] 5 days [113]	24 hours [115]
	TLR-4 (Toll like receptor 4)	1 day [124]	
	TNF-α (Tumor necrosis factor)	6 hours [90,98,110] 12 hours [48] 1 day [50,89,99,113,123,124] 2 days [50,121] 36 hours [123] 3 days [50,87,89,113,118,135,137] 5 days [113] 7 days [126]	24 hours [115]
	TRAP (Tartrate-resistant acid phosphatase)	6 hours [98] 1 day [99,123] 36 hours [123]	
	VEGF (Vascular endothelial growth factor)	6 hours [98] 12 hours [48] 1 day [99,113,123,124] 36 hours [123] 2 days [88] 3 days [113,137] 4 days [134,136] 5 days [113]	
Cytokines (ELISA)	Arg-1 (Arginase 1)	1 day [114] 3 days [114] 7 days [114]	
	CCL-2 (C-C Motif Chemokine Ligand 2) / MCP-1 (monocyte chemoattractant protein 1)	1 day [139] 2 days [100] 3 days [89,135,139]	
	CCL3 (C-C Motif Chemokine Ligand 3) / MIP-1α (macrophage inflammatory protein 1-alpha)	24 hours [139] 48 hours [100] 3 days [116,135,139]	

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Table 5 (*continued*)

Method	Factor/assay	Monoculture	Co-culture
	CCL4 (C-C Motif Chemokine Ligand 4) / MIP-1β	1 day [139] 2 days [100] 3 days [139]	
	CCL5 (C-C Motif Chemokine Ligand 5) / RANTES	2 days [100] 3 days [135]	
	IL-1β (Interleukin 1 beta)	6 hours [50] 12 hours [109] 1 day [50,98] 2 days [50,121] 3 days [50,135]	12 hours [109] 1 day [115] 4 days [107]
	IL-4 (Interleukin 4)	4 days [134,136]	
	IL-6 (Interleukin 6)	6 hours [48,110] 1 day [98,114] 2 days [88,100,121] 3 days [114,116,135] 4 days [134,136] 7 days [114]	4 days [107]
	IL-10 (Interleukin 10)	3 hours [126] 6 hours [110,126] 12 hours [126] 24 hours [114,126] 2 days [88,126] 3 days [114,116,126] 4 days [134,136] 7 days [114]	1 day [115] 4 days [107]
	iNOS (Inducible nitric oxide synthase)	3 hours [126] 6 hours [126] 12 hours [126] 1 day [114,126] 2 days [126] 3 days [114,126] 7 days [114]	
	TGF-β1 (Transforming growth factor beta 1)		4 days [107]
	TNF-α (tumor necrosis factor)	3 hours [113] 6 hours [48,50,110,126] 12 hours [109,126] 1 day [50,98,126] 2 days [50,88,121,126] 3 days [50,113,116,126] 4 days [134,136]	12 hours [109]
Western Blot	CD86 (Cluster of differentiation 86)	6 hours [98]	
	IkBα (Inhibitor of nuclear factor kappa B)	6 hours [98] 7 days [123]	
Immunofluorescence	Arg-1 (Arginase 1)	24 hours [126] 4 days [134]	
	CCR-7 (C-C Motif Chemokine Receptor 7)	4 days [134]	
	CD206 (Cluster of differentiation 206)	24 hours [136] 4 days [136]	24 hours [115]
	IL-10 (Interleukin 10)	24 hours [126]	
	iNOS (Inducible nitric oxide synthase)	24 hours [126,136] 4 days [136]	
	TNF-α (tumor necrosis factor)	24 hours [126]	
Cell surface markers (flow cytometry)	CCR-7 (C-C Motif Chemokine Receptor 7)	24 hours [123] 3 days [88] 4 days [134,136]	
	CD80 (Cluster of differentiation 80)	3 days [87]	
	CD163 (Cluster of differentiation 163)	24 hours [123]	
	CD206 (Cluster of differentiation 206)	3 days [87,88] 4 days [134,136] 7 days [186]	
Oxidative molecules	ROS (Reactive oxygen species)	2 hours [50] 6 hours [98] 3 days [113]	
Microarray	Cytokine array (40 factors)	7 days [100]	
	Gene expression array (>10 factors and 111 factors)	3 days [139] 4 days [136]	
	Whole genome analysis	3 days [113]	

N.I. not indicated.

Table 6
Methodologies used to evaluate skeletal/bone cells, including the factors and the time points at which they are commonly evaluated.

Method	Factor/assay	Monoculture	Co-culture
Morphology	Fluorescence/confocal microscopy	1 hour [99] 4 hour [99] 1 day [99,126] 3 days [126] 7 days [109,126] 21 days [112]	1 day [90,112] 3 days [109,110] 4 days [107] 7 days [136] 14 days [136]
	SEM (Scanning electron microscopy)	1 day [99,113,136] 2 days [118] 3 days [99] 4 days [136] 7 days [109]	7 days [112]
Cell viability & proliferation	Resazurin-based assays, live-dead imaging, etc.	6 hour [50] 1 day [86,89,99,109,112,118,126] 3 days [50,86,89,99,118,126] 5 days [86,89,99,112] 7 days [50,89,109,112,118,126] 14 days [50,109,112] 21 days [109,112]	1 day [90,124,135] 3 days [90,124,135] 5 days [124] 6 days [122] 7 days [90,135]
Gene expression (PCR)	ALP (Alkaline phosphatase)	3 days [89] 7 days [89,113,126,137] 14 days [113] 21 days [122]	1 day [50,124] 3 days [50,89,99,110,123,135] 7 days [88,114,118,123,137] 14 days [118,134,136]
	BMP-2 (Bone morphogenetic protein 2)	1 day [89] 3 days [87,89] 7 days [89]	1 day [50] 3 days [48,50,99,110] 7 days [88] [116] 14 days [134]
	BMPR-1a (Bone morphogenetic protein receptor type 1A)		3 days [98,123]
	BMPR-2 (Bone Morphogenetic Protein Receptor Type 2)		3 days [98,123]
	COL-1 (Collagen 1)	3 days [87,89] 7 days [89,113,137] 14 days [113]	1 day [50] 3 days [48,50,89,99,110,123,133,135] 7 days [88,118,121,123,186] 14 days [118,136]
	IBSP (Integrin binding sialoprotein)		3 days [48,110,133] 7 days [123]
	OCN (Osteocalcin)	3 days [89] 7 days [89,113,126,137] 14 days [113] 21 days [122]	1 day [90,124] 3 days [48,89,99,110,123,133,135] 7 days [114,118,121,123] 14 days [118,134,136]
	OPG (Osteoprotegerin)		3 days [99,123,135]
	OPN (Osteopontin)	7 days [137]	1 day [50,90] 3 days [50,133] 7 days [116,118,123] 14 days [118,121,134]
	OSX (Osterix) / SP7 (SP7 transcription factor-2)	7 days [126] 21 days [122]	3 days [99]
	RUNX-2 (Runt-related transcription factor 2)	3 days [87] 7 days [113,126,137] 14 days [113] 21 days [122]	1 day [90,99,124] 3 days [48,89,110] 7 days [88,114,116,118,121] 14 days [118,136]
	SMAD1 (Mothers against decapentaplegic homologue 1)		3 days [98,99,123]
	SMAD4 (Mothers against decapentaplegic homologue 4)		3 days [98,99,123]
	SMAD5 (Mothers against decapentaplegic homologue 5)		3 days [98,99,123]
Alkaline phosphatase (ALP)	ALP assay (Alkaline phosphatase) (quantitative)	6 hours [50] 3 days [50,88,89,113] 7 days [50,89,99,109,112,113,116,126] 10 days [123] 14 days [50,109,112,113] 21 days [112]	3 days [113] 7 days [88,113,116,118,126,137] 10 days [123,124] 14 days [113,118]
	ALP staining (Alkaline phosphatase) (qualitative)	7 days [88] 21 days [122]	7 days [88,118,136] 14 days [118,134,136] 3 weeks [121]

(*continued on next page*)

Table 6 (*continued*)

Method	Factor/assay	Monoculture	Co-culture
Calcium deposits indication of mineralization	Alizarin Red	7 days [89,99,109] 14 days [113] [99] 21 days [113,122] 28 days [116]	7 days [88,112,126,136,137] 10 days [123] 14 days [48,88,98,110,112,133,134,136] 21 days [112,121,124] 28 days [116]
Western Blot	β-catenin		3 days [98] 7 days [133]
	ALP (Alkaline phosphatase)		3 days [48,98,110] 7 days [133] 14 days [123]
	Axin-2		3 days [98] 7 days [133]
	COL-1 (Collagen 1)		3 days [48,98,110] 7 days [121,133]
	OCN (Osteocalcin)		7 days [121]
	OPN (Osteopontin)		3 days [98] 7 days [133] 14 days [123]
	RUNX-2 (Runt-related transcription factor 2)	21 days [122]	3 days [48,110] 7 days [121,133]
Immunofluorescence	ALP (Alkaline phosphatase)		3 days [110] 7 days [136] 14 days [134,136]
	COL-1 (Collagen 1)		7 days [89]
	OCN (Osteocalcin)		14 days [136]
	OPN (Osteopontin)		1 day [90]
	RUNX-2 (Runt-related transcription factor 2)		7 days [89]
Migration	Real-time cell analysis		8 hours [139]
	Scratch assay		12 hours [118] 24 hours [118]
	Transwell assays		8 hours [118] 24 hours [100]
Microarray	Gene expression array (17 factors)		3 days [186] 7 days [186]

The methods recurring in the literature are explained below in separated subsections depending on whether they are used for both skeletal and immune cells or for either family of cells.

4.2.2. Methods used for both skeletal cells and immune cells

Cell morphology. By studying the morphology and attachment of cells, one can obtain information about their responses to a biomaterial. In the case of macrophages, their polarization can result in dramatic changes of cell shape *in vitro*. For example, macrophages with an anti-inflammatory M2 profile exhibit an elongated shape, whereas pro-inflammatory M1 macrophages display a rounded morphology [110,140,141].

Cell morphology on a biomaterial is generally assessed using microscopy, which often involves fluorescent labelling of the cells. A common example is the cytoplasmic staining CellTracker™, which is a fluorescent dye that passes the cell membrane and is retained within the cells. This method allows for live monitoring of the cells for several generations. CellTracker™ can be used to stain immune cells and skeletal cells in different colours before being seeded on a biomaterial, allowing for convenient visualisation of both cell types [107,112]. Alternatively, the cytoskeleton or cell nucleus can be stained using, for example, phalloidin and 4′,6-diamidino-2-phenylindole (DAPI) staining, respectively. While CellTracker™ is intended to be used over the culture time, both cytoskeleton and cell nucleus staining are typically performed after fixation at the end point of a study.

Another commonly employed method is scanning electron microscopy (SEM). Although requiring additional sample preparation steps and only allowing end-point analysis, SEM provides higher resolution than fluorescence microscopy. SEM has also been used to visualize the morphology of two cell types on a biomaterial [103] even though it does not come with the advantage of clearly visualizing the different parts of the cells as is otherwise easily done with counterstaining in fluorescence microscopy.

Nowadays, with advanced image analysis techniques, images can be post-processed to obtain quantitative data. For accurate analyses, image stitching can overcome the inaccuracy of selecting a representative area/image by composing a full map of a sample, although requiring long acquisition times and post-processing. While SEM reaches resolutions of 10-100 nm [142] and provides information regarding the microstructure of both cell and biomaterial, coupling SEM to focused ion beam (FIB) allows for further analysis of the cell-biomaterial interphase [143,144]. While both SEM and FIB-SEM may provide valuable insights, such in-depth characterisation is often not needed to evaluate the osteoimmunological properties of biomaterials.

Cell viability and cell proliferation. There are several methods to evaluate cell viability, the most common ones being colorimetric metabolic assays, which are often resazurin-based reagents or fluorescent labelling of living and dead cells. There are several resazurin-based assays on the market (*e.g.* MTT, Alamar Blue and Cell Counting Kit – 8), which are all based on assessing the reduction of a substrate during cell metabolism. By plotting the metabolic activity or cell number over time, information regarding cell proliferation is obtained. To evaluate cell viability by means of fluorescence labelling, a LIVE/DEAD staining method (*e.g.* cal-

cein acetoxymethyl ester (calcein-AM) and propidium iodide) is generally used.

Noteworthy, many of these reagents measure the metabolic activity of cells, rather than the cell number. To correlate the metabolic activity to a cell number, a standard curve with serial dilutions of cells is often used. A concern when using such assays is the assumption that all cells have the same metabolic state, which might not always be the case. In other words, differences observed may be attributed to changes in cell number, changes in cell metabolism, or a combination thereof. Hence, combinations with qualitative methods (*e.g.* cell staining) might provide more complete and accurate results. Finally, for some biochemical reagents it is claimed that repeated measurements can be performed. Whereas this would bring the great advantage of reducing the number of samples needed and being able to track the same sample, our experience is that the cells' signal may decrease over time. Therefore, one should perform preliminary studies to assess that the cells of interest are not affected by being repetitively exposed to a biochemical reagent, thereby influencing the results.

Gene expression. Quantitative real-time polymerase chain reaction (qPCR, also called quantitative PCR or real-time PCR), either involving a reverse transcription step (qRT-PCR) or not (qPCR), combines the amplification of mRNA and detection into a single step. This is achieved by using a variety of different fluorescent chemistries that allow to detect the target DNA, and are characterized by the point in time (or PCR cycle) where the target amplification is first detected (referred to as cycle threshold, C_t) [145]. In the context of osteoimmunology, it is relevant to determine the polarization of macrophages and the differentiation of skeletal cells into the osteogenic lineage. Specifically, for the immune cells, qRT-PCR/qPCR are used to determine mRNA encoding inflammatory factors such as TNF-α (associated with the pro-inflammatory M1 phenotype) or IL-10 (associated with the anti-inflammatory M2 phenotype). For the skeletal cells, typical markers for osteogenic differentiation include, but are not limited to, ALP, RUNX-2, COL-1, OCN and OPN.

There is no doubt that qPCR is widely used in experimental research due to its accurate and unprecedented sensitivity to quantify mRNA. However, such extreme sensitivity also implies that false positives can originate from contaminations. Experimentally, the process is long and tedious, with several delicate steps (*e.g.* cell lysis, RNA extraction, DNA transcription) where the conservation of the target DNA is crucial to produce reliable results [146].

Cell surface markers. The detection and localization of a wide variety of antigens on the cell surface can be evaluated using immunofluorescent labelling and analysed using microscopy or flow cytometry. Flow cytometry is a technique that can be used to characterize and sort cells, based on their physical and chemical characteristics. In short, a sample of cells in solution passes one or multiple lasers, after which light scattering differentiates the cells by their size and granularity together with a fluorescence signal. The fluorescence signal is provided by immunofluorescence stainings and can therefore also be assessed by imaging. For example, M1 pro-inflammatory macrophages can be recognized by high expression of the cell surface marker CD68, while M2 anti-inflammatory macrophages can be recognized by CD206 (Table 1). For skeletal cells/MSCs, imaging of immunofluorescent staining has also been used to evaluate ALP and OCN.

Some of the limitations of the analysis of cell surface markers is that it is challenging to compare samples obtained in different experiments, although standardizing the samples by the unit of fluorescence intensity can potentially allow such comparisons [147]. While immunofluorescence only requires a fluorescence microscope and provides images relatively easy to interpret, flow cytometry requires sophisticated equipment and produces large amounts of information to process.

Western blot. Western blot can be used to assess protein production down to picogram level. With this analytical technique, a mixture of proteins is separated based on their molecular weight using gel electrophoresis and characterized using antibodies specific to the protein of interest. A protein of interest for the immune cells is IκBα (inhibitor of NF-κB). For the skeletal cells, some typically analysed proteins are ALP, COL-1, OPN or RUNX-2.

Although Western blot has a huge potential, several limitations have been reported for this technique. For instance, the off-target interaction of antibodies with other proteins and the large inter-operator variability due to the technical demand of the techniques should be kept in mind [148].

Microarrays. High-throughput methods such as microarrays have been used to evaluate a large group of proteins or expressed genes. The advantage of this method is that many proteins are evaluated in parallel, consuming small quantities of samples and reagents. In osteoimmunology studies, proteins expressed by immune cells have been evaluated using microarrays to screen multiple cytokines in a semiquantitative manner [100,139,149]. Similarly, microarrays have been used to evaluate the gene expression profile [136] and even to analyse the whole genome of immune cells [113].

The few producers of necessary equipment and buffers as well as the experimental complexity, not only in terms of experimental design and set-up, but also in terms of data analysis, should be named among the limitations of this technique. In addition, this technology has limited accessibility and high cost [150] [151].

4.2.3. Methods specific for immune cells

Enzyme-linked immunosorbent assay (ELISA). ELISA is a technique based on the specific recognition of an antigen, usually by an antibody. ELISA tests can be performed using commercially available kits that are accompanied by a well-defined protocol and sensitivity limits. Although multiple types of ELISAs are on the market, the antibody-antigen detection is typically based on colorimetric analysis. ELISAs are often used to evaluate the cytokine release profile of inflammatory cells, for instance to determine macrophage polarization. An M1 pro-inflammatory profile is characterized by the release of for example IL-1β, IL-6 and TNF-α, while M2 anti-inflammatory macrophages can be identified by IL-10 and TGF-β (Table 1).

ELISA is widely used due to its high specificity and selectivity to detect a wide variety of markers, using straightforward protocols. Some of the disadvantages are the relatively long experimental procedure and the need to fit the target values of the sample of interest into those of the ELISA kit detection range, which might require certain optimization.

Oxidative compounds. Some immune cells have the ability to release reactive oxygen intermediates (*e.g.* ROS, superoxides, peroxides and, hydroxyl radicals) or reactive nitrogen oxides (*i.e.* nitric oxide, NO, and peroxynitrite, ONOO-) [152]. The antimicrobial potential of these compounds is linked to the irreversible damage created to the DNA due to the modification by oxidation of some cellular components. The effect is however local and can be reduced by quenching the oxidative species with a sufficient amount of anti-oxidative molecules [153]. ROS levels can be determined by a variety of biochemical assays that assess the oxidation of a substrate, which can be measured by colorimetric, fluorimetric or luminescence analysis, for example by using a plate reader or flow cytometry.

While these assays are straight-forward to perform, they are also challenging because the oxidative intermediates are released for a short period of time, reaching a peak only a few minutes after the start of the reaction. This can be overcome by monitoring the kinetics of the signal with an early start.

4.2.4. Methods specific for skeletal cells

Alkaline phosphatase activity (ALP). ALP activity in osteoblasts correlates well to their differentiation state and for this reason has been routinely used as an early marker of osteoblast differentiation *in vitro* [154]. In particular, ALP is an enzyme that regulates the local concentration of calcium and phosphate, which is important for bone mineralization. The three main possibilities to assess this differentiation marker are immunofluorescent staining, imaging ALP-stained cells or performing colorimetric ALP activity assay with cell lysates. The ALP activity assay is based on the dephosphorylation of a phosphatase substrate (*e.g.* p-nitrophenyl phosphate) by ALP.

ALP quantification, although being a valuable and straightforward assay, requires cell lysis to release the intracellular ALP, making it an end-point method. Moreover, ALP values require a correlation to cell numbers, thus involving an additional measurement to normalize the obtained ALP values. These normalized values are often obtained by measuring total cell number or total protein content in the same lysates prepared for ALP quantification.

Alizarin Red S. Another later-stage differentiation marker is extracellular calcium deposition (mineralization). This can be determined using methods such as the Alizarin Red S staining, which binds to calcium forming a poorly soluble salt [155]. This calcium-alizarin red S appears as a bright red stain at the bottom of the well where cells are cultured. The results can either be analysed qualitatively by imaging the cells or quantitatively by extracting the stain and quantifying its absorbance.

Even though Alizarin Red S is a commonly used method to determine osteoblast maturation and matrix deposition and mineralization, concerns have been raised regarding its specificity and therefore its relevance. To obtain more conclusive results, it has been suggested to use this staining (or the other common alternative, von Kossa staining) in combination with other methods [156,157]. In addition, although not typically used in the studies selected for this review, more recently, Raman and infrared spectroscopy have been explored as tools for matrix deposition and mineralization analysis [158–160]. As these are label-free and non-destructive techniques, these methods have attracted increasing attention over the past years.

Migration. Within the context of osteoimmunology, it is important to unravel whether the chemokines released by the immune cells would attract MSCs into the damaged area. The assay basically consists of placing transwells (with a pore size large enough to allow MSCs migration, generally 8 µm), immersed in a well containing a biomaterial and inflammatory cells, which may cause the release of chemoattractive factors in the medium. The capability of cells to migrate can also be evaluated with a scratch assay, which in brief involves scraping a monolayer of cells and evaluating the migration of the cells repopulating the area [118].

Although these assays come with advantage of simplicity, one can argue that the significance of the results is limited. In the case of transwells, the cells have to cross a membrane that has poor physiological relevance (membrane's thickness is of hundreds of micrometres and pores of tens of micrometres). Moreover, time-lapse data are difficult to obtain with conventional microscopy and live cell imaging of this process is complex [161]. Some limitations to highlight regarding the scratch test are its low reproducibility (cell monolayer is scraped manually [86,118]) as well as its inability to evaluate non-adherent cells and to measure cell chemotaxis [161].

4.2.5. Discussion of the materials evaluated and the methods used

Among the articles considered in this review, most of the studies on osteoimmunology were performed with ceramics and polymers, and a few with composites and metals. The ceramics that were evaluated are hydroxyapatite, tricalcium phosphate, biphasic calcium phosphate and bioactive glass. A variety of different polymers were assessed, of which PEEK and chitosan materials were assayed several times. Regarding the physicochemical properties, chemistry and roughness are the properties that are more commonly evaluated. Finally, there are also some articles that evaluated only a material type with a fixed physicochemical composition.

All three methodological approaches (direct co-culture and indirect co-culture by means of transwells or CM) were used to evaluate the OIM properties of polymers and composites. In contrast, the OIM properties of ceramics and metals were mainly evaluated with CM. There is no clear reason accounting for the methodological choice since any material could in principle be evaluated with any of the approaches. The choice may instead have to do with the familiarity that a research group has with a certain methodology or may be linked to existing work, for example to be able to compare the outcome with previous results.

As can be visualized in Table 4, most of the assays performed with immune cells, which had the aim to analyse the cellular viability, morphology, gene expression, protein characterization (ELISA, Western blot, flow cytometry and protein array) and oxidative compounds, were performed in monoculture. For the skeletal cells, the assays evaluating the differentiation (gene expression, ALP and mineralization) or migration of skeletal cells were almost always performed in co-culture with immune cells. In fact, in studies on osteoimmunology, co-cultures are crucial to determine whether the exposure of immune cells with a biomaterial caused a release of factors that would in turn result in the differentiation of skeletal cells. For this reason, any monoculture study with immune cells could actually be considered as a preliminary evaluation needed to understand the environment created by immune cells that in turn caused a specific response to skeletal cells.

Up to now, all the studies described have been performed using conventional cell culture methods. However, there are other methodologies that are currently used to evaluate biomaterials, which in the future could be adapted to evaluate the OIM properties of biomaterials. These more recent approaches are explained in the next section.

5. Advanced approaches to assess the biological properties of biomaterials

As mentioned in the introduction, the recognition of the importance of osteoimmunology has led to a shift in the traditional evaluation of biomaterials for bone repair. The focus is no longer solely put on osteogenesis and osseointegration, but instead also on the immune system and in particular its interaction with the skeletal system. Even though this insight has increased the physiological relevance of *in vitro* evaluations, the typical experimental set-ups used to assess the biological properties of biomaterials, and with that, their immunomodulatory capacity, are still strongly centred on conventional approaches that involve standard tissue culture plastic. Nowadays, driven by the poor correlation between *in vitro* and *in vivo* biomaterial assessments [162], the added value of mimicking the natural *in vivo* microenvironment is widely acknowledged and fuelling the development of alternative *in vitro* platforms that capture the physiological characteristics of bone and

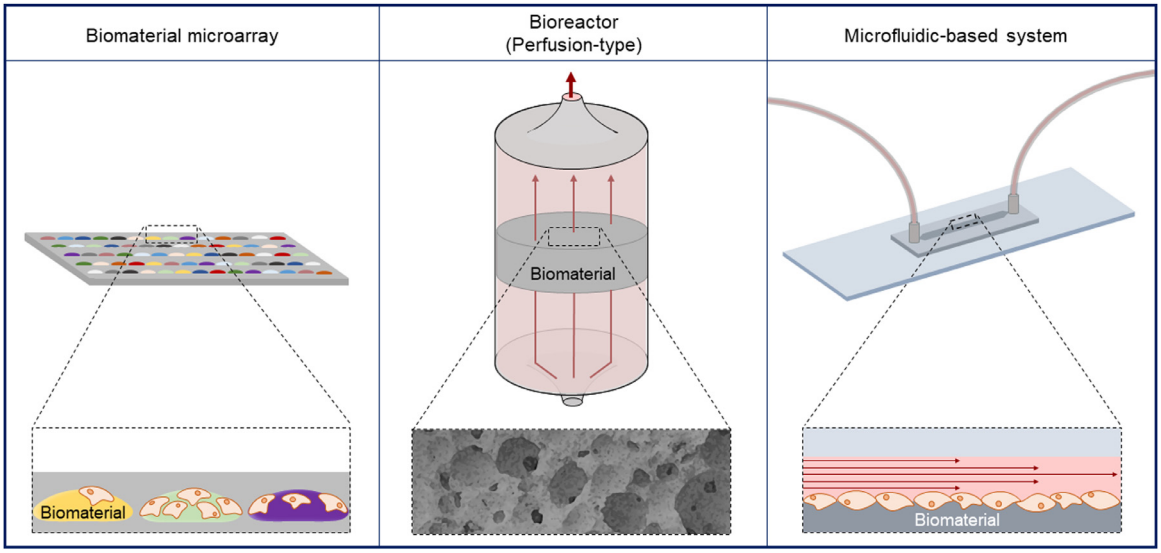

Fig. 3. Schematic of the advanced methods that can be used to evaluate biomaterials: biomaterial microarrays, bioreactors, microfluidic-based methods.

the bone environment. Moreover, stimulated by the rapid advances in the biomaterial field, the demand for high-throughput and cost-effective screening methods is increasing.

The goal of this section is to highlight advanced methods that are currently available and/or being explored to assess the biological properties of biomaterials for bone repair. The methods covered are microarrays, bioreactors and microfluidic-based platforms, in particular those inspired by organ-on-chip technology for the latter (Fig. 3). Although not widely applied to the field of osteoimmunology yet, the significant advances made in each of these methods until now show promising for translation to the field of osteoimmunology in the future.

5.1. Biomaterial microarrays

As mentioned in Section 4.2.2 'Methods used for both skeletal cells and immune cells', typically, microarrays are used to measure large amounts of biological samples (*e.g.* DNA, proteins) simultaneously. However, microarrays have significantly evolved and are no longer only used to screen a myriad of biological samples, but also tailored to evaluate a multitude of biomaterials at the same time. These so-called biomaterial microarrays are generated by dispensing the biomaterials in a uniform arrangement on a microarray surface (Fig. 3). This surface is a key factor in the success of the approach and should not only be non-toxic, but also prevent non-specific adhesion of cells and be compatible with the differences among the biomaterials of interest. Typically, the biomaterials are dispensed on the microarray surface using contact or non-contact printing methods, the latter referring to whether the printing tip comes in contact with the microarray surface (*e.g.* microstamping and nano-tip) or not (*e.g.* piezoelectric or thermal ink-jet).

One of the first biomaterial microarrays was proposed more than 15 years ago by Anderson et al. [163]. Over 1,700 cell-material interactions were screened, using an array with the size of a typical microscopy slide. The biomaterial array, containing acrylate-based biomaterials, was seeded with either human embryonic stem cells or mouse myoblasts and tested for each biomaterial's ability to support cell growth. The results showed selective and cell-type specific support on the different biomaterials, which the authors mentioned could be of particular interest when working with multicellular tissue-engineered constructs.

Since this early work, biomaterial microarrays have been applied to a number of biomaterial-cell combinations, which also include studies relevant to biomaterials for bone repair. For example, Khan *et al.*, developed a biomaterial microarray containing 135 polymer blends to identify cell-compatible polymers that were able to support several human skeletal cell types [164]. Very recently and for the first time, a biomaterial microarray approach was used to study the OIM properties of concave and convex nanomaterials [90]. In that work, RAW 264.7 cells were firstly grown on the different microarrays to prepare CM. This CM was subsequently used to culture, among others, MSCs and assess their proliferation and expression of osteogenic-related markers. Although not within the scope of this review, we would like to highlight the polysaccharide-based array containing 36 biomaterials that was implanted into an animal model to capture the effect of the immune response [165].

Even though biomaterial microarrays may offer a suitable tool for simultaneous screening of a large number of cell-biomaterial interactions, there are certain factors that should not be overlooked. Firstly, for high-throughput screening, an automated, rapid and accurate patterning of biomaterials on the array is required, which is not always that evident. In addition, although it would be desirable to miniaturize the biomaterials for high-throughput purposes, scaling down the biomaterials may be limited to the technical processing of biomaterials. Another non-trivial but very important factor is that crosstalk between different biomaterials should ideally be avoided, this to prevent confounding effects from soluble factors originating from neighbouring cells, biomolecule-loaded biomaterials, or biomaterial debris. This implies that the microarrays should be carefully designed and/or optimized to distinguish between cellular responses caused by the actual biomaterial of interest or other factors. Moreover, for overall success, the high-throughput set-up should be accompanied by suitable data collection and analysis methods, which allow assessment of the whole array. For more details on biomaterial microarrays and suggestions on how to address the above-mentioned challenges, the reader is referred to two excellent reviews [166,167].

5.2. Dynamic cell culture conditions

One way of providing more *in vivo*-like conditions to cells grown with/in/on biomaterials is by offering dynamic cell culture conditions. In the context of screening the biological properties of biomaterials, this can be visualized as a flow of cell culture medium throughout or along a biomaterial to which cells are ad-

hered (Fig. 3). Such dynamic conditions may not only allow a continuous (or otherwise predetermined) supply of nutrients, oxygen and removal of waste, but also come with the great advantage of providing mechanical stimulation. The latter is especially interesting when considering the mechano-responsiveness of bone cells, particularly to fluid-induced shear stress and deformation. To create such a more physiologically relevant biomaterial testing environment, bioreactors or microfluidic systems can be used.

5.2.1. Bioreactors

For orthopaedic applications, three main types of bioreactors are typically explored [168]. These are the spinner flasks, rotating wall vessels and perfusion systems. While the spinner flasks and rotating wall vessels are mainly effective at providing a homogeneous medium solution along the outer surface of a biomaterial, a perfusion system offers the possibility to perfuse media throughout a porous material, which more effectively exposes the cells to flow-induced shear stress. Apart from that, design features that allow additional mechanical stimulation (*e.g.* compression and tension) of the biomaterial-cell construct may be incorporated [169]. For example, upon stimulation by local mechanical strain, mineralization of the ECM may be accelerated [170,171]. It has also been shown that the shear-stress that is intrinsically caused by the fluid moving along the bioreactor can enhance MSCs to mature towards bone-depositing osteoblasts [172]. It is worth mentioning that, generally, the main driving force for using bioreactors is to facilitate cell cultures throughout entire (porous) materials, rather than to characterize the biomaterial. However, few studies have specifically put emphasis on the characterization of biomaterials using bioreactors and the potential benefits this can offer for *in vitro* evaluations.

For instance, some studies have shown significant differences between cells grown in/on biomaterials cultured in bioreactors compared to cells grown under static cell culture conditions. To give an example, MC3T3-E1 cells cultured on polycaprolactone scaffolds that had different scaffold architectures showed only minimal differences in cell response when evaluated under static conditions [173]. However, when the cells were grown under dynamic conditions, osteogenic differentiation was correlated to scaffold architecture. In another study, Kluge *et al.* implemented a versatile bioreactor system to simultaneously test a variety of biomaterials and enable mechanical stretching [174]. In a relevant study by Seifert *et al.*, a bioreactor system was used to evaluate the crosstalk between immune cells and mesenchymal stem cells that were seeded in an agarose matrix. The results showed enhanced proliferation of MSCs in the presence of immune cells and suppressive effects of MSCs on pro-inflammatory cytokine release [175].

Even though bioreactors provide the opportunity for more *in vivo*-like cell culture conditions, several challenges remain. For instance, maintaining cells on a biomaterial under dynamic conditions is more complex than when using a traditional well plate, not only requiring additional or specialized equipment, but also highly interdisciplinary expertise. Apart from the cell maintenance, the evaluation of results may also be complicated, particularly when aiming to decouple the effects of the biomaterial, different cell types and the dynamic culture conditions. This is especially challenging from a practical point of view, as being able to make conclusive statements requires multiple controls. Moreover, depending on the bioreactor of choice, the type of biomaterials that can be analysed is limited. For example, if perfusion is performed through a porous scaffold, the architecture and interconnectivity of the pores will affect the flow distribution, making it difficult to precisely control the stimulation by fluid-flow and therefore to optimize and study the consequent cell response. From the practical point of view, bioreactors may require a large amount of cell culture media and biomaterials.

5.2.2. Microfluidic-based systems

Another way to provide dynamic cell culture conditions is by using microfluidic-based platforms, which, as recently highlighted by Mestres *et al.*, offer a promising approach to screen biomaterials [176]. Microfluidic technology allows cells to be cultured on biomaterials while being geometrically confined by channels of only hundreds of micrometres, thereby providing a more physiologically relevant microenvironment compared to classical macroscale cultures (*i.e.* static well plate cultures or macroscale bioreactors). In addition, microfluidic technology enables controlled perfusion of cells and offers the advantage of adjusting relevant microenvironmental parameters, such as fluid shear stress, biochemical concentration gradients and environmental cues. Apart from the shear stress, which is intrinsically created upon fluid flow within microfluidic channels, microfluidic systems provide the opportunity to incorporate other mechanical stimuli, for example compressive strain by using flexible membranes or magnets [177,178].

Although this field is still in its infancy, multiple studies have already successfully integrated biomaterials in microfluidic systems and shown the possibility for biological characterization [111,179–182]. Recently, medical grade titanium was integrated into a microfluidic system and subsequently characterized for its biological properties over a period of 10 days [182]. Cell proliferation and differentiation studies with MC3T3-E1 cells revealed an increase in cell proliferation, but not differentiation, of cells grown in this micrometric dynamic environment, suggesting that proliferation was the dominating process in detriment of differentiation. Interestingly, this trend was not found when the cells were grown on the biomaterial under static conditions on standard tissue culture plastic. Overall, this work illustrated the importance of optimizing *in vitro* cell culture conditions and how this may affect biomaterial testing outcomes. Another example is given by Barata *et al.*, who integrated micropatterned polylactic acid on-chip, which allowed evaluation of the effect of biomaterial geometries on human MG-63 osteosarcoma cell morphology and distribution, both under perfusion and diffusion flow regimes [181].

To date, multiple works have reported on the evaluation of bone cells or immune cells in microfluidic systems. For bone cells, this is particularly in the context of bone cell function, bone regeneration, cancer metastasis to bone and vascularization [183]. For immune cells, most work has focused on the interaction of immune cells with tumour or endothelial cells and inflammation, including single-cell analysis [184,185]. However, no studies have been reported that combine microfluidics with biomaterials and osteoimmunology.

In addition to providing a highly controlled cell culture environment that can mimic physiological conditions more closely, microfluidic approaches also require lower amounts of reagents and cells, making it a cost-effective technique. However, apart from the already mentioned points related to working under dynamic conditions, such as the increased complexity in experimental set-up, operation of the system and interpretation of the results, working on such a small scale brings other challenges. For example, the typical biochemical assays and instruments used for analysis are often targeting macroscale cultures, meaning that optimization may be necessary to obtain data from the low amount of cells and volumes associated with the micron-scale. In addition, integration of certain biomaterials may be more demanding than others. The integration may be particularly difficult for highly porous or fragile materials, as the micrometric channels could be blocked by particles that detach from the biomaterial. Moreover, for heterogeneous biomaterials it may be difficult to include a biomaterial with small

enough dimensions that is representative of the whole chemistry or structure [176].

5.3. Promise of the advanced methods in the context of osteoimmunology

Even though these advanced methods have not been widely applied to assess the biological properties of biomaterials in the context of osteoimmunology yet, their success to evaluate biomaterials could be potentially expanded to the complex field of osteoimmunology. In fact, in the majority of cases, the already existing approaches could be translated to study OIM properties of biomaterials in a relatively easy manner, namely by selecting different or additional relevant cell types and by taking the traditional experimental approaches (as described in Section 4.1 'Experimental approaches') into account.

Although these advanced approaches show great promise and may offer an advanced tool to study osteoimmunology under more physiologically relevant conditions, several general challenges exist, limiting their rapid and widespread use. These challenges are mainly related to the lack of standardization, which is on its turn linked to the fact that using these methods to screen biomaterials is a fairly new concept that is not fully explored yet. In most cases, these platforms have to be adapted to a specific need or research question, requiring specific solutions for experimental set-up, data acquisition and data analysis, which could lead to different versions of the same approach. This hinders the exchange among different research groups, as well as gaining general interest from the scientific community. Moreover, for these methods to advance and accelerate their routine use, it should be proven that they have additional value over the classical methods. This will require testing of multiple biomaterials and comparing the results to evaluations performed using the classical *in vitro* methods and subsequently correlating these with *in vivo* results.

6. Concluding remarks

The success of implanted biomaterials is dependent on the osteoimmunological response they elicit. Simply put, a biomaterial intended to fill a critical size bone defect should induce bone formation which, in its early stages, involves a spatiotemporally limited inflammatory response. In contrast, during later stages of bone resorption and remodelling, an induction of chronic inflammatory responses must be avoided. Understanding the complex interaction between biomaterials, skeletal cells and immune cells is therefore paramount for the biomaterial field to advance and move towards new biomaterial designs that support the synergy between both cell types and enhance healing.

In this review, we discussed the experimental approaches that are nowadays used to assess the OIM properties of biomaterials *in vitro*. Based on the articles under study, we observed that the versatility of studies in the field of osteoimmunology has resulted in a long list of possible cell culture approaches and methodologies to analyse the cell response. The lack of consensus in this nascent field makes it challenging to standardize experimental set-ups and to extrapolate results regardless of the type of biomaterial or cell system studied. By gathering the information relevant to the different stages of experimental design, we aimed to provide the reader with an easy-to-use guide to the topic, intending to increase understanding and facilitate the planning of an experiment on osteoimmunology.

From the different possible approaches to culture immune and skeletal cells with a biomaterial, the most common one is to use a CM. This approach avoids the complexity of co-culture studies, either directly or using transwells; however, it overlooks the direct and dynamic interactions between both cell types. In fact, the assessment of the dynamic interactions between immune and skeletal cells in contact with biomaterials could be the cornerstone for the future of studies on osteoimmunology. Additionally, the role of angiogenesis in inflammation and remodelling reinforce the need for such dynamic and multisystem approaches.

Traditional approaches using tissue culture plastic to evaluate biomaterials may in the future be replaced by emerging advanced approaches such as microarrays, bioreactors, and microfluidic-based systems. Such approaches allow multiple measurements at once and can provide dynamic cell culture environment, offering more physiologically relevant screening conditions. Although not yet applied to the field of osteoimmunology, research efforts so far have demonstrated the added value that these methods can offer. Despite the several challenges that remain, which are mainly related to the lack of standardization of and experience with these methods, we foresee that increasing the physiological relevance of *in vitro* biomaterial screening will help to design optimal biomaterials for enhanced healthcare applications.

Declaration of Competing Interest

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2021.05.038.

References

[1] J.J. Jandinski, Osteoclast activating factor is now interleukin-1 beta: historical perspective and biological implications, J. Oral Pathol. Med. 17 (1988) 145–152, doi:10.1111/j.1600-0714.1988.tb01515.x.
[2] F.E. Dewhirst, P.P. Stashenko, J.E. Mole, T. Tsurumachi, Purification and partial sequence of human osteoclast-activating factor: identity with interleukin 1 beta, J. Immunol. 135 (1985) 2562–2568 http://www.ncbi.nlm.nih.gov/pubmed/3875658.
[3] M. Horowitz, A. Vignery, R.K. Gershon, R. Baron, Thymus-derived lymphocytes and their interactions with macrophages are required for the production of osteoclast-activating factor in the mouse, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 2181–2185, doi:10.1073/pnas.81.7.2181.
[4] J.E. Horton, L.G. Raisz, H.A. Simmons, J.J. Oppenheim, S.E. Mergenhagen, Bone resorbing activity in supernatant fluid from cultured human peripheral blood leukocytes, Science (80-.). 177 (1972) 793–795. https://doi.org/10.1126/science.177.4051.793.
[5] J.R. Arron, Y. Choi, Bone versus immune system, Nature 408 (2000) 535–536, doi:10.1038/35046196.
[6] M.C. Walsh, N. Kim, Y. Kadono, J. Rho, S.Y. Lee, J. Lorenzo, Y. Choi, OSTEOIMMUNOLOGY: Interplay Between the Immune System and Bone Metabolism, Annu. Rev. Immunol. 24 (2006) 33–63, doi:10.1146/annurev.immunol.24.021605.090646.
[7] E.Thomas Pashuck, M.M. Stevens, Designing regenerative biomaterial therapies for the clinic, Sci. Transl. Med. 4 (2012) 160sr4-160sr4, doi:10.1126/scitranslmed.3002717.
[8] I. Roato, D. Caldo, L. D'Amico, P. D'Amelio, L. Godio, S. Patanè, F. Astore, G. Grappiolo, M. Boggio, R. Scagnelli, L. Molfetta, R. Ferracini, Osteoclastogenesis in peripheral blood mononuclear cell cultures of periprosthetic osteolysis patients and the phenotype of T cells localized in periprosthetic tissues, Biomaterials 31 (2010) 7519–7525, doi:10.1016/j.biomaterials.2010.06.027.

[9] M. Tsukasaki, H. Takayanagi, Osteoimmunology: evolving concepts in bone–immune interactions in health and disease, Nat. Rev. Immunol. 19 (2019) 626–642, doi:10.1038/s41577-019-0178-8.
[10] J. Lee, H. Byun, S.K. Madhurakkat Perikamana, S. Lee, H. Shin, Current advances in immunomodulatory biomaterials for bone regeneration, Adv. Healthc. Mater. 8 (2019) 1–20, doi:10.1002/adhm.201801106.
[11] H. Takayanagi, Osteoimmunology: Shared mechanisms and crosstalk between the immune and bone systems, Nat. Rev. Immunol. 7 (2007) 292–304, doi:10.1038/nri2062.
[12] L. Chung, D.R. Maestas, F. Housseau, J.H. Elisseeff, Key players in the immune response to biomaterial scaffolds for regenerative medicine, Adv. Drug Deliv. Rev. 114 (2017) 184–192, doi:10.1016/j.addr.2017.07.006.
[13] L. Tätting, O. Sandberg, M. Bernhardsson, J. Ernerudh, P. Aspenberg, Isolated metaphyseal injury influences unrelated bones: A flow cytometric study of tibia and humerus in mice, Acta Orthop 88 (2017) 223–230, doi:10.1080/17453674.2016.1274587.
[14] G. Eghbali-Fatourechi, S. Khosla, A. Sanyal, W.J. Boyle, D.L. Lacey, B.L. Riggs, Role of RANK ligand in mediating increased bone resorption in early postmenopausal women, J. Clin. Invest. 111 (2003) 1221–1230, doi:10.1172/jci17215.
[15] H. Tawfeek, B. Bedi, J.-Y. Li, J. Adams, T. Kobayashi, M.N. Weitzmann, H.M. Kronenberg, R. Pacifici, Disruption of PTH Receptor 1 in T Cells Protects against PTH-Induced Bone Loss, PLoS One 5 (2010) e12290, doi:10.1371/journal.pone.0012290.
[16] V.A. Triant, T.T. Brown, H. Lee, S.K. Grinspoon, Fracture prevalence among human immunodeficiency virus (HIV)-infected versus non-HIV-infected patients in a large U.S. healthcare system, J. Clin. Endocrinol. Metab. 93 (2008) 3499–3504, doi:10.1210/jc.2008-0828.
[17] G. Schett, J.P. David, The multiple faces of autoimmune-mediated bone loss, Nat. Rev. Endocrinol. 6 (2010) 698–706, doi:10.1038/nrendo.2010.190.
[18] G. Mori, P. D'Amelio, R. Faccio, G. Brunetti, Bone-immune cell crosstalk: Bone diseases, J. Immunol. Res. 2015 (2015), doi:10.1155/2015/108451.
[19] K. Schmidt-Bleek, B.J. Kwee, D.J. Mooney, G.N. Duda, Boon and bane of inflammation in bone tissue regeneration and its link with angiogenesis, Tissue Eng. - Part B Rev. 21 (2015) 354–364, doi:10.1089/ten.teb.2014.0677.
[20] C. Tzioupis, P.V. Giannoudis, Prevalence of long-bone non-unions, Injury 38 (2007) S3–S9, doi:10.1016/S0020-1383(07)80003-9.
[21] N. Kohli, S. Ho, S.J. Brown, P. Sawadkar, V. Sharma, M. Snow, E. García-Gareta, Bone remodelling in vitro: Where are we headed?: -A review on the current understanding of physiological bone remodelling and inflammation and the strategies for testing biomaterials in vitro, Bone 110 (2018) 38–46, doi:10.1016/j.bone.2018.01.015.
[22] E. Zhao, H. Xu, L. Wang, I. Kryczek, K. Wu, Y. Hu, G. Wang, W. Zou, Bone marrow and the control of immunity, Cell. Mol. Immunol. 9 (2012) 11–19, doi:10.1038/cmi.2011.47.
[23] M. Timlin, D. Toomey, C. Condron, C. Power, J. Street, P. Murray, D. Bouchier-Hayes, Fracture hematoma is a potent proinflammatory mediator of neutrophil function, J. Trauma - Inj. Infect. Crit. Care. 58 (2005) 1223–1229, doi:10.1097/01.TA.0000169866.88781.F1.
[24] A.R. Pettit, M.K. Chang, D.A. Hume, L.-J. Raggatt, Osteal macrophages: a new twist on coupling during bone dynamics, Bone 43 (2008) 976–982, doi:10.1016/j.bone.2008.08.128.
[25] B.N. Brown, B.D. Ratner, S.B. Goodman, S. Amar, S.F. Badylak, Macrophage polarization: an opportunity for improved outcomes in biomaterials and regenerative medicine, Biomaterials 33 (2012) 3792–3802, doi:10.1016/j.biomaterials.2012.02.034.
[26] C.J. Ferrante, S.J. Leibovich, Regulation of Macrophage Polarization and Wound Healing, Adv. Wound Care. 1 (2012) 10–16, doi:10.1089/wound.2011.0307.
[27] M. Ponzetti, N. Rucci, Updates on osteoimmunology: What's new on the cross-talk between bone and immune system, Front. Endocrinol. (Lausanne). 10 (2019) 236, doi:10.3389/fendo.2019.00236.
[28] A. Mantovani, S.K. Biswas, M.R. Galdiero, A. Sica, M. Locati, Macrophage plasticity and polarization in tissue repair and remodelling, J. Pathol. 229 (2013) 176–185, doi:10.1002/path.4133.
[29] M.M. McDonald, A. Morse, K. Mikulec, L. Peacock, P.A. Baldock, P.J. Kostenuik, D.G. Little, Matrix metalloproteinase-driven endochondral fracture union proceeds independently of osteoclast activity, J. Bone Miner. Res. 28 (2013) 1550–1560, doi:10.1002/jbmr.1889.
[30] C. Nich, S.B. Goodman, Role of macrophages in the biological reaction to wear debris from joint replacements, J. Long. Term. Eff. Med. Implants. 24 (2014) 259–266, doi:10.1615/jlongtermeffmedimplants.2014010562.
[31] B. ten Harkel, T. Schoenmaker, D.I. Picavet, N.L. Davison, T.J. de Vries, V. Everts, The foreign body giant cell cannot resorb bone, but dissolves hydroxyapatite like osteoclasts, PLoS One 10 (2015) e0139564, doi:10.1371/journal.pone.0139564.
[32] M.G. Dorrington, I.D.C. Fraser, NF-κB signaling in macrophages: dynamics, crosstalk, and signal integration, Front. Immunol. 10 (2019) 705, doi:10.3389/fimmu.2019.00705.
[33] P. Varela, S. Sartori, R. Viebahn, J. Salber, G. Ciardelli, Macrophage immunomodulation: an indispensable tool to evaluate the performance of wound dressing biomaterials, J. Appl. Biomater. Funct. Mater. (2019) 17, doi:10.1177/2280800019830355.
[34] M.L. Novak, T.J. Koh, Macrophage phenotypes during tissue repair, J. Leukoc. Biol. 93 (2013) 875–881, doi:10.1189/jlb.1012512.
[35] S. Gordon, Alternative activation of macrophages, Nat. Rev. Immunol. 3 (2003) 23–35, doi:10.1038/nri978.
[36] P. Krzyszczyk, R. Schloss, A. Palmer, F. Berthiaume, The role of macrophages in acute and chronic wound healing and interventions to promote pro-wound healing phenotypes, Front. Physiol. 9 (2018), doi:10.3389/fphys.2018.00419.
[37] A. Mantovani, A. Sica, S. Sozzani, P. Allavena, A. Vecchi, M. Locati, The chemokine system in diverse forms of macrophage activation and polarization, Trends Immunol 25 (2004) 677–686, doi:10.1016/j.it.2004.09.015.
[38] K.L. Spiller, R.R. Anfang, K.J. Spiller, J. Ng, K.R. Nakazawa, J.W. Daulton, G. Vunjak-Novakovic, The role of macrophage phenotype in vascularization of tissue engineering scaffolds, Biomaterials 35 (2014) 4477–4488, doi:10.1016/j.biomaterials.2014.02.012.
[39] Y. Yao, X.H. Xu, L. Jin, Macrophage polarization in physiological and pathological pregnancy, Front. Immunol. 10 (2019) 792, doi:10.3389/fimmu.2019.00792.
[40] Z. Chen, T. Klein, R.Z. Murray, R. Crawford, J. Chang, C. Wu, Y. Xiao, Osteoimmunomodulation for the development of advanced bone biomaterials, Mater. Today. 19 (2016) 304–321, doi:10.1016/j.mattod.2015.11.004.
[41] L. Chung, D.R. Maestas, F. Housseau, J.H. Elisseeff, Key players in the immune response to biomaterial scaffolds for regenerative medicine, Adv. Drug Deliv. Rev. 114 (2017) 184–192, doi:10.1016/j.addr.2017.07.006.
[42] J. Kim, W.A. Li, W. Sands, D.J. Mooney, Effect of pore structure of macroporous poly(lactide-co-glycolide) scaffolds on the in vivo enrichment of dendritic cells, ACS Appl. Mater. Interfaces. 6 (2014) 8505–8512, doi:10.1021/am501376n.
[43] E.M. Sussman, M.C. Halpin, J. Muster, R.T. Moon, B.D. Ratner, Porous implants modulate healing and induce shifts in local macrophage polarization in the foreign body reaction, Ann. Biomed. Eng. 42 (2014) 1508–1516, doi:10.1007/s10439-013-0933-0.
[44] Z. Chen, A. Bachhuka, F. Wei, X. Wang, G. Liu, K. Vasilev, Y. Xiao, Nanotopography-based strategy for the precise manipulation of osteoimmunomodulation in bone regeneration, Nanoscale 9 (2017) 18129–18152, doi:10.1039/c7nr05913b.
[45] K.A. Barth, J.D. Waterfield, D.M. Brunette, The effect of surface roughness on RAW 264.7 macrophage phenotype, J. Biomed. Mater. Res. - Part A. 101 A (2013) 2679–2688, doi:10.1002/jbm.a.34562.
[46] J. Fink, R. Fuhrmann, T. Scharnweber, R.P. Franke, Stimulation of monocytes and macrophages: possible influence of surface roughness, Clin. Hemorheol. Microcirc. (2008) 205–212, doi:10.3233/CH-2008-1090.
[47] G. Mestres, M. Espanol, W. Xia, C. Persson, M.-P. Ginebra, M.K. Ott, Inflammatory Response to Nano- and Microstructured Hydroxyapatite, PLoS One 10 (2015) e0120381, doi:10.1371/journal.pone.0120381.
[48] J.M. Sadowska, F. Wei, J. Guo, J. Guillem-Marti, M.-P. Ginebra, Y. Xiao, Effect of nano-structural properties of biomimetic hydroxyapatite on osteoimmunomodulation, Biomaterials 181 (2018) 318–332, doi:10.1016/j.biomaterials.2018.07.058.
[49] Z. Chen, A. Bachhuka, S. Han, F. Wei, S. Lu, R.M. Visalakshan, K. Vasilev, Y. Xiao, Tuning chemistry and topography of nanoengineered surfaces to manipulate immune response for bone regeneration applications, ACS Nano 11 (2017) 4494–4506, doi:10.1021/acsnano.6b07808.
[50] A. Diez-Escudero, M. Espanol, M. Bonany, X. Lu, C. Persson, M.-P. Ginebra, Heparinization of beta tricalcium phosphate: osteo-immunomodulatory effects, Adv. Healthc. Mater. 7 (2018) 1700867, doi:10.1002/adhm.201700867.
[51] J.A. Jones, D.T. Chang, H. Meyerson, E. Colton, K.K. Il, T. Matsuda, J.M. Anderson, Proteomic analysis and quantification of cytokines and chemokines from biomaterial surface-adherent macrophages and foreign body giant cells, J. Biomed. Mater. Res. - Part A. 83 (2007) 585–596, doi:10.1002/jbm.a.31221.
[52] R. Sridharan, D.J. Kelly, F. O'Brien, Substrate stiffness modulates the crosstalk between MSCs and macrophages, J. Biomech. Eng. (2020), doi:10.1115/1.4048809.
[53] O. Veiseh, J.C. Doloff, M. Ma, A.J. Vegas, H.H. Tam, A.R. Bader, J. Li, E. Langan, J. Wyckoff, W.S. Loo, S. Jhunjhunwala, A. Chiu, S. Siebert, K. Tang, J. Hollister-Lock, S. Aresta-Dasilva, M. Bochenek, J. Mendoza-Elias, Y. Wang, M. Qi, D.M. Lavin, M. Chen, N. Dholakia, R. Thakrar, I. Lacík, G.C. Weir, J. Oberholzer, D.L. Greiner, R. Langer, D.G. Anderson, Size- and shape-dependent foreign body immune response to materials implanted in rodents and non-human primates, Nat. Mater. 14 (2015) 643–651, doi:10.1038/nmat4290.
[54] F.O. Novais, R.C. Santiago, A. Báfica, R. Khouri, L. Afonso, V.M. Borges, C. Brodskyn, M. Barral-Netto, A. Barral, C.I. de Oliveira, Neutrophils and macrophages cooperate in host resistance against leishmania braziliensis infection, J. Immunol. 183 (2009) 8088–8098, doi:10.4049/jimmunol.0803720.
[55] K. Nathan, L.Y. Lu, T. Lin, J. Pajarinen, E. Jämsen, J.-F. Huang, M. Romero-Lopez, M. Maruyama, Y. Kohno, Z. Yao, S.B. Goodman, Precise immunomodulation of the M1 to M2 macrophage transition enhances mesenchymal stem cell osteogenesis and differs by sex, Bone Joint Res 8 (2019) 481–488, doi:10.1302/2046-3758.810.BJR-2018-0231.R2.
[56] R. Huang, X. Wang, Y. Zhou, Y. Xiao, RANKL-induced M1 macrophages are involved in bone formation, Bone Res 5 (2017) 17019, doi:10.1038/boneres.2017.19.
[57] J. Sadowska, M.-P. Ginebra, Inflammation and biomaterials: Role of the immune response in bone regeneration by synthetic bone grafts, J. Mater. Chem. B. 8 (2020) 2418–2430, doi:10.1039/D0TB01379J.
[58] S. Franz, S. Rammelt, D. Scharnweber, J.C. Simon, Immune responses to implants – A review of the implications for the design of immunomodulatory biomaterials, Biomaterials 32 (2011) 6692–6709, doi:10.1016/j.biomaterials.2011.05.078.
[59] H. Ito, Chemokines in mesenchymal stem cell therapy for bone repair: A novel concept of recruiting mesenchymal stem cells and the possible cell sources, Mod. Rheumatol. 21 (2011) 113–121, doi:10.1007/s10165-010-0357-8.

[60] J.J. El-Jawhari, E. Jones, P.V. Giannoudis, The roles of immune cells in bone healing; what we know, do not know and future perspectives, Injury 47 (2016) 2399–2406, doi:10.1016/j.injury.2016.10.008.
[61] L. Chiossone, R. Conte, G.M. Spaggiari, M. Serra, C. Romei, F. Bellora, F. Becchetti, A. Andaloro, L. Moretta, C. Bottino, Mesenchymal stromal cells induce peculiar alternatively activated macrophages capable of dampening both innate and adaptive immune responses, Stem Cells 34 (2016) 1909–1921, doi:10.1002/stem.2369.
[62] A. Rutkovskiy, K.-O. Stensløkken, I.J. Vaage, Osteoblast Differentiation at a Glance, Med. Sci. Monit. Basic Res. 22 (2016) 95–106, doi:10.12659/msmbr.901142.
[63] J. Zhou, F. Wang, Y. Ma, F. Wei, Vitamin D3 contributes to enhanced osteogenic differentiation of MSCs under oxidative stress condition via activating the endogenous antioxidant system, Osteoporos. Int. 29 (2018) 1917–1926, doi:10.1007/s00198-018-4547-0.
[64] G. Chen, C. Deng, Y.P. Li, TGF-β and BMP signaling in osteoblast differentiation and bone formation, Int. J. Biol. Sci. 8 (2012) 272–288, doi:10.7150/ijbs.2929.
[65] A. Javed, H. Chen, F.Y. Ghori, Genetic and transcriptional control of bone formation, Oral Maxillofac. Surg. Clin. North Am. 22 (2010) 283–293, doi:10.1016/j.coms.2010.05.001.
[66] C. Zhang, Transcriptional regulation of bone formation by the osteoblast-specific transcription factor Osx, J. Orthop. Surg. Res. 5 (2010) 37, doi:10.1186/1749-799X-5-37.
[67] T. Schinke, G. Karsenty, Transcriptional control of osteoblast differentiation and function, in: Princ, Bone Biol (2002) 83–91, doi:10.1016/b978-012098652-1.50107-4.
[68] L.J. Raggatt, N.C. Partridge, Cellular and molecular mechanisms of bone remodeling, J. Biol. Chem. 285 (2010) 25103–25108, doi:10.1074/jbc.R109.041087.
[69] J.P. Bilezikian, T.L. Clemens, T.J. Martin, C.J. Rosen, Principles of bone biology, Second, Academic Press, 2019, doi:10.1016/C2015-1-01622-2.
[70] N.A. Sims, J.H. Gooi, Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption, Semin. Cell Dev. Biol. 19 (2008) 444–451, doi:10.1016/j.semcdb.2008.07.016.
[71] M. Zhou, S. Li, J.L. Pathak, Pro-inflammatory Cytokines and Osteocytes, Curr. Osteoporos. Rep. 17 (2019) 97–104, doi:10.1007/s11914-019-00507-z.
[72] V. Everts, J.M. Delaissé, W. Korper, D.C. Jansen, W. Tigchelaar-Gutter, P. Saftig, W. Beertsen, The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation, J. Bone Miner. Res. 17 (2002) 77–90, doi:10.1359/jbmr.2002.17.1.77.
[73] M.C. Horowitz, Y. Xi, K. Wilson, M.A. Kacena, Control of osteoclastogenesis and bone resorption by members of the TNF family of receptors and ligands, Cytokine Growth Factor Rev 12 (2001) 9–18, doi:10.1016/S1359-6101(00)00030-7.
[74] S.L. Teitelbaum, Bone Resorption by Osteoclasts, Science (80-.) 289 (2000) 1504–1508, doi:10.1126/science.289.5484.1504.
[75] S.L. Teitelbaum, Osteoclasts; culprits inflammatory osteolysis, Arthritis Res. Ther. 8 (2006) 201, doi:10.1186/ar1857.
[76] G. Spence, N. Patel, R. Brooks, W. Bonfield, N. Rushton, Osteoclastogenesis on hydroxyapatite ceramics: the effect of carbonate substitution, J. Biomed. Mater. Res. A. 92 (2010) 1292–1300, doi:10.1002/jbm.a.32373.
[77] J. Salbach, S. Kliemt, M. Rauner, T.D. Rachner, C. Goettsch, S. Kalkhof, M. von Bergen, S. Möller, M. Schnabelrauch, V. Hintze, D. Scharnweber, L.C. Hofbauer, The effect of the degree of sulfation of glycosaminoglycans on osteoclast function and signaling pathways, Biomaterials 33 (2012) 8418–8429, doi:10.1016/j.biomaterials.2012.08.028.
[78] J. Salbach-Hirsch, J. Kraemer, M. Rauner, S.A. Samsonov, M.T. Pisabarro, S. Moeller, M. Schnabelrauch, D. Scharnweber, L.C. Hofbauer, V. Hintze, The promotion of osteoclastogenesis by sulfated hyaluronan through interference with osteoprotegerin and receptor activator of NF-κB ligand/osteoprotegerin complex formation, Biomaterials 34 (2013) 7653–7661, doi:10.1016/j.biomaterials.2013.06.053.
[79] A.-M. Negrescu, M.-G. Necula, A. Gebaur, F. Golgovici, C. Nica, F. Curti, H. Iovu, M. Costache, A. Cimpean, In Vitro Macrophage Immunomodulation by Poly(ε-caprolactone) Based-Coated AZ31 Mg Alloy, Int. J. Mol. Sci. 22 (2021) 909, doi:10.3390/ijms22020909.
[80] A. Diez-Escudero, E. Torreggiani, G. Di Pompo, M. Espanol, C. Persson, G. Ciapetti, N. Baldini, M. Ginebra, Effect of calcium phosphate heparinization on the in vitro inflammatory response and osteoclastogenesis of human blood precursor cells, J. Tissue Eng. Regen. Med. 13 (2019) term.2872, doi:10.1002/term.2872.
[81] R.J. Miron, D.D. Bosshardt, OsteoMacs: Key players around bone biomaterials, Biomaterials 82 (2016) 1–19, doi:10.1016/j.biomaterials.2015.12.017.
[82] G. Kaur, J.M. Dufour, Cell lines: Valuable tools or useless artifacts., Spermatogenesis. 2 (2012) 1–5. https://doi.org/10.4161/spmg.19885.
[83] B. Taciak, M. Białasek, A. Braniewska, Z. Sas, P. Sawicka, Ł. Kiraga, T. Rygiel, M. Król, Evaluation of phenotypic and functional stability of RAW 264.7 cell line through serial passages, PLoS One 13 (2018) 1–13, doi:10.1371/journal.pone.0198943.
[84] M.C. Nielsen, M.N. Andersen, H.J. Møller, Monocyte isolation techniques significantly impact the phenotype of both isolated monocytes and derived macrophages in vitro, Immunology 159 (2020) 63–74, doi:10.1111/imm.13125.
[85] S. Mechiche Alami, S.C. Gangloff, D. Laurent-Maquin, Y. Wang, H. Kerdjoudj, Concise Review: In Vitro Formation of Bone - Like Nodules Sheds Light on the Application of Stem Cells for Bone Regeneration, Stem Cells Transl. Med. 5 (2016) 1587–1593, doi:10.5966/sctm.2015-0413.
[86] Y. Zhou, S. Han, L. Xiao, P. Han, S. Wang, J. He, J. Chang, C. Wu, Y. Xiao, Accelerated host angiogenesis and immune responses by ion release from mesoporous bioactive glass, J. Mater. Chem. B. 6 (2018) 3274–3284, doi:10.1039/C8TB00683K.
[87] C. Yang, C.C. Zhao, X.Y. Wang, M.C. Shi, Y.L. Zhu, L.G. Jing, C.T. Wu, J. Chang, Stimulation of osteogenesis and angiogenesis by micro/nano hierarchical hydroxyapatite via macrophage immunomodulation, Nanoscale 11 (2019) 17699–17708, doi:10.1039/c9nr05730g.
[88] M. Wang, Y. Yu, K. Dai, Z. Ma, Y. Liu, J. Wang, C. Liu, Improved osteogenesis and angiogenesis of magnesium-doped calcium phosphate cement: Via macrophage immunomodulation, Biomater. Sci. 4 (2016) 1574–1583, doi:10.1039/c6bm00290k.
[89] S. Cheng, D. Zhang, M. Li, X. Liu, Y. Zhang, S. Qian, F. Peng, Osteogenesis, angiogenesis and immune response of Mg-Al layered double hydroxide coating on pure Mg, Bioact. Mater. 6 (2021) 91–105, doi:10.1016/j.bioactmat.2020.07.014.
[90] S. Ni, D. Zhai, Z. Huan, T. Zhang, J. Chang, C. Wu, Nanosized concave pit/convex dot microarray for immunomodulatory osteogenesis and angiogenesis, Nanoscale 12 (2020) 16474–16488, doi:10.1039/d0nr03886e.
[91] T. Yao, Y. Asayama, Animal-cell culture media: History, characteristics, and current issues, Reprod. Med. Biol. 16 (2017) 99–117, doi:10.1002/rmb2.12024.
[92] M. Arora, Cell culture media: a review, Mater. Methods. 3 (2013) 1–29, doi:10.13070/mm.en.3.175.
[93] A.H. Ryu, W.L. Eckalbar, A. Kreimer, N. Yosef, N. Ahituv, Use antibiotics in cell culture with caution: genome-wide identification of antibiotic-induced changes in gene expression and regulation, Sci. Rep. 7 (2017) 1–9, doi:10.1038/s41598-017-07757-w.
[94] R.I. Freshney, Culture of Animal Cells, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2010, doi:10.1002/9780470649367.
[95] K. Meszaros, S. Aberle, M. White, J.B. Parent, Immunoreactivity and bioactivity of lipopolysaccharide-binding protein in normal and heat-inactivated sera, Infect. Immun. 63 (1995) 363–365, doi:10.1128/iai.63.1.363-365.1995.
[96] A. Bruinink, U. Tobler, M. Hälg, J. Grünert, Effects of serum and serum heat-inactivation on human bone derived osteoblast progenitor cells, J. Mater. Sci. Mater. Med. 15 (2004) 497–501, doi:10.1023/B:JMSM.0000021127.62879.a1.
[97] A. Nimura, T. Muneta, K. Otabe, H. Koga, Y.J. Ju, T. Mochizuki, K. Suzuki, I. Sekiya, Analysis of human synovial and bone marrow mesenchymal stem cells in relation to heat-inactivation of autologous and fetal bovine serums, BMC Musculoskelet. Disord. (2010) 11, doi:10.1186/1471-2474-11-208.
[98] Z. Chen, S. Ni, S. Han, R. Crawford, S. Lu, F. Wei, J. Chang, C. Wu, Y. Xiao, Nanoporous microstructures mediate osteogenesis by modulating the osteoimmune response of macrophages, Nanoscale 9 (2017) 706–718, doi:10.1039/C6NR06421C.
[99] L. Bai, Z. Du, J. Du, W. Yao, J. Zhang, Z. Weng, S. Liu, Y. Zhao, Y. Liu, X. Zhang, X. Huang, X. Yao, R. Crawford, R. Hang, D. Huang, B. Tang, Y. Xiao, A multifaceted coating on titanium dictates osteoimmunomodulation and osteo/angio-genesis towards ameliorative osseointegration, Biomaterials 162 (2018) 154–169, doi:10.1016/j.biomaterials.2018.02.010.
[100] H.R. Caires, T. Esteves, P. Quelhas, M.A. Barbosa, M. Navarro, C.R. Almeida, Macrophage interactions with polylactic acid and chitosan scaffolds lead to improved recruitment of human mesenchymal stem/stromal cells: a comprehensive study with different immune cells, J. R. Soc. Interface. 13 (2016) 20160570, doi:10.1098/rsif.2016.0570.
[101] C.E.A. Jochems, J.B.F. Van der Valk, F.R. Stafleu, V. Baumans, The use of fetal bovine serum: Ethical or scientific problem? ATLA Altern. to Lab. Anim. 30 (2002) 219–227, doi:10.1177/026119290203000208.
[102] M. Guha, N. Mackman, LPS induction of gene expression in human monocytes, Cell. Signal. 13 (2001) 85–94, doi:10.1016/S0898-6568(00)00149-2.
[103] S.L. Weinstein, M.R. Gold, A.L. DeFranco, Bacterial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages, Proc. Natl. Acad. Sci. 88 (1991) 4148–4152, doi:10.1073/pnas.88.10.4148.
[104] M. Dzietko, M. Hahnemann, O. Polley, M. Sifringer, U. Felderhoff-Mueser, C. Bührer, Effects of PMA (PHORBOL-12-MYRISTATE-13-ACETATE) on the Developing Rodent Brain, Biomed Res. Int. 2015 (2015) 1–9, doi:10.1155/2015/318306.
[105] X.Y. Li, Y. Wang, Z.G. Wang, Y.X. Qi, L.L. Li, P.B. Zhang, X.S. Chen, Y.B. Huang, Composite PLA/PEG/nHA/Dexamethasone Scaffold Prepared by 3D Printing for Bone Regeneration, Macromol. Biosci. 18 (2018), doi:10.1002/mabi.201800068.
[106] P.C. Qiu, M.B. Li, K. Chen, B. Fang, P.F. Chen, Z.B. Tang, X.F. Lin, S.W. Fan, Periosteal matrix-derived hydrogel promotes bone repair through an early immune regulation coupled with enhanced angio- and osteogenesis, Biomaterials (2020) 227, doi:10.1016/j.biomaterials.2019.119552.
[107] I. Fasolino, M.G. Raucci, A. Soriente, C. Demitri, M. Madaghiele, A. Sannino, L. Ambrosio, Osteoinductive and anti-inflammatory properties of chitosan-based scaffolds for bone regeneration, Mater. Sci. Eng. C. (2019) 105, doi:10.1016/j.msec.2019.110046.
[108] V. Bordoni, G. Reina, M. Orecchioni, G. Furesi, S. Thiele, C. Gardin, B. Zavan, G. Cuniberti, A. Bianco, M. Rauner, L.G. Delogu, Stimulation of bone formation by monocyte-activator functionalized graphene oxide: In vivo, Nanoscale 11 (2019) 19408–19421, doi:10.1039/c9nr03975a.
[109] S. Behera, D. Naskar, S. Sapru, P. Bhattacharjee, T. Dey, A.K. Ghosh, M. Mandal, S.C. Kundu, Hydroxyapatite reinforced inherent RGD containing silk fibroin composite scaffolds: Promising platform for bone tissue engineering, Nanomedicine 13 (2017) 1745–1759, doi:10.1016/j.nano.2017.02.016.

[110] J.M. Sadowska, F. Wei, J. Guo, J. Guillem-Marti, Z. Lin, M.P. Ginebra, Y. Xiao, The effect of biomimetic calcium deficient hydroxyapatite and sintered β-tricalcium phosphate on osteoimmune reaction and osteogenesis, Acta Biomater 96 (2019) 605–618, doi:10.1016/j.actbio.2019.06.057.
[111] J.H. Lee, H. Wang, J.B. Kaplan, W.Y. Lee, Effects of Staphylococcus epidermidis on osteoblast cell adhesion and viability on a Ti alloy surface in a microfluidic co-culture environment, Acta Biomater 6 (2010) 4422–4429, doi:10.1016/j.actbio.2010.05.021.
[112] P. Bhattacharjee, T.K. Maiti, D. Bhattacharya, S.K. Nandi, Effect of different mineralization processes on in vitro and in vivo bone regeneration and osteoblast-macrophage cross-talk in co-culture system using dual growth factor mediated non-mulberry silk fibroin grafted poly (€-caprolactone) nanofibrous scaffold, Colloids Surfaces B Biointerfaces 156 (2017) 270–281, doi:10.1016/j.colsurfb.2017.05.043.
[113] A. Gao, Q. Liao, L. Xie, G. Wang, W. Zhang, Y. Wu, P. Li, M. Guan, H. Pan, L. Tong, P.K. Chu, H. Wang, Tuning the surface immunomodulatory functions of polyetheretherketone for enhanced osseointegration, Biomaterials 230 (2020) 119642, doi:10.1016/j.biomaterials.2019.119642.
[114] Y. Sun, S. Liu, Y. Fu, X.X. Kou, D.Q. He, G.N. Wang, C.C. Fu, Y. Liu, Y.H. Zhou, Mineralized collagen regulates macrophage polarization during bone regeneration, J. Biomed. Nanotechnol. 12 (2016) 2029–2040, doi:10.1166/jbn.2016.2296.
[115] J. Ding, B. Chen, T. Lv, X. Liu, X. Fu, Q. Wang, L. Yan, N. Kang, Y. Cao, R. Xiao, Bone marrow mesenchymal stem cell-based engineered cartilage ameliorates polyglycolic acid/polylactic acid scaffold-induced inflammation through M2 polarization of macrophages in a pig model, Stem Cells Transl. Med. 5 (2016) 1079–1089, doi:10.5966/sctm.2015-0263.
[116] R. Sridharan, K.J. Genoud, D.J. Kelly, F.J. O'Brien, Hydroxyapatite particle shape and size influence MSC osteogenesis by directing the macrophage phenotype in collagen-hydroxyapatite scaffolds, ACS Appl. Bio Mater. 3 (2020) 7562–7574, doi:10.1021/acsabm.0c00801.
[117] P.L. Graney, S.-I. Roohani-Esfahani, H. Zreiqat, K.L. Spiller, In vitro response of macrophages to ceramic scaffolds used for bone regeneration, J. R. Soc. Interface. 13 (2016) 20160346, doi:10.1098/rsif.2016.0346.
[118] F. Zhao, W. Xie, W. Zhang, X. Fu, W. Gao, B. Lei, X. Chen, 3D Printing nanoscale bioactive glass scaffolds enhance osteoblast migration and extramembranous osteogenesis through stimulating immunomodulation, Adv. Healthc. Mater. 7 (2018) 1800361, doi:10.1002/adhm.201800361.
[119] S. Bourne, L.D.K. Buttery, I.D. Xynos, V. Episkopou, R.M. Winston, S.P.F. Hughes, J.M. Polak, Selective differentiation of osteoblasts and in vitro bone formation from murine embryonic stem cells, Second Smith Nephew Int. Symp. - Tissue Eng. 2000 Adv. Tissue Eng. Biomater. Cell Signal. 7 (2000) 132.
[120] F. Langenbach, J. Handschel, Effects of dexamethasone, ascorbic acid and β-glycerophosphate on the osteogenic differentiation of stem cells in vitro, Stem Cell Res. Ther. 4 (2013) 117, doi:10.1089/ten.teb.2011.0199.
[121] C.L. Li, L. Yang, X.H. Ren, M. Lin, X.L. Jiang, D.N. Shen, T.T. Xu, J. Ren, L.J. Huang, W. Qing, J.J. Zheng, Y.D. Mu, Groove structure of porous hydroxyapatite scaffolds (HAS) modulates immune environment via regulating macrophages and subsequently enhances osteogenesis, J. Biol. Inorg. Chem. 24 (2019) 733–745, doi:10.1007/s00775-019-01687-w.
[122] G. Bassi, F. Guilloton, C. Menard, M. Di Trapani, F. Deschaseaux, L. Sensebé, H. Schrezenmeier, R. Giordano, P. Bourin, M. Dominici, K. Tarte, M. Krampera, Effects of a ceramic biomaterial on immune modulatory properties and differentiation potential of human mesenchymal stromal cells of different origin, Tissue Eng. - Part A. 21 (2015) 767–781, doi:10.1089/ten.tea.2014.0269.
[123] Z.T. Chen, X.L. Mao, L.L. Tan, T. Friis, C.T. Wu, R. Crawford, Y. Xiao, Osteoimmunomodulatory properties of magnesium scaffolds coated with beta-tricalcium phosphate, Biomaterials 35 (2014) 8553–8565, doi:10.1016/j.biomaterials.2014.06.038.
[124] L. Zhang, J. Ke, Y. Wang, S. Yang, R.J. Miron, Y. Zhang, An in vitro investigation of the marked impact of dendritic cell interactions with bone grafts, J. Biomed. Mater. Res. - Part A. 105 (2017) 1703–1711, doi:10.1002/jbm.a.36048.
[125] D. Xue, E. Chen, H. Zhong, W. Zhang, S. Wang, M.U. Joomun, T. Yao, Y. Tan, S. Lin, Q. Zheng, Z. Pan, Immunomodulatory properties of graphene oxide for osteogenesis and angiogenesis, Int. J. Nanomedicine. 13 (2018) 5799–5810, doi:10.2147/IJN.S170305.
[126] X.T. He, R.X. Wu, X.Y. Xu, J. Wang, Y. Yin, F.M. Chen, Macrophage involvement affects matrix stiffness-related influences on cell osteogenesis under three-dimensional culture conditions, Acta Biomater 71 (2018) 132–147, doi:10.1016/j.actbio.2018.02.015.
[127] L.R. Smith, S. Cho, D.E. Discher, Stem cell differentiation is regulated by extracellular matrix mechanics, Physiology 33 (2018) 16–25, doi:10.1152/physiol.00026.2017.
[128] D. Benayahu, Y. Wiesenfeld, R. Sapir-Koren, How is mechanobiology involved in mesenchymal stem cell differentiation toward the osteoblastic or adipogenic fate? J. Cell. Physiol. 234 (2019) 12133–12141, doi:10.1002/jcp.28099.
[129] Y. Lv, C. Lin, High mobility group box 1-immobilized nanofibrous scaffold enhances vascularization, osteogenesis and stem cell recruitment, J. Mater. Chem. B. 4 (2016) 5002–5014, doi:10.1039/C6TB00826G.
[130] P.M. Mountziaris, S.N. Tzouanas, A.G. Mikos, Dose effect of tumor necrosis factor-alpha on in vitro osteogenic differentiation of mesenchymal stem cells on biodegradable polymeric microfiber scaffolds, Biomaterials 31 (2010) 1666–1675, doi:10.1016/j.biomaterials.2009.11.058.
[131] P.M. Mountziaris, E.D. Lehman, I. Mountziaris, D.C. Sing, K. Kasper, A.G. Mikos, Effect of temporally patterned TNF-α delivery on in vitro osteogenic differentiation of mesenchymal stem cells cultured on biodegradable polymer scaffolds, J Biomater Sci Polym 24 (2013) 1794–1813, doi:10.1080/09205063.2013.803455.Effect.
[132] M.A.M. Vis, K. Ito, S. Hofmann, Impact of culture medium on cellular interactions in in vitro co-culture systems, 8 (2020) 1–8. https://doi.org/10.3389/fbioe.2020.00911.
[133] Z. Chen, L. Chen, R. Liu, Y. Lin, S. Chen, S. Lu, Z. Lin, Z. Chen, C. Wu, Y. Xiao, The osteoimmunomodulatory property of a barrier collagen membrane and its manipulation: Via coating nanometer-sized bioactive glass to improve guided bone regeneration, Biomater. Sci. 6 (2018) 1007–1019, doi:10.1039/c7bm00869d.
[134] C. Yang, L. Ouyang, W. Wang, B. Chen, W. Liu, X. Yuan, Y. Luo, T. Cheng, K.W.K. Yeung, X. Liu, X. Zhang, Sodium butyrate-modified sulfonated polyetheretherketone modulates macrophage behavior and shows enhanced antibacterial and osteogenic functions during implant-associated infections, J. Mater. Chem. B. 7 (2019) 5541–5553, doi:10.1039/c9tb01298b.
[135] B. Yuan, M.G. Raucci, Y. Fan, X. Zhu, X. Yang, X. Zhang, M. Santin, L. Ambrosio, Injectable strontium-doped hydroxyapatite integrated with phosphoserine-tethered poly(epsilon-lysine) dendrons for osteoporotic bone defect repair, J. Mater. Chem. B. 6 (2018) 7974–7984, doi:10.1039/c8tb02526f.
[136] W. Liu, J.H. Li, M.Q. Cheng, Q.J. Wang, K.W.K. Yeung, P.K. Chu, X.L. Zhang, Zinc-Modified Sulfonated Polyetheretherketone Surface with Immunomodulatory Function for Guiding Cell Fate and Bone Regeneration, Adv. Sci. 5 (2018), doi:10.1002/advs.201800749.
[137] Z. Zhang, Y. Xie, H. Pan, L. Huang, X. Zheng, Influence of patterned titanium coatings on polarization of macrophage and osteogenic differentiation of bone marrow stem cells, J. Biomater. Appl. 32 (2018) 977–986, doi:10.1177/0885328217746802.
[138] M. Wang, F. Chen, J. Wang, X. Chen, J. Liang, X. Yang, X. Zhu, Y. Fan, X. Zhang, Calcium phosphate altered the cytokine secretion of macrophages and influenced the homing of mesenchymal stem cells, J. Mater. Chem. B. 6 (2018) 4765–4774, doi:10.1039/c8tb01201f.
[139] M.L. Wang, F.Y. Chen, J. Wang, X.N. Chen, J. Liang, X. Yang, X.D. Zhu, Y.J. Fan, X.D. Zhang, Calcium phosphate altered the cytokine secretion of macrophages and influenced the homing of mesenchymal stem cells, J. Mater. Chem. B. 6 (2018) 4765–4774, doi:10.1039/c8tb01201f.
[140] F.Y. McWhorter, T. Wang, P. Nguyen, T. Chung, W.F. Liu, Modulation of macrophage phenotype by cell shape, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 17253–17258, doi:10.1073/pnas.1308887110.
[141] H.-S. Lee, E. Al, Correlating macrophage morphology and cytokine production resulting from biomaterial contact, J Biomed Mater Res A 101 (2013) 203–212, doi:10.1038/jid.2014.371.
[142] S. Thiberge, A. Nechushtan, D. Sprinzak, O. Gileadi, V. Behar, O. Zik, Y. Chowers, S. Michaeli, J. Schlessinger, E. Moses, Scanning electron microscopy of cells and tissues under fully hydrated conditions, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 3346–3351, doi:10.1073/pnas.0400088101.
[143] T. Hasegawa, T. Yamamoto, H. Hongo, Z. Qiu, M. Abe, T. Kanesaki, K. Tanaka, T. Endo, P.H.L. de Freitas, M. Li, N. Amizuka, Three-dimensional ultrastructure of osteocytes assessed by focused ion beam-scanning electron microscopy (FIB-SEM), Histochem. Cell Biol. 149 (2018) 423–432, doi:10.1007/s00418-018-1645-1.
[144] A. Diez-Escudero, M. Espanol, E.B. Montufar, G. Di Pompo, G. Ciapetti, N. Baldini, M.-P. Ginebra, Focus ion beam/scanning electron microscopy characterization of osteoclastic resorption of calcium phosphate substrates, Tissue Eng. Part C Methods. 23 (2017) 118–124, doi:10.1089/ten.tec.2016.0361.
[145] M.L. Wong, J.F. Medrano, Real-time PCR for mRNA quantitation, Biotechniques 39 (2005) 75–85, doi:10.2144/05391RV01.
[146] C. Favrot, Polymerase chain reaction: advantages and drawbacks, Congr. Latinoam. Dermatologia Vet. 3 (2015).
[147] O.M. Delmonte, T.A. Fleisher, Flow cytometry: Surface markers and beyond, J. Allergy Clin. Immunol. 143 (2019) 528–537, doi:10.1016/j.jaci.2018.08.011.
[148] R. Ghosh, J.E. Gilda, A.V Gomes, Accuracy of Western Blots, Expert Rev. Proteomics. 11 (2014) 549–560, doi:10.1586/14789450.2014.939635.The.
[149] S.N. King, S.E. Hanson, X. Chen, J. Kim, P. Hematti, S.L. Thibeault, In vitro characterization of macrophage interaction with mesenchymal stromal cell-hyaluronan hydrogel constructs, J. Biomed. Mater. Res. - Part A. 102 (2014) 890–902, doi:10.1002/jbm.a.34746.
[150] P. Jaluria, K. Konstantopoulos, M. Betenbaugh, J. Shiloach, A perspective on microarrays: current applications, pitfalls, and potential uses, Microb. Cell Fact. 6 (2007) 1–14, doi:10.1186/1475-2859-6-4.
[151] G. Russo, C. Zegar, A. Giordano, Advantages and limitations of microarray technology in human cancer, Oncogene 22 (2003) 6497–6507, doi:10.1038/sj.onc.1206865.
[152] B. Han, D.C. Dubois, K.M.K. Boje, S.J. Free, R.R. Almon, Quantification of iNOS mRNA with reverse transcription polymerase chain reaction directly from cell lysates, Nitric Oxide - Biol. Chem. 3 (1999) 281–291, doi:10.1006/niox.1999.0240.
[153] G. Mestres, C.F. Santos, L. Engman, C. Persson, M.K. Ott, Scavenging effect of Trolox released from brushite cements, Acta Biomater 11 (2015) 459–466, doi:10.1016/j.actbio.2014.09.007.
[154] J.E. Aubin, K. Tursken, J.N.M. Heersche, Osteoblastic cell lineage, in: Cell. Mol. Biol. Bone, Elsevier, 1993, pp. 1–45, doi:10.1016/B978-0-08-092500-4.50005-X.
[155] H. Puchtler, S.N. Meloan, M.S. Terry, On the history and mechanism of alizarin and alizarin red S stains for calcium, J. Histochem. Cytochem. 17 (1969) 110–124.

[156] S. Boonrungsiman, E. Gentleman, R. Carzaniga, N.D. Evans, D.W. McComb, A.E. Porter, M.M. Stevens, The role of intracellular calcium phosphate in osteoblast-mediated bone apatite formation, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 14170–14175, doi:10.1073/pnas.1208916109.
[157] S. Mechiche Alami, H. Rammal, C. Boulagnon-Rombi, F. Velard, F. Lazar, R. Drevet, D. Laurent Maquin, S.C. Gangloff, J. Hemmerlé, J.C. Voegel, G. Francius, P. Schaaf, F. Boulmedais, H. Kerdjoudj, Harnessing Wharton's jelly stem cell differentiation into bone-like nodule on calcium phosphate substrate without osteoinductive factors, Acta Biomater 49 (2017) 575–589, doi:10.1016/j.actbio.2016.11.042.
[158] H. Rammal, M. Dubus, N.B. Bercu, E. Mathieu, C. Terryn, M. Molinari, S.C. Gangloff, N. Nassif, C. Mauprivez, F. Quilès, H. Kerdjoudj, Mechanobiologically induced bone-like nodules: matrix characterization from micro to nanoscale, Nanomedicine Nanotechnology, Biol. Med. 29 (2020) 102256, doi:10.1016/j.nano.2020.102256.
[159] F. Ravera, E. Efeoglu, H.J. Byrne, Vibrational spectroscopy for in vitro monitoring stem cell differentiation, Molecules 25 (2020) 5554, doi:10.3390/molecules25235554.
[160] A. Ghita, F.C. Pascut, V. Sottile, C. Denning, I. Notingher, Applications of Raman micro-spectroscopy to stem cell technology: label-free molecular discrimination and monitoring cell differentiation, EPJ Tech. Instrum. 2 (2015) 6, doi:10.1140/epjti/s40485-015-0016-8.
[161] C.R. Justus, N. Leffler, M. Ruiz-Echevarria, L.V. Yang, *In vitro* cell migration and invasion assays, J. Vis. Exp. (2014) 1–8, doi:10.3791/51046.
[162] G. Hulsart-Billström, J.I. Dawson, S. Hofmann, R. Müller, M.J. Stoddart, M. Alini, H. Redl, A. El Haj, R. Brown, V. Salih, J. Hilborn, S. Larsson, R.O.C. Oreffo, A surprisingly poor correlation between in vitro and in vivo testing of biomaterials for bone regeneration, Eur. Cells Mater. 31 (2016) 312–322 https://doi.org/https://doi.org/doi:, doi:10.22203/ecm.v031a20.
[163] D.G. Anderson, S. Levenberg, R. Langer, Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells, Nat. Biotechnol. 22 (2004) 863–866, doi:10.1038/nbt981.
[164] F. Khan, R.S. Tare, J.M. Kanczler, R.O.C. Oreffo, M. Bradley, Strategies for cell manipulation and skeletal tissue engineering using high-throughput polymer blend formulation and microarray techniques, Biomaterials 31 (2010) 2216–2228, doi:10.1016/j.biomaterials.2009.11.101.
[165] M.B. Oliveira, M.P. Ribeiro, S.P. Miguel, A.I. Neto, P. Coutinho, I.J. Correia, J.F. Mano, In vivo high-content evaluation of three-dimensional scaffolds biocompatibility, Tissue Eng. - Part C Methods. 20 (2014) 851–864, doi:10.1089/ten.tec.2013.0738.
[166] M.B. Oliveira, J.F. Mano, High-throughput screening for integrative biomaterials design: Exploring advances and new trends, Trends Biotechnol. 32 (2014) 627–636, doi:10.1016/j.tibtech.2014.09.009.
[167] J. Sarkar, A. Kumar, Recent advances in biomaterial-based high-throughput platforms, Biotechnol. J. 16 (2021) 2000288, doi:10.1002/biot.202000288.
[168] J. Rauh, F. Milan, K.P. Günther, M. Stiehler, Bioreactor systems for bone tissue engineering, Tissue Eng. - Part B Rev. 17 (2011) 263–280, doi:10.1089/ten.teb.2010.0612.
[169] J.R. Vetsch, R. Müller, S. Hofmann, The evolution of simulation techniques for dynamic bone tissue engineering in bioreactors, J. Tissue Eng. Regen. Med. (2015) 903–917, doi:10.1002/term.
[170] K. Song, T. Liu, Z. Cui, X. Li, X. Ma, Three-dimensional fabrication of engineered bone with human bio-derived bone scaffolds in a rotating wall vessel bioreactor, J. Biomed. Mater. Res. - Part A. 86 (2008) 323–332, doi:10.1002/jbm.a.31624.
[171] Z.Y. Zhang, S.H. Teoh, W.S. Chong, T.T. Foo, Y.C. Chng, M. Choolani, J. Chan, A biaxial rotating bioreactor for the culture of fetal mesenchymal stem cells for bone tissue engineering, Biomaterials 30 (2009) 2694–2704, doi:10.1016/j.biomaterials.2009.01.028.
[172] D. Marolt, I.M. Campos, S. Bhumiratana, A. Koren, P. Petridis, G. Zhang, P.F. Spitalnik, W.L. Grayson, G. Vunjak-Novakovic, Engineering bone tissue from human embryonic stem cells, Proc. Natl. Acad. Sci. 109 (2012) 8705–8709, doi:10.1073/pnas.1201830109.
[173] M. Bartnikowski, T.J. Klein, F.P.W. Melchels, M.A. Woodruff, Effects of scaffold architecture on mechanical characteristics and osteoblast response to static and perfusion bioreactor cultures, Biotechnol. Bioeng. 111 (2014) 1440–1451, doi:10.1002/bit.25200.
[174] J.A. Kluge, E. Al, Bioreactor System Using Noninvasive Imaging and Mechanical Stretch for Biomaterial Screening, Ann Biomed Eng (2011) 1390–1402, doi:10.1161/CIRCULATIONAHA.110.956839.
[175] M. Seifert, A. Lubitz, J. Trommer, D. Könnig, G. Korus, U. Marx, H.-D. Volk, G. Duda, G. Kasper, K. Lehmann, M. Stolk, C. Giese, Crosstalk between Immune Cells and Mesenchymal Stromal Cells in a 3D Bioreactor System, Int. J. Artif. Organs. 35 (2012) 986–995, doi:10.1177/039139881203501104.
[176] G. Mestres, R.A. Perez, N.L. D'Elia, L. Barbe, Advantages of microfluidic systems for studying cell-biomaterial interactions - Focus on bone regeneration applications, Biomed. Phys. Eng. Express. 5 (2019) 032001, doi:10.1088/2057-1976/ab1033.
[177] W.Y. Sim, S.W. Park, S.H. Park, B.H. Min, S.R. Park, S.S. Yang, A pneumatic micro cell chip for the differentiation of human mesenchymal stem cells under mechanical stimulation, Lab Chip 7 (2007) 1775–1782, doi:10.1039/b712361m.
[178] S.H. Song, J. Choi, H. Il Jung, A microfluidic magnetic bead impact generator for physical stimulation of osteoblast cell, Electrophoresis 31 (2010) 2762–2770, doi:10.1002/elps.201000201.
[179] A. Hartmann, M. Stamp, R. Kmeth, S. Buchegger, B. Stritzker, B. Saldamli, R. Burgkart, M.F. Schneider, A. Wixforth, A novel tool for dynamic cell adhesion studies-the De-Adhesion Number Investigator DANI, Lab Chip 14 (2014) 542–546, doi:10.1039/C3LC50916H.
[180] M.E.M. Stamp, A.M. Jötten, P.W. Kudella, D. Breyer, F.G. Strobl, T.M. Geislinger, A. Wixforth, C. Westerhausen, Exploring the limits of cell adhesion under shear stress within physiological conditions and beyond on a chip, Diagnostics 6 (2016) 38, doi:10.3390/diagnostics6040038.
[181] D. Barata, E. Provaggi, C. Van Blitterswijk, P. Habibovic, Development of a microfluidic platform integrating high-resolution microstructured biomaterials to study cell-material interactions, Lab Chip 17 (2017) 4134–4147, doi:10.1039/c7lc00802c.
[182] S.S.D. Carter, L. Barbe, M. Tenje, G. Mestres, Exploring microfluidics as a tool to evaluate the biological properties of a titanium alloy under dynamic conditions, Biomater. Sci. 8 (2020) 6309–6321, doi:10.1039/d0bm00964d.
[183] A. Mansoorifar, R. Gordon, R.C. Bergan, L.E. Bertassoni, Bone-on-a-Chip: Microfluidic Technologies and Microphysiologic Models of Bone Tissue, Adv. Funct. Mater. 31 (2021) 2006796, doi:10.1002/adfm.202006796.
[184] A. Polini, L.L. del Mercato, A. Barra, Y.S. Zhang, F. Calabi, G. Gigli, Towards the development of human immune-system-on-a-chip platforms, Drug Discov. Today. 24 (2019) 517–525, doi:10.1016/j.drudis.2018.10.003.
[185] N. Sinha, N. Subedi, J. Tel, Integrating immunology and microfluidics for single immune cell analysis, Front. Immunol. 9 (2018) 1–16, doi:10.3389/fimmu.2018.02373.
[186] S.N. King, S.E. Hanson, X. Chen, J. Kim, P. Hematti, S.L. Thibeault, In vitro characterization of macrophage interaction with mesenchymal stromal cell-hyaluronan hydrogel constructs, J Biomed Mater Res A 102 (2014) 890–902, doi:10.1002/jbm.a.34746.
[187] S. Ansari, C. Chen, M.M. Hasani-Sadrabadi, B. Yu, H.H. Zadeh, B.M. Wu, A. Moshaverinia, Hydrogel elasticity and microarchitecture regulate dental-derived mesenchymal stem cell-host immune system cross-talk, Acta Biomater 60 (2017) 181–189, doi:10.1016/j.actbio.2017.07.017.