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Review article

Interplay between biomaterials and the immune system: Challenges and opportunities in regenerative medicine

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ABSTRACT

The use of biomaterials for tissue engineering and regenerative medicine applications has increased dramatically over recent years. However, the clinical uptake of a wide variety of biomaterials remains limited due to adverse effects commonly exhibited by patients, which are caused by the host immune response. Despite this, current *in vitro* evaluation standards (ISO-10993) for assessing the host response to biomaterials have limitations in predicting the likelihood of *in vivo* biomaterial acceptance. Furthermore, endotoxin contamination of biomaterials is rarely considered, despite its ability to significantly affect the performance of biomaterials and engineered tissues. This review highlights the importance of the immune response to biomaterials and discusses existing challenges and opportunities in the development and standardised assessment of the immune response to biomaterials, including the importance of endotoxin levels. In addition, the properties of biomaterials that impact the host immune response and the exploitation of immunomodulatory biomaterials in regenerative medicine are explored. Finally, a standardised *in vitro* pathway of evaluating the immune response to biomaterials (hydrogels) and their regenerative potential is proposed, aiming to ensure safety and consistency, while reducing costs and the use of animals in the biomaterials research for tissue engineering and regenerative medicine.

Statement of significance

This review presents a critical analysis of the role of the interactions between the immune system and biomaterials in determining the therapeutic success of biomaterial-based approaches. No such review addressing the lack of understanding of biomaterial-immune system interactions during the developmental and pre-clinical stages of biomaterials, including the impact of the endotoxin levels of biomaterials on the immune response, is published. As there is a lack of *in vitro* regulations to evaluate the immune response to biomaterials, a standardised *in vitro* pathway to evaluate the immune response to biomaterials (hydrogels) and their immunomodulatory and regenerative potential for use in tissue engineering/regenerative medicine applications is presented. The aim of the proposed pathway of biomaterial evaluation is to ensure safety and consistency in the biomaterials research community, while reducing costs and animal use (through the concept of the 3R's - reduction, refinement, and replacement of animals).

1. Introduction

Current tissue engineering and regenerative medicine strategies aim to restore the function of damaged tissues [1]. The current gold standard is autografting (utilising a patient's tissue to enhance healing), however, limitations such as the limited harvest of certain tissues as well as lengthy surgical time and donor site morbidity have warranted the search for alternative options [2]. The use of biomaterials for this purpose presents an attractive option, with the number of biomaterials being developed and studied increasing rapidly. Biomaterials play a major role in the estimated $400Bn worldwide medical device market, however, biomaterials are foreign bodies, thus adverse immune reactions to biomaterials pose a fundamental challenge that can drastically reduce the quality of life for patients, explaining their current poor clinical uptake [1,2]. These adverse reactions commonly disrupt the healing process, resulting in immense pain for the patient, excessive inflam-

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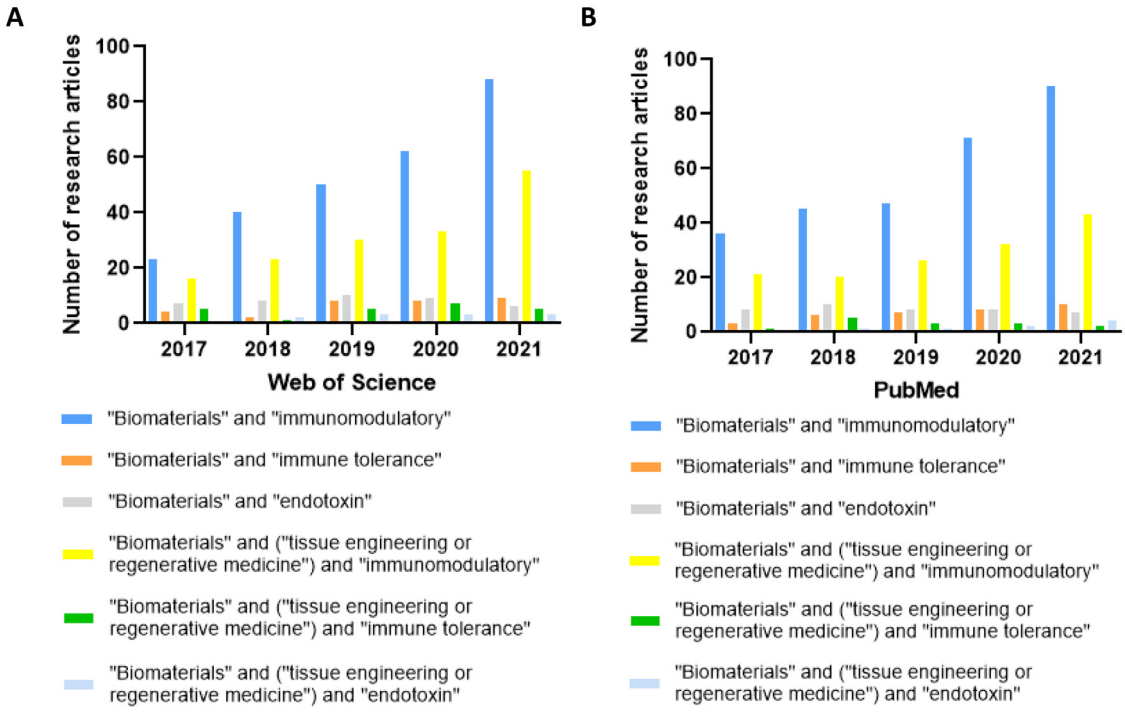

Fig. 1. *Number of publications (research articles) involving biomaterials in selected contexts*. The number of publications (research articles) involving biomaterials and the accompanying keywords from searches in (**A)** Web of Science, and (**B)** PubMed during the years 2017–2021 (indicated with keywords of "biomaterials" and "immunomodulatory" and with the addition of ("tissue engineering or regenerative medicine")). Data obtained October 2022.

mation, tissue destruction, and can lead to the rejection of medical devices. The lack of detailed understanding of the interactions between biomaterials and the immune system represents a major barrier to developing effective biomaterial-based therapies and tissue engineering approaches [1,2]. Tissue engineered heart valves (TEHVs) are an example of products that have failed in clinical trials due to adverse foreign body responses and immune rejection, highlighting the importance and need to thoroughly characterise the immune response to biomaterials prior to clinical translation in order to allow accurate predictions of the host response to biomaterials [3].

The design of biomaterials has largely been based on their physical and mechanical properties such as mechanical stiffness/strength, porosity, and degradability; however, recently it has been recognised that the host immune response to biomaterials is a critical determinant of successful clinical outcomes [4]. In previous decades, "immune-evasive" or "inert" biomaterials have been deployed to prevent unwanted inflammation, however, the focus is now shifting towards utilising the properties of biomaterials to modulate the host immune response (Fig. 1). This can be evidenced by the continuous and significant increase of scientific publications (research articles) related to *immunomodulatory biomaterials for tissue engineering and/or regenerative medicine* in the last 5 years published in world-class knowledge and scientific databases such as Web of Science (Fig. 1A) and PubMed (Fig. 1B). This highlights the increasing interest of researchers in different scientific disciplines in modulating the immune response to biomaterials for specific regenerative or tissue engineering applications. Biomaterials can be utilised to attempt to promote immune tolerance, for example as potential therapies for autoimmune diseases or to prevent the rejection of implanted grafts, however, only a few publications in this context have been documented to date. In both scientific databases, the investigation of endotoxin levels is poorly explored within the biomaterial field for these applications, as seen by the limited number of publications.

Immunomodulatory biomaterials can shift the host response to an implant (from one resulting in scar tissue formation or fibrous encapsulation) towards one of tissue integration and functional remodelling [5–7]. A deeper understanding of the interplay between biomaterials and the immune system will facilitate the production of effective immunomodulatory biomaterials that can deliver long term benefits to patients affected by organ/graft rejection, autoimmune diseases, chronic inflammatory diseases, and the immune-mediated rejection of biologics [1,2,5]. Therefore, repairing and engineering new tissues requires a well-established pathway to evaluate the immune response to these biomaterials.

Currently, *in vitro* assessment protocols for evaluating biomaterials tend to follow the International Organisation of Standardisation (ISO) standards [2,8]. However, a multicentre analysis concluded that the current *in vitro* assessments of biomaterials are not suitable in predicting their acceptance *in vivo*, highlighting the need to improve *in vitro* protocols for evaluating biomaterials and develop *in vitro* assays that allow accurate predictions of biomaterial acceptance *in vivo* [2,9]. This would substantially reduce experimental time and resources (through the concept of the 3R's – reduction, refinement, and replacement of animals) and decrease the amount of unsuitable biomaterials that reach the clinic, ultimately improving the safety of patients [2].

Ideally, a pathway of assessing the immune response to biomaterials would be standardised, allowing for comparisons between biomaterials, which would require incorporation into the ISO standards for the biological evaluation of medical devices (ISO-10993). This remains the food and drug administration (FDA) regulatory standard that biomaterials must pass to allow their use in clinics [2,8]. Given the pivotal role of the immune response to biomaterials in determining clinical outcomes, immune testing must be considered in evaluation protocols. Evaluating the immune response to biomaterials may allow the determination of the likelihood of their acceptance *in vivo*, which would mitigate undesirable outcomes at the clinical level [2]. This review investigates the central role of the

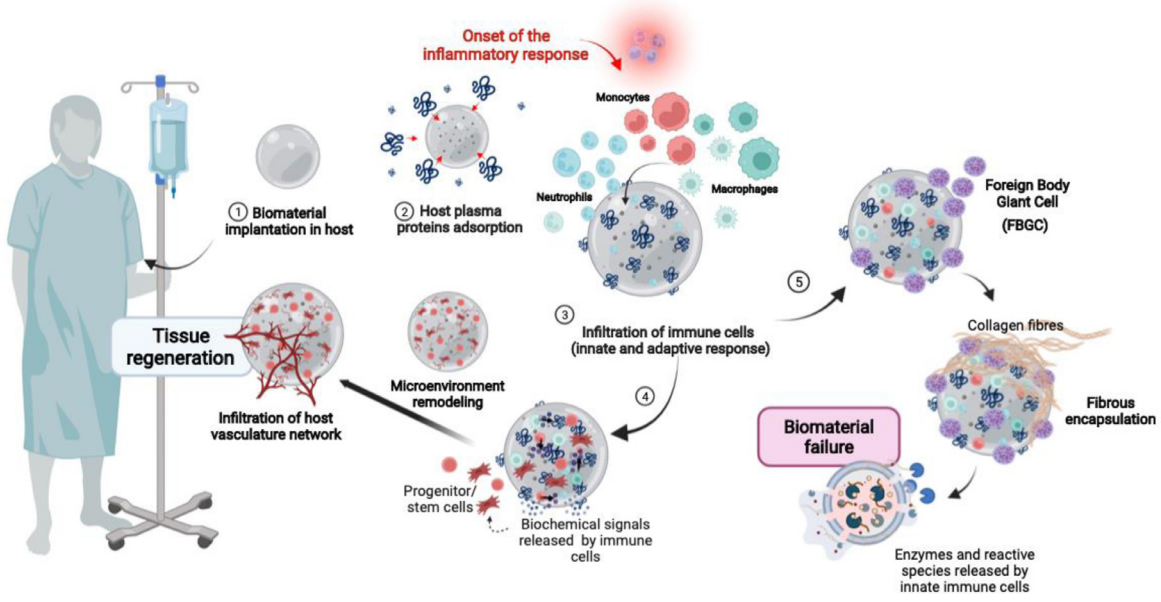

Fig. 2. *Host immune response to implanted biomaterial leads to foreign body response (FBR) or tissue regeneration.* A schematic representation of the host response to biomaterial implantation; host plasma protein adherence, neutrophil recruitment, monocyte recruitment and macrophage adhesion, leading to either macrophage fusion, foreign body giant cell (FBGC) formation and fibrous encapsulation, or microenvironmental remodelling and tissue regeneration. Created in Biorender.com.

interactions between biomaterials and the immune system during the developmental and preclinical stages of biomaterials and evidences the necessity of evaluating the immune response to biomaterials *in vitro* to allow the prediction of the likelihood of a biomaterial to be accepted *in vivo*. Furthermore, the importance of considering the impact of the levels of endotoxin in biomaterials will also be emphasised.

2. Immune response to implanted biomaterials

When a biomaterial is implanted into a living host, a foreign body response (FBR) is initiated, whereby the host immune system recognises the biomaterial as foreign, commencing a complex cascade of events mediated by a variety of components [1,2,5–7,10–12]. This can ultimately culminate in the fibrous encapsulation of the biomaterial (leading to biomaterial failure) and the unwanted degradation of the biomaterial due to the release of enzymes and reactive species by immune cells (if the acute inflammatory response is unresolved), or alternatively, in an ideal scenario, can lead to microenvironmental remodelling and tissue regeneration (Fig. 2) [5–7].

Initially, host plasma proteins adhere to the biomaterial surface, creating a chemoattractant gradient for the subsequent recruitment of immune cells, leading to the onset of the inflammatory response. These proteins include the complement family of proteins, which become activated following biomaterial interaction and are potent immune cell recruiters (particularly C3a and C5a) as they opsonise the implanted biomaterial, leading to the initiation of the innate immune response. The host immune response following biomaterial implantation is essential to allow tissue regeneration and healing, however, an excessive host response can ultimately result in biomaterial failure. The body's response to biomaterial implantation may be mostly determined by the nature and extent of initial protein adsorption, thus preventing excessive protein (including complement) adsorption may be a potential therapeutic target in preventing biomaterial rejection/FBR [6,13–15].

During acute inflammation, the first cells recruited (within hours) to an implanted biomaterial are circulating polymorphonuclear leukocytes (mainly neutrophils), where they infiltrate the surrounding tissue to clear debris and activate host responses [1,2,5–7,10–12]. Generally, neutrophils are cleared within a few days in a healthy wound, however, they have been shown to persist around implanted biomaterials for several weeks, although the potential consequences of this are poorly understood. Studies have suggested that neutrophils do play a role in the host immune response to implanted biomaterials, with excessive neutrophil recruitment and persistence and the presence of neutrophil extracellular traps (NETs) potentially resulting in the fibrous encapsulation of the biomaterial, preventing integration between tissue and biomaterial, and thus impairing tissue regeneration and leading to biomaterial failure. However, studies have demonstrated conflicting results, thus the precise function of neutrophils remains to be elucidated [6,16]. Neutrophils secrete various cytokines and chemokines to regulate the immune response, including interleukin (IL)-8 (which further activates neutrophils themselves), monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1β, resulting in the recruitment and activation of monocytes, macrophages, dendritic cells (DCs), and lymphocytes [16].

Monocytes circulating in the blood are activated in the transition from acute to chronic inflammation, where they migrate to the injury site (via chemoattractant factors) and differentiate into macrophages following extravasation of the tissue [1,2,5–7,10,11]. Macrophages play a crucial role in early wound healing through the release of enzymes important for tissue reorganisation and angiogenesis. Macrophages display an "M1-like" (pro-inflammatory) phenotype during acute inflammation, secreting cytokines such as IL-1α, IL-1β, IL-6, IL-8, and tumour necrosis factor (TNF)-α. "M2-like" (anti-inflammatory) macrophages (more specifically a subtype of M2 - M2c) are vital in promoting tissue remodelling during chronic inflammation, secreting cytokines such as IL-4, IL-10 and transforming growth factor (TGF)-β), which mediate tissue regeneration and wound healing [1,5,10]. As well as exhibiting anti-fibrotic functions, it is important to note that TGF-β also plays a central role in the development of fibrosis, with this dual function of TGF-β potentially dependent on its cell source and immune context [12]. It should also be noted that the binary M1/M2 nomenclature of macrophages is considered an over-simplification,

as macrophage polarisation likely consists of a spectrum of phenotypes that are induced and modulated by the local microenvironment. A greater understanding of macrophage heterogeneity (as well as of other immune cells) and its role in immune responses could perhaps enable the design of therapies that can effectively target specific cell subtypes [10,17].

Although M1 macrophages play a role in the initial vascularisation steps, their persistence in later stages may lead to tissue damage (due to their secretion of pro-inflammatory molecules). Conversely, M2 macrophages should be the predominant phenotype during tissue repair, however, their excessive presence may result in the fibrous encapsulation of the biomaterial implant as opposed to efficient healing. Thus, the sequential transition from M1 to M2 macrophage phenotype is crucial in determining the host outcome of biomaterial implantation [5–7,10]. Lymphocytes are involved in the chronic phase of inflammation, with T-helper (Th)1 and Th2 cells mediating macrophage polarisation into M1 and M2 phenotypes, respectively [1,5,10]. The mechanism of "frustrated phagocytosis", occurring because of macrophages attempting to increase their phagocytic ability to degrade large implants, results in the fusion of macrophage membranes and the subsequent formation of foreign body giant cells (FBGCs). Mast cell and Th2 cell-mediated release of IL-4 and IL-13 also stimulate the formation of FBGCs [1,5,10–12]. FBGCs can remain at the implantation site for the whole lifetime of the biomaterial implant, giving rise to degradative species that subsequently degrade the implanted biomaterial. The ongoing progression of inflammation results in the formation of a fibrotic collagenous capsule covering the biomaterial surface (due to the overproduction of collagen by fibroblasts), resulting in its isolation from the host tissue, and ultimately leading to the failure of the biomaterial implant [5,7,10].

If inflammation is unresolved and persistent, and cells are continually and excessively recruited to an implanted biomaterial, the sustained, chronic pro-inflammatory environment will lead to the development of FBGCs and subsequent rejection of the biomaterial [6,7]. Contamination of biomaterials with endotoxin can lead to the development of this excessive pro-inflammatory environment, which will be discussed in more detail in the next section [18]. However, if the acute inflammatory phase is resolved and inflammation is not excessive and does not persist, immunomodulatory molecules and biochemical signals released by immune cells (particularly macrophages as described) can lead to the remodelling of the local microenvironment through the modulation of host progenitor/stem cells, as well as fibroblasts and endothelial cells. Fibroblasts synthesise new extra-cellular matrix (ECM) through their production of collagen and other macromolecules (such as proteoglycans), while endothelial cells organise into new blood vessels to allow the exchange of nutrients with the newly formed tissue. Thus, the desired transition from the development of host vasculature to subsequent tissue integration/generation and efficient tissue repair/wound healing can be achieved, ultimately leading to the successful regeneration of functional tissue [6,7].

3. Impact of endotoxin contamination of biomaterials on the host immune response

The contamination of biomaterials with bacteria is ubiquitous, and bacterial toxins can be potent activators of the immune system and can cause severe disease in humans [18–20]. Exotoxins are proteins that are secreted by bacteria or released upon bacterial lysis and can elicit significant pathogenicity. However, unlike endotoxin (or lipopolysaccharide (LPS)), exotoxins are heat labile and are also filterable, thus exotoxins do not pose a major problem when using biomaterials for tissue engineering/regenerative medicine applications [19,20]. Endotoxin is found in the outer membrane of Gram-negative bacteria, consisting of lipid A (a lipid moiety), an oligosaccharide core, and O-specific antigen (a sequence of repetitive subunits) (Fig. 3A) [18], is difficult to excrete from the body (with an average molecular mass of 10-20kDa), and is also heat-stable (up to 180°C) and UV stable [18,21,22]. Endotoxin is present in all natural biomaterials as these are obtained from non-sterile environments, thus are contaminated with bacteria and therefore endotoxin, while contamination in synthetic biomaterials likely arises due to contaminated laboratory reagents or equipment [18,21,22]. Endotoxin will remain in biomaterials unless specific treatment is undertaken to remove it, however, removing endotoxin is extremely challenging. Although a combination of treatments such as acidic and alkaline solutions, organic solvents, detergents, or ultrasonication can allow endotoxin to be removed from biomaterials, such methods may cause damage to the biomaterial and/or affect its performance. Furthermore, techniques such as phase separation, ultrafiltration, chromatography, and adsorption exist for biomaterials that are in solution, however, these are commonly limited due to the pH or viscosity of the solution. Endotoxin removal spin columns and resins are commercially available to remove endotoxin from proteins, however, these may lead to loss of product and again may affect the performance of biomaterials. Consequently, endotoxin-free products have been developed by companies to address this issue [18,21–24].

When bacteria die, the lysis of their cell membranes causes the release of large amounts of endotoxin (smaller quantities of endotoxin are also released during cell division). Following the implantation of a biomaterial into a host, endotoxin (that is present due to the contamination of biomaterials) enters the bloodstream where it exerts its effects on host cells [18]. Endotoxin can also induce an immune response in tissues themselves, through the activation of DCs for example (the typical danger sensors of the immune system). The mechanism of endotoxin is mainly facilitated through Toll-like receptor (TLR)-4 (Fig. 3B), which is expressed in an abundance of cell types; including immune cells such as neutrophils, monocytes, macrophages and DCs, as well as various other cell types including mesenchymal stem/stromal cells (MSCs), which are commonly deployed in regenerative medicine/tissue engineering approaches [18,21,22]. The host immune system can detect and elicit strong immune responses in response to extremely small concentrations (~1 ng/ml) of endotoxin, which results in the production of pro-inflammatory molecules such as IL-1, IL-6, TNF-α, and type I interferons (IFNs) through the TLR-4 pathway, culminating in the initiation of the acute inflammatory response. The persistence of this strong inflammatory response can be detrimental to the success of tissue engineering applications, through mediating the development of chronic inflammation as described and subsequent host rejection of implanted grafts/medical devices. Furthermore, high concentrations of endotoxin can result in fever, hypotension, chronic diseases (such as respiratory distress syndrome) and can also induce septic shock, highlighting the necessity for its impact to be seriously considered [18,21,22].

In the last decade, there has been rapid advancement in the biofabrication field due to the need for novel biomaterials to become available for commercial and clinical use [18,23]. Much focus to date has been on utilising biomaterials to mimic the target tissue's composition and provide an optimal microenvironment for cellular differentiation and proliferation, with endotoxin contamination often being overlooked (Fig. 1). This is likely due to unawareness of the impact of endotoxin contamination, and lack of details on the endotoxin levels in commercially available products [18]. Although the potential impact of endotoxin contamination of biomaterials has been documented for several years, the recent rapid growth of the use of biomaterials in the tissue engineering field means that this problem is now more prevalent than ever before. However, surprisingly, only a few studies have investigated the effect of endotoxin on engineered tissues to be

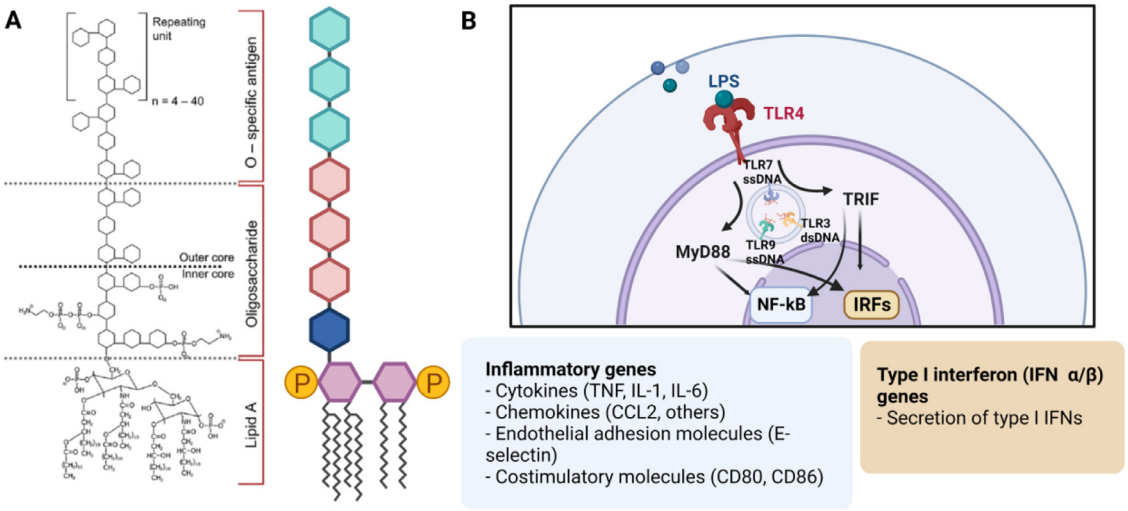

Fig. 3. The structure of endotoxin and the cellular mechanism of endotoxin**. (A)** The structure of endotoxin - Lipid A, core oligosaccharide and O-specific antigen. (**B)** The cellular mechanism of endotoxin. Adapted from [18]. Created in Biorender.com.

transplanted into patients, despite endotoxin significantly affecting the performance of these tissues [18,25].

Sustained high endotoxin levels in gelatin were shown to inhibit the formation of bone tissue in rats while causing significant cell senescence, whereas low levels of endotoxin in biomaterials have been shown to promote bone formation, highlighting that differing levels of endotoxin can alter the performance of engineered tissues [26,27]. Endotoxin levels have also been shown to affect the performance of gelatin methacryloyl hydrogels aiming to regenerate cartilage tissue. MSCs were encapsulated in the hydrogels, and their chondrogenic differentiation was evaluated by measuring the production of glycosaminoglycans (GAGs). A significantly higher amount of GAGs were produced in low endotoxin gelatin methacryloyl than in high endotoxin gelatin methacryloyl (eliciting a more biologically relevant performance) [28]. Furthermore, endotoxin has demonstrated significant inhibitory effects on dermal wound healing, meaning biomaterials containing high levels of endotoxin may result in limited integration into the skin and elicit severe inflammation at implantation sites [29]. A significant inflammatory response was elicited from all tissues displaying high levels of endotoxin, regardless of the tissue investigated and the implantation site. Endotoxin has also been shown to delay the FBR due to the prolonged presence of granulocytes and the altering of macrophage function (through the production of IL-10), demonstrating the different immunomodulatory effects exhibited by endotoxin and its ability to significantly impact biomaterial function [30].

A recent study investigating the immune response to marine-derived collagen and gelatin extracts for tissue engineering applications [31] considered the effect of endotoxin, evaluating the levels of endotoxin in the biomaterials prior to assessing the subsequent immune response. The biomaterials were found to be contaminated with endotoxin, with the levels of endotoxin found to be above the guidelines of regulatory authorities. However, the study used LPS as a control and found no cytokine production at the lowest concentration of LPS tested (3.13 ng/ml), which was higher than the endotoxin levels found in the biomaterials. The authors attributed their observed results to the nature of the biomaterials and not the effect of endotoxin. In this study, shark collagen was suggested to be the least immunogenic material as it induced a low expression of pro-inflammatory cytokines and inducible nitric oxide synthase (encoded by Nos2) and a high expression of Arginase 1 (encoded by Arg1). Shark gelatin demonstrated the highest level of pro-inflammatory expression, however also exhibited a high expression of IL-10 and arginase, both of which are markers of M2-like macrophages. Additionally, the biomaterials all exhibited a transient recruitment of neutrophils when injected into the peritoneal cavity of mice, highlighting their potential as candidate biomaterials for regenerative medicine applications [31].

The strong inflammatory response elicited in response to the contamination of biomaterials with endotoxin has led to strict regulation from regulatory authorities on the levels of endotoxin in medical devices; 0.5 EU/ml or 20 EU/device for products that contact the cardiovascular and lymphatic system, and 0.06 EU/ml or 2.15 EU/device for products that contact cerebrospinal fluid. However, vast numbers of medical devices are continually recalled due to their levels of endotoxin being too high [18,21]. Furthermore, preclinical, and pathophysiological models (used to study the pathophysiological processes in diseases), do not require such regulation of endotoxin levels, despite endotoxin being known to affect cellular behaviour, cellular interactions, and the therapeutic efficacy of treatments. The neglect of the issues relating to the endotoxin contamination of biomaterials, particularly by material scientists in the discovery and early development stages, may lead to incorrect conclusions and subsequent halting or staggering of biomaterial development and translation to applications. Commercially available assays such as the Limulus amoebocyte lysate (LAL) assay allow the levels of endotoxin in biomaterials to be measured and assessed. This assay should be utilised in conjunction with the use of endotoxin-free products (such as endotoxin-free water) to ensure sufficiently low/no endotoxin contamination of biomaterial products, as well as in preclinical and pathophysiological models [18,32,33].

4. Ability of biomaterial scaffolds to modulate the immune response

Biomaterials can be developed into porous 3D scaffolds, which play a central role in tissue engineering applications by; providing mechanical support, allowing the perfusion of oxygen and nutrients, and providing biochemical signals that modulate cellular behaviour (attachment, migration, proliferation, and differentiation) [4,34]. Scaffolds can be engineered to mimic the natural ECM of tissues, providing biological, chemical, and mechanical cues that allow the direct infiltration and manipulation of host immune cells within the body and the creation of a local microenvironment for tissue repair and regeneration [4,34,35]. Furthermore, immunomodulatory molecules (such as cytokines and growth factors)

Table 1
Summary of studies of selected hydrogels used for immunomodulation in tissue engineering/regenerative medicine applications. FBR = foreign body response, PEG = poly(ethylene glycol), MSCs = mesenchymal stem/stromal cells, PVA = poly(vinyl alcohol), VEGF = vascular endothelial growth factor, PDGF = platelet derived growth factor, HA = hyaluronic acid.

Hydrogel	Study outcome	Potential limitations
Alginate	Hydrogels of spheres with larger diameters induced less FBR and fibrosis than hydrogels with smaller diameters.	*In vivo*, suggested loss of viability of encapsulated islets [38].
	Covalent modification of hydrogels with triazole substantially reduced FBR in rodents and non-human primates by inhibiting macrophage recruitment and fibrous deposition.	Expression profile from the surrounding environment of implanted microcapsules was not significantly different, and there was no enrichment for any specific macrophage subtype [39].
PEG	Hydrogels of lower stiffness reduced macrophage activation and caused a less severe FBR.	Degree of hydrogel swelling may also have played a role in the observed results, particularly for the *in vivo* environment [40].
	In vitro, encapsulating murine MSCs in hydrogels attenuated the fibrotic response of the FBR by downregulating macrophage activation *In vivo*, encapsulated MSCs reduced the thickness of fibrous capsules in mice.	*In vivo* effect diminished with osteogenic differentiation [41].
PVA	Delivery of dexamethasone, VEGF, and PDGF from hydrogels promoted angiogenesis, blood vessel maturation and prevented FBR.	Capillary density may affect biosensor lag time and sensitivity [42].
Gelatin	Encapsulating MSCs and macrophages in hydrogels helped the generation of skin tissue and wound healing in mice.	*In vitro* and *in vivo* results of the recruitment of MSCs and macrophages were different. Effect of high numbers of MSCs recruited to hydrogels on macrophage recruitment was not shown [43].
Collagen-HA	Significant expansion of the secretory profile of proangiogenic and immunomodulatory paracrine factors of MSCs encapsulated in the hydrogel platform.	Predominant effects were seen when comparing collagen to gelatin materials. Differences in paracrine activity observed may be due to hydrogel stiffness [44].
Pullulan-collagen	Seeding MSCs on the hydrogels improved their viability and augmented their proangiogenic, fibromodulatory and immunomodulatory effects *in vivo*.	Changing the timing/frequency of treatment following injury may lead to different effects on wound healing [45].
HA	Coculture of MSCs with autologous chondrocytes in hydrogels significantly increased the rate of chondrogenesis.	Results may be specific to this engineered hydrogel system, as matrix interactions may control this response. Specific mechanism of interactions between MSCs and articular chondrocytes is not understood [46].

can be incorporated into scaffolds, providing spatiotemporal control of the scaffold microenvironment to ensure that the desired immune response is elicited [1,4,5,10,34–36]. Scaffolds can also be retrieved after implantation/injection into the body to allow the immune response to be monitored [36].

Hydrogels are a form of biomaterial that have great potential to be utilised for immunomodulatory applications in tissue engineering/regenerative medicine [37]. Hydrogels possess unique and highly desirable physicochemical properties including; high-water content, drug-loading capability, biocompatibility, biodegradability, and ease of construction and manipulation, rendering them attractive options to be deployed for immunomodulation [37]. Hydrogels can serve as scaffolds in the body as they can form ECM-like architectures that mimic the nature of tissues and can provide the necessary cues (chemical, mechanical, and spatial) to cells and host tissue [4]. Additionally, hydrogels can be injected into the body in a minimally invasive manner, thus minimising unwanted host inflammatory responses (as opposed to invasive surgery through implantation), and can also be designed to display specific properties depending on their application, such as self-healing, shape-memory, and stimuli-responsiveness [4,35–37]. Furthermore, the aforementioned ease of design and fabrication of engineered hydrogels in creating a 3D microenvironment means that hydrogels can serve as effective models for the study of immunomodulation [33,37]. A summary of studies of selected hydrogels used for immunomodulation in tissue engineering/regenerative medicine applications and some potential limitations of these studies (highlighted by the authors) is displayed in Table 1.

Biomaterials possess various properties that affect the immune response elicited to them by the host, including; biomaterial type, surface chemistry, surface topography, biodegradability, size and shape, and mechanical properties (Fig. 4) [10]. These properties of biomaterials can be tuned to elicit the desired immunomodulatory effects [1,4,5,10,35].

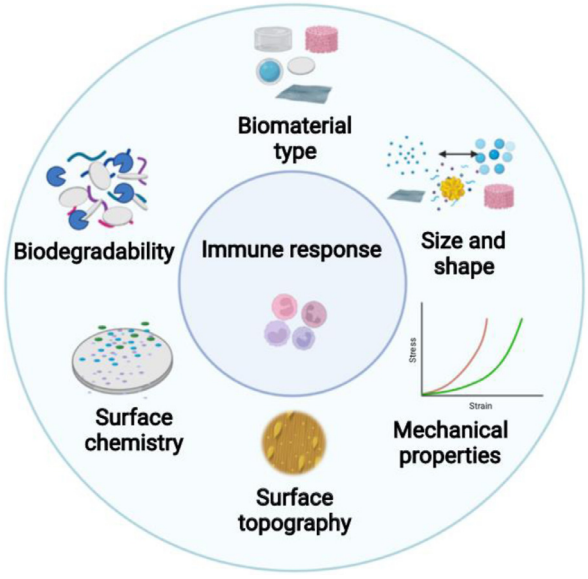

Fig. 4. *Properties of biomaterial scaffolds that impact the host immune response.* Biomaterial type, surface chemistry, surface topography, biodegradability, size and shape, and mechanical properties [10]. Created in Biorender.com.

4.1. Biomaterial type

Depending on the type of biomaterial used (natural, synthetic, or composite), the host immune response to the biomaterial dif-

fers. Natural polymers such as collagen, hyaluronic acid (HA), alginate, and chitosan for example resemble molecules that naturally occur within the body and release mainly non-toxic compounds, therefore tend to be biocompatible. Additionally, naturally derived biomaterials are bioactive and can present adhesion regions for cells and thus degrade easily through enzymatic degradation, activating less adverse FBR compared with synthetic biomaterials. For example, collagen is naturally degraded by collagenase and other proteases, which allows cells in the tissue to mediate its local degradation in a controlled manner [47]. However, natural biomaterials may possess weak mechanical properties that may limit their use in various applications [10,48]. Synthetic materials such as polyethylene (PE), poly(ethylene glycol) (PEG), poly(lactide-*co*-glycolide) (PLG), and poly(lactic acid-*co*-glycolic acid) (PLGA) possess controllable biomechanical and biodegradation properties and can easily be designed to control host protein attachment, rendering them attractive options to be utilised in tissue engineering [49]. However, synthetic materials may mediate the development of chronic inflammation and thus the fibrous isolation and encapsulation of biomaterial implants [10,48].

Host immune responses are elicited in response to both natural and synthetic biomaterials but to varying extents, which can depend on the specific bulk or surface properties of the materials. For example, ECM scaffolds (obtained from biological tissues) induced macrophages to produce increased levels of cluster of differentiation (CD)206 (a pro-regenerative marker) compared to synthetic scaffolds composed of either PE or PEG. Additionally, neutrophil recruitment was higher in the synthetic scaffolds due to them displaying a higher stiffness compared to the naturally derived biomaterial [50].

Composite biomaterials can combine the properties of more than one material to optimise their biological performance. Composites can reflect the desired properties of their constituent materials while providing additional properties (that do not normally exist in either material comprising the composite), allowing the modulation of cell behaviour. Composite materials can additionally be designed to possess adhesion regions and incorporate growth factors into their structure to achieve better cell attachment, migration, proliferation, and differentiation [10,51]. For example, PEG-collagen composites (with a macroporous structure) increased the mobility and migration of DCs and T cells compared to scaffolds comprised of PEG alone (where no T cell attachment or migration was observed). Additionally, the only cytokine/chemokine secretion observed was on the PEG hydrogels with high mechanical strength [52].

4.2. Surface chemistry

The surface chemistry of biomaterials plays an important role in mediating immune cell recruitment and activation. Functional groups on the surface of biomaterials can control protein and cell adhesion and thus the immune response elicited towards them [10]. Biomaterials tend to be hydrophobic, and proteins bind to hydrophobic surfaces stronger than they do to hydrophilic surfaces [10,53,54]. Thus, hydrophilic molecules such as PEG are often incorporated into tissue engineering scaffolds to counteract the immunogenicity of hydrophobic surfaces [5]. Conversely, it was shown that surface hydrophobicity (or wettability) may not correspond with the effect of functional groups and that the surface chemical structure was the predominant factor affecting fibrous capsule thickness *in vivo* [10].

Several studies have shown that biomaterial surfaces with -NH_2 and -OH groups induced more protein and immune cell migration to the biomaterial implantation site and thicker fibrous capsules surrounding the implant compared to biomaterial surfaces with -COOH and -CF groups [10,55,56]. Biomaterial surface chemistry has also been demonstrated to affect macrophage attachment, polarisation, and secretion of immunoregulatory molecules. Furthermore, the surface charge of biomaterials has also been shown to differentially affect the host immune response, however, results reported in the literature on the effect of surface charge are conflicting. Thus, there is no optimal surface chemistry and therefore surface chemistry should be modulated to elicit the desired immunomodulatory effect depending on the application [5,10].

4.3. Surface topography

The surface topography (size, shape, and texture) of biomaterials affects cellular morphology, adhesion, motility, proliferation, differentiation, and cytokine secretion [4,5,10]. Regulating the surface topography of biomaterials allows the control of macrophage migration, differentiation, polarisation, and function and therefore the nature of the immune response elicited by the host [5,10]. Additionally, the pore scaffold architecture (pore density, size, and shape) has also been shown to affect cellular behaviour (cell migration, proliferation, and polarisation), as well as modulate the formation of cartilage and bone and angiogenesis [4,5]. Thus, the porosity of biomaterials should aim to be optimised in the design of scaffolds to sufficiently influence macrophage function (to promote a pro-regenerative environment) whilst ensuring that the mechanical strength and therefore the integrity of the scaffold is not compromised [5].

The surface topography of biomaterials is an effective modulator of cellular behaviour as the components of the ECM interact with cells at the micro/nanoscale, and scaffolds can be engineered to mimic these interactions by designing their surface topography with these same dimensions [10,54]. A recent study investigated the influence of surface patterns in the micro/nanoscale on human macrophage adhesion and phenotype by designing a platform with many patterns in various shapes, dimensions, and with changing cell attachment properties. An increased attachment of macrophages was observed on micropillars with a 5 μm diameter and cell attachment was observed in between the micropatterns on low cell adhesion surfaces, whereas macrophages adsorbed micropillars through phagocytosis on high cell adhesion surfaces [57]. Again, contradictory results are observed in the literature regarding the effect of the surface topography of biomaterials on the host immune response. Further research on these immunomodulatory effects is required to facilitate the design of biomaterials with optimal surface topography properties to elicit the desired outcomes [5].

4.4. Biodegradability

The degradation of biomaterials is another property that can influence the host immune response. For example, HA scaffolds were enzymatically degraded into small fragments of varying molecular weight (MW), and when incubated with DCs and T cells, increased activation of DCs and higher T cell proliferation was observed with low MW fragments [58]. In addition, low MW HA was shown to induce a pro-inflammatory macrophage phenotype, whereas high MW HA promoted an anti-inflammatory macrophage phenotype [59].

Biodegradability and tissue regeneration must be coordinated to ensure that the biomaterial scaffold maintains adequate strength and can successfully sustain the mechanical stresses of the scaffold microenvironment. Additionally, the by-products of scaffold degradation must be non-toxic to ensure an unwanted host immune response is avoided, and must also be extractable through normal bodily function [4]. If scaffold degradation is faster than native tissue regeneration and wound healing, cells will be deprived of ECM-like structure, the newly formed tissue may be non-

functional/defective, and the degradation by-products may not be sufficiently removed from the body [4]. However, scaffold degradation being too slow may lead to the encapsulation of the scaffold, initiating a host immune response and thus insufficient integration or rejection from host tissue [4,60].

The degradation rate of biomaterial scaffolds is controlled by various factors including the material composition, the scaffold microenvironment and structure, bulk/surface modification, and the mechanical environment [4]. Additionally, the cross-linking of a biomaterial scaffold can also affect its rate of degradation and thus influence the recruitment of immune cells and the inflammatory response [4,10]. For example, cross-linking collagen scaffolds with glutaraldehyde resulted in a ten-fold increase in neutrophil recruitment after 28 days following subcutaneous implantation into mice compared with collagen scaffolds crosslinked with hexamethylene diisocyanate (due to the differing degradation rates of the scaffolds) [61]. Furthermore, cross-linking agents aiming to strengthen scaffolds can promote pro-inflammatory immune cell phenotypes [4]. Thus, the degradation rate of biomaterial scaffolds should be designed to optimise the desired immunomodulatory effects.

4.5. Size and shape

The size and shape of a biomaterial scaffold affects the behaviour of host immune cells and the subsequent immune response elicited [4,10]. The importance of the geometry of the scaffold in modulating its biocompatibility was highlighted *in vivo* in a murine FBR model resembling that of humans. Alginate hydrogels with spherical shapes of differing diameters were inserted into mice, and spheres with larger diameters ($>$ 1.5 mm) were shown to be more biocompatible, inducing less FBR and fibrosis compared to spheres with smaller diameters [38]. Additionally, the size of implanted scaffolds was also shown to influence FBR and fibrosis, subsequently impacting the therapeutic effects of transplanted islets [38]. Thus, the biocompatibility of biomaterial scaffolds *in vivo* can be significantly improved by tuning the scaffold geometry [4,10,35,38].

4.6. Mechanical properties

Along with the chemical and surface properties, the bulk features of biomaterial scaffolds such as their mechanical properties also modulate the behaviour of host immune cells [10]. For example, macrophage phenotype and activation were shown to change in response to biomaterial stiffness. Gel-coated collagens of higher stiffness (323 kPa) induced M1 macrophages with low phagocytic ability, whereas scaffolds of lower stiffness (11 kPa and 88 kPa) induced M2 macrophages with an increased phagocytic ability [62]. However, the effects of stiffness on macrophage phenotype appear conflicting. Collagen gels of a lower stiffness (30 Pa) activated M1 macrophages more than collagen gels of a higher stiffness (100 Pa), whereas collagen scaffolds with a higher stiffness mediated M2 macrophage polarisation and an increased production of IL-10 and decreased production of TNF-α [63]. Additionally, PEG hydrogels of a lower stiffness reduced macrophage activation, resulting in a less severe FBR [40]. Thus, again like various other biomaterial properties, the immunomodulatory effects exerted by the mechanical properties of biomaterial scaffolds require further investigation to allow these to be optimised.

5. Immunomodulatory approaches using biomaterial scaffolds for tissue engineering/regenerative medicine applications

Immunomodulation can be achieved using a variety of different strategies including modifying the physical/chemical properties of biomaterials and incorporating immunomodulatory molecules (such as cytokines) and cells (such as MSCs) into biomaterial scaffolds (Fig. 5) [1].

5.1. Bio-chemical modification of biomaterial scaffolds

Chemically modifying scaffolds is an effective way of modulating immune cell behaviour to direct the host immune response and ameliorate FBR [4]. For example, the covalent modification of alginate hydrogels with triazole substantially reduced FBR in rodents and non-human primates by inhibiting macrophage recruitment and fibrous deposition around the hydrogels [39]. In addition, incorporating molecules such as cytokines and growth factors into biomaterial scaffolds is another effective immunomodulatory method [1,5,11,35]. PEG hydrogels incorporating immobilised TGF-β or IL-10 suppressed the maturation of DCs even under LPS or pro-inflammatory cytokine stimulation [65]. The controlled, sequential delivery of interferon (IFN)-γ and IL-4 from scaffolds mediated the transition of macrophages from M1 to M2 phenotype and enhanced the vascularisation of bone scaffolds [66]. Furthermore, the combined delivery of dexamethasone with vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) has also been shown to prevent FBR and increase blood vessel maturation and angiogenesis [5,42,67].

5.2. Inducing immune tolerance in transplantation

Scaffolds can also be utilised to establish a local tolerogenic microenvironment to promote immune tolerance in islet transplantation [64,68,69]. Scaffolds have been thoroughly investigated as a tool to improve islet allograft engraftment and survival; however, the primary role of these scaffolds is to aid the attachment, survival, and function of transplanted islets without promoting islet-specific immune tolerance. Additionally, systemic immunosuppression is commonly administered daily to prevent or delay islet rejection within biomaterial scaffolds. Therefore, scaffolds that support both islet engraftment and promote immune tolerance have been investigated in preclinical models. PLG scaffolds have been deployed to co-deliver islets and TGF-β, which resulted in a decreased infiltration of innate immune cells (including DCs and macrophages) and prolonged graft survival. However, all transplanted islets were eventually rejected (Fig. 5C) [64]. Another study explored the transplantation of islets within scaffolds loaded with IL-33, where frequencies of regulatory T cells (Tregs) were observed within the scaffold and graft survival was significantly enhanced. However, $<$ 15 % of mice displayed long term survival [68]. Incorporating additional immunomodulatory molecules may enhance the efficacy of these treatments.

5.3. Use of granular hydrogels for immunomodulation

As injectable hydrogels require hydrogel degradation to occur before tissue formation, granular hydrogels or microporous annealed particle (MAP) scaffolds have recently been developed [70,71]. MAP scaffolds are injectable, *in situ* crosslinked microporous scaffolds, comprised of microgel building blocks that facilitate simultaneous tissue regeneration and biomaterial degradation, something has never been achieved before using injectable scaffolds. MAP scaffolds are also highly tuneable, allowing the incorporation of a wide range of molecules and have thus been utilised in various biomedical applications. Formation by microfluidic technology allows the modulation of the physicochemical properties of the building blocks, allowing the downstream properties of the MAP scaffold to be controlled [70].

Injection of a MAP scaffold *in vivo* promoted cell migration and resulted in rapid cutaneous tissue regeneration and tissue forma-

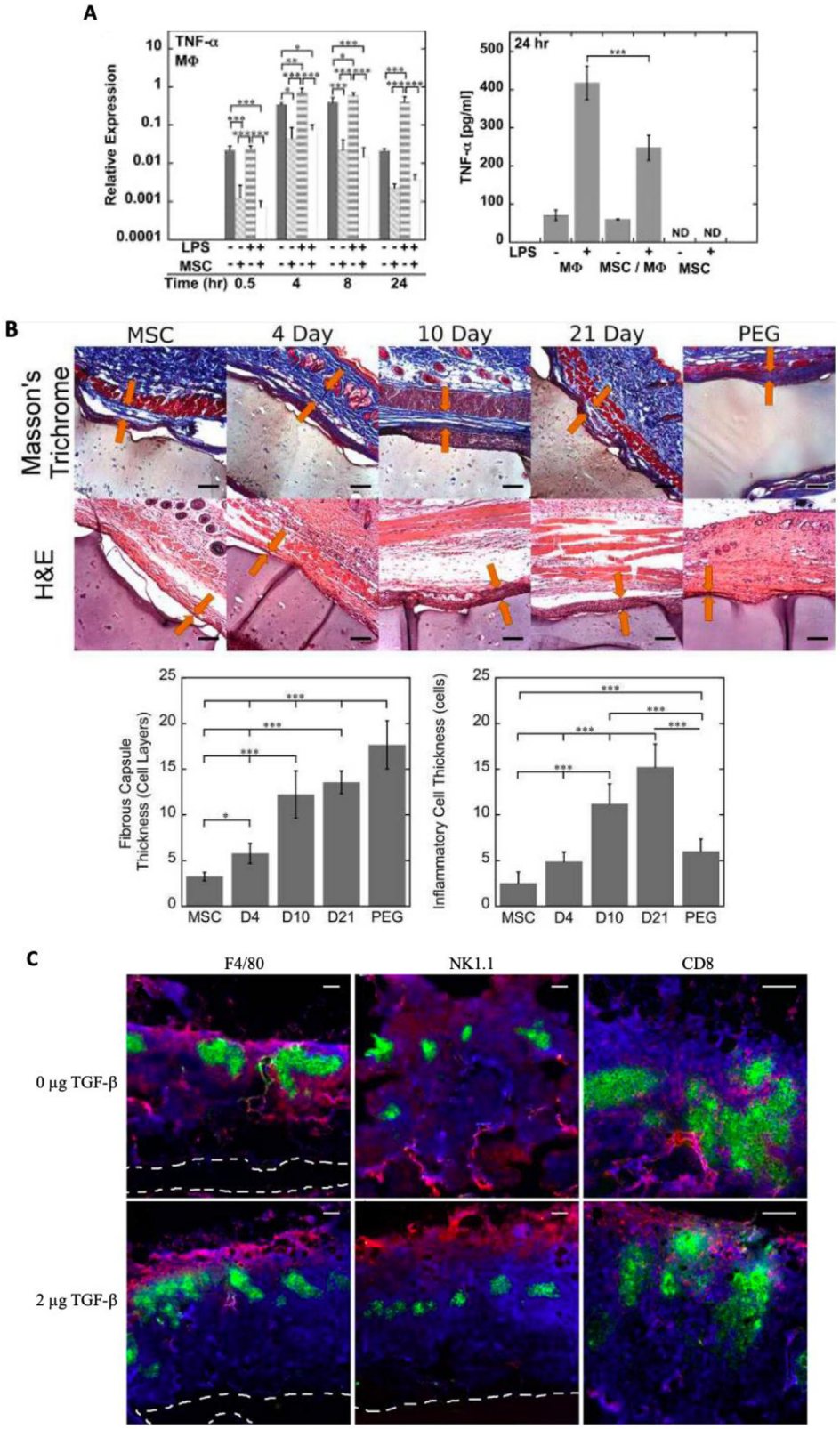

Fig. 5. *Immunomodulatory approaches using biomaterials scaffolds*. **(A)** Effect of mesenchymal stem/stromal cells (MSCs) on macrophage activation *in vitro*. Relative gene expression and cytokine secretion of the pro-inflammatory cytokine tumour necrosis factor (TNF)-α, in macrophage and MSC monocultures and co-culture (encapsulated in poly(ethylene glycol) (PEG) hydrogels) in the absence and presence of lipopolysaccharide (LPS) [41]. (**B**) *In vivo* response to PEG hydrogels encapsulated with MSCs and osteogenically differentiating MSCs. Hydrogels were subcutaneously implanted into immunocompetent C57bl/6 mice for 28 days, and Masson's Trichrome and H&E staining were used for PEG hydrogels containing; MSCs, MSCs differentiated for 4 days, MSCs differentiated for 10 days, MSCs differentiated for 21 days, and no MSCs (PEG). Arrows indicate fibrous capsule (in Masson's Trichrome section) and the layer of inflammatory cells at the material/host interface (in H&E sections). Scale bars = 100 μm [41]. (**C**) Immunofluorescence imaging of leukocyte infiltration into allogeneic islet grafts implanted on poly(lactide-co-glycolide) (PLG) scaffolds with and without transforming growth factor (TGF)-β. Detection of insulin (green), nuclei (blue) and F4/80, NK1.1, or cluster of differentiation (CD)8 (red) in histological sections of transplanted islet grafts on scaffolds containing 0 μg and 2 μg of TGF-β at day 7. Scale bars = 100 μm [64]. Reprinted with copyright 2022 Elsevier.

tion and a reduced host immune response compared to a non-porous control [70]. With the aim of promoting more extensive tissue ingrowth prior to scaffold degradation, another study attempted to slow scaffold degradation by switching the chirality of the crosslinking peptides of the MAP scaffold from $_{L}$- to $_{D}$-amino acids [71]. Surprisingly, $_{D}$-peptide crosslinked MAP hydrogel ($_{D}$-MAP) resulted in quicker biomaterial degradation *in vivo*, yet elicited significant tissue regeneration to healed cutaneous wounds and significant antigen-specific immunity against the $_{D}$-peptides, with an adaptive immune response required for skin regeneration [71]. To tune hydrogel properties such as strength and rigidity, thiol-norbornene click reaction was deployed to synthesise HA hydrogel microparticles (HMPs), which were then annealed into a porous scaffold by tetrazine-norbornene click reaction. Injection of these porous MAP scaffolds into an ischemic stroke model demonstrated the biocompatibility of this chemistry *in vivo*, exhibiting reduced inflammation and astrogliosis compared to alternative crosslinking chemistries [72]. A further study investigated the fate of neural progenitor cells *in vitro* and concluded that the chosen post-modification of the engineered MAP scaffolds can be utilised to either promote neurogenesis or enhance stemness, demonstrating the potential of modulating the phenotypes of MAP scaffolds for the intended application [73].

Controlled, scaffold-mediated delivery of exogenous growth factors is a powerful tool to improve tissue integration with biomaterial scaffolds and enhance tissue regeneration, however, the predetermined nature of this delivery presents limitations, including the ability to meet each specific wound's spatiotemporal regenerative needs [74]. To overcome this, heparin microislands, which are spatially isolated heparin-containing microparticles that can organise endogenous signals, were incorporated into MAP scaffolds, which resulted in significantly improved wound healing in a diabetic wound model (epidermal regeneration and revascularisation) compared to two clinically relevant controls [74]. A further translational hurdle posed to growth factor releasing scaffolds is the limited shelf stability of solubilised protein. To address this, one study presented a lyophilised MAP scaffold that can be rehydrated effectively before use. Loading these scaffolds with epidermal growth factor (EGF) following lyophilisation maintained scaffold properties and resulted in faster reepithelialisation *in vivo,* demonstrating the clinical translatability of MAP scaffolds and potential to be utilised in a wide variety of regenerative applications [75].

5.4. Exploiting MSCs as immunomodulators

MSCs are widely used in tissue engineering/regenerative medicine approaches due to their differentiating capacity and regenerative and immunomodulatory properties [10]. The immunomodulatory functions of MSCs are mainly deployed via interacting with immune cells through cell-to-cell contact and by paracrine mechanisms. Their secretome is comprised of an extensive repertoire of cytokines, chemokines, and growth factors that modulate the function of immune cells, including molecules such as TNF-α, TGF-β, IFN-γ, prostaglandin (PG)E2, indoleamine-pyrrole 2,3-dioxygenase (IDO), and fibroblast growth factor (FGF), along with many others [76]. The paracrine factors of MSCs are encapsulated in cell-secreted extracellular vesicles (EVs), which are defined as exosomes, microvesicles, or apoptotic bodies depending on their cell of origin and size, and with their paracrine actions varying depending on the source of MSCs, the target cells, and the local microenvironment [76].

Encapsulating murine MSCs in PEG hydrogels reduced the fibrotic response of the FBR compared to acellular hydrogels via the downregulation of M1 macrophage activation *in vitro* (Fig. 5A). *In vivo*, encapsulated MSCs again reduced the thickness of fibrous capsules in mice compared to acellular hydrogels, however, this effect declined with osteogenic differentiation (Fig. 5B) [41]. Thus, the crosstalk between MSCs and immune cells requires further investigation to allow the modulation of macrophage phenotype and attenuation of the FBR to improve tissue engineering outcomes [41]. The encapsulation of MSCs and macrophages in gelatin hydrogels has been shown to aid in the generation of skin tissue and wound healing in mice [77], and in graft-versus-host disease models, alginate-MSC combinations significantly increased the survival rate of mice [43]. Additionally, the immunomodulatory capacity of MSCs can be enhanced by cytokines within a stable hydrogel microenvironment. For example, PGE2 controlled-release hydrogels created a sufficient microenvironment to sustain the immune privilege properties of MSCs and restore cardiac function in rats [78]. Furthermore, MSCs encapsulated in a collagen-HA-based hydrogel platform demonstrated a significant increase of their secretory profile of proangiogenic and immunomodulatory paracrine factors, and seeding MSCs on pullulan-collagen hydrogels improved their viability and augmented their proangiogenic, fibromodulatory and immunomodulatory effects *in vivo* [44,45].

Recently, the focus of using of MSCs in tissue repair and immunomodulation has shifted towards macrophage and T cell regulation [79]. MSCs themselves can secrete cytokines to modulate cellular behaviour, for example, their secretion of TGF-β can promote Treg and macrophage induction [80,81]. Furthermore, MSC composite scaffolds can regulate macrophage activation and reduce the FBR through crosstalk with inflammatory cells [41]. Thus, a greater understanding of the immunoregulatory effects of MSCs will continue to aid in developing effective tissue engineering/regenerative medicine approaches. MSCs have been effectively deployed in various applications such as cartilage, bone, and myocardial regeneration, however, in many studies the mechanism of MSC repair is unclear [79]. For example, coculture of MSCs with autologous chondrocytes in HA hydrogels demonstrated a significantly higher rate of chondrogenesis, and bone marrow MSC-based engineered cartilage ameliorated poly(glycolic acid)/poly(lactic acid) (PGA/PLA) scaffold-induced inflammation and improved cartilage tissue regeneration through the M2 polarisation of macrophages [46,82]. However, the underlying mechanism of immune regulation of MSCs by biomaterials has not been investigated and is not understood [79]. Additionally, collagen scaffolds enhanced the production of trophic factors, modified their fibrogenic and immunomodulatory phenotypes, and promoted the cardioprotective effects of MSCs [83]. Understanding the MSC-mediated mechanism of tissue repair will further enhance the therapeutic efficacy of MSCs in tissue engineering applications [79].

5.4.1. The use of MSC-derived extracellular vesicles (EVs) for immunomodulation

MSC-derived EVs (particularly exosomes) are emerging as novel, cell-free alternative therapies for regenerative applications and immune-related diseases [84]. MSC-derived EVs can maintain the desirable immunodulatory properties of their parent cells, while their cell-free nature gives them a more favourable safety profile in comparison to cellular-based therapies. The therapeutic efficacy of EVs has been investigated in several disease models, including their ability to induce macrophage polarisation. For example, MSC-derived EVs demonstrated the ability to inhibit the inflammatory response in osteoarthritis (OA) by mediating M2 macrophage infiltration into OA cartilage defects, while reducing M1 macrophage infiltration and down-regulating the expression of TNF-α and IL-1β [85]. However, further research is required to gain a greater understanding of the mechanisms of EVs and their interactions with immune cells. Furthermore, despite their therapeutic potential, limitations of MSC-derived EVs exist, including their inability to replicate MSC cell-to-cell contact, the challenge of producing

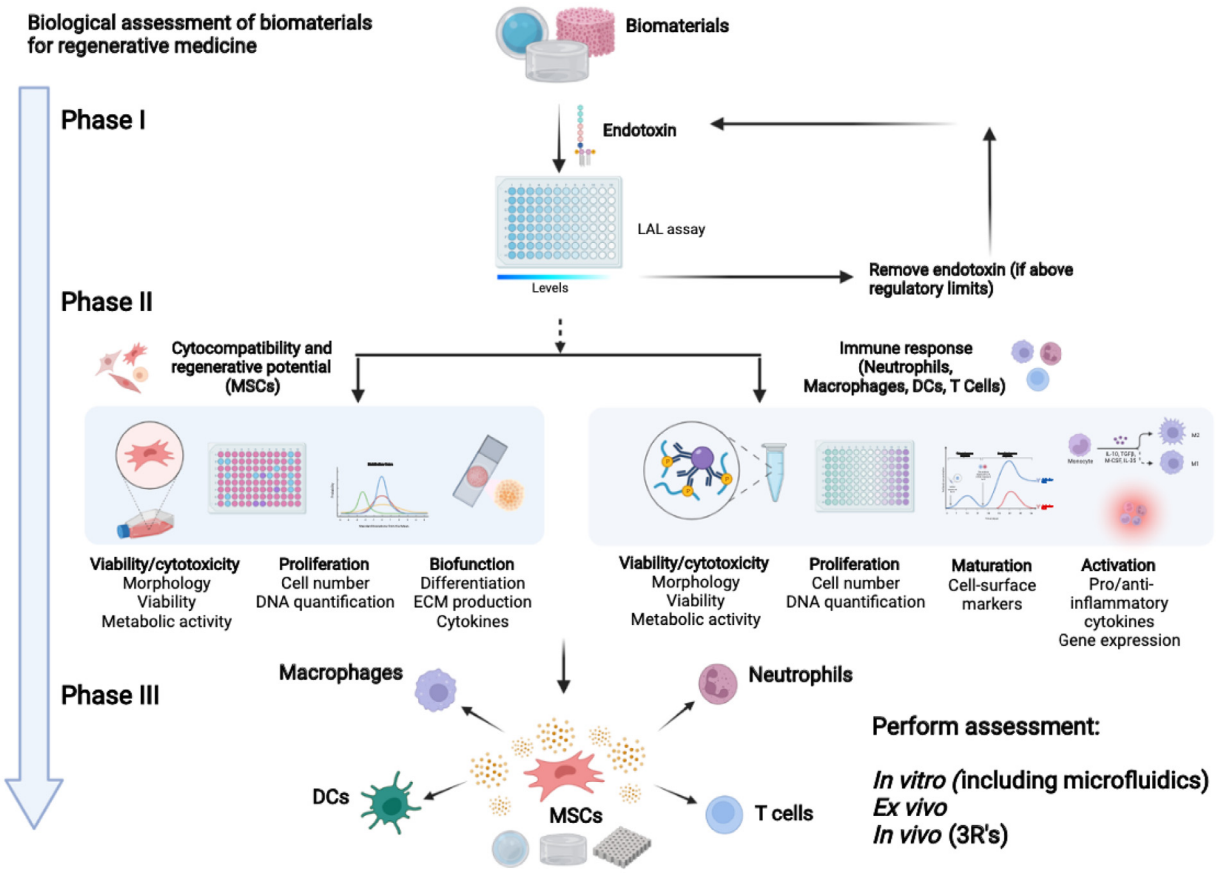

Fig. 6. *A pathway to measure the immune response to biomaterials (hydrogels) in vitro and regenerative potential for tissue engineering/regenerative medicine applications***.** Phase I – determine endotoxin content of biomaterials (e.g., Limulus amoebocyte lysate (LAL) assay). If endotoxin levels are above regulatory limits, remove endotoxin and perform LAL assay again. Phase II – determine immune response using peripheral blood mononuclear cells (PBMCs) (focussing on neutrophils, macrophages (differentiated from monocytes via monocyte-colony stimulating factor, M-CSF), dendritic cells (DCs) and T cells) by assessing viability/cytotoxicity (morphology, viability, metabolic activity), proliferation (cell number, DNA quantification), maturation (cell-surface marker expression) and activation (pro/anti-inflammatory cytokine production and gene expression). For assessing hydrogels for tissue engineering/regenerative medicine applications, determine cytocompatibility and regenerative potential (viability/cytotoxicity, proliferation and biofunction) by assessing morphology, viability, metabolic activity, cell number, DNA quantification, differentiation, extra-cellular matrix (ECM) production, and cytokine production of mesenchymal stem/stromal cells (MSCs). Phase III – If biomaterials are modified for immunomodulation (e.g., MSCs/cytokines are incorporated into hydrogels, or properties of biomaterials (e.g., surface chemistry/topography of hydrogels) are changed), determine the subsequent immune response (as in Phase II). This assessment should first be performed *in vitro* (including the use of microfluidic technology) and *ex vivo* (e.g., tissue explants) to predict the likelihood of biomaterial acceptance before validation *in vivo,* hence the 3R's (reduction, refinement, and replacement of animals). Created in Biorender.com.

large quantities of EVs with high purity, and their rapid clearance from the body. Investigating the effects of biomaterials on MSC-derived EVs, including the possibility of encapsulating EVs in biomaterials to achieve a sustained release in a controlled manner, may represent a promising approach to increase the efficacy of potential cell-free, EV-based immunomodulatory therapeutics [84].

6. A pathway to evaluate the immune response to biomaterials (hydrogels) *in vitro* and regenerative potential for tissue engineering/regenerative medicine applications

Currently, *in vitro* assessments that have been used to evaluate biomaterials have been heavily focused on non-immune cell cytotoxicity and cytocompatibility and are general across all medical devices. There have been limited assessments of the immune response elicited to biomaterials *in vitro,* therefore the current techniques are limited in their ability to predict the acceptance of biomaterials *in vivo* [2]. Thus, we propose a pathway to evaluate the immune response to biomaterials (hydrogels) *in vitro* and their regenerative potential for tissue engineering/regenerative medicine applications, with the end goal of incorporating the *in vitro* assessment of the immune response into ISO-10993, providing a standardised means of evaluating the likelihood of biomaterial acceptance *in vivo* (Fig. 6).

The evaluation of different forms of biomaterials (i.e., other than hydrogels), for example other porous scaffolds, nanoparticles, films etc., or evaluation for a different purpose (i.e., other than tissue engineering/regenerative medicine), for example autoimmune diseases, may follow a similar pathway to the proposed evaluation to determine the host immune response and subsequent likelihood of biomaterial acceptance *in vivo*. However, the widely applied use of hydrogels in the tissue engineering field has motivated the proposed pathway that has been suggested for tissue engineering/regenerative medicine applications [37].

6.1. In vitro *evaluation of the immune response and regenerative potential of biomaterials*

The cells to focus on in the evaluation of the immune response and regenerative potential of biomaterials have been suggested and described in Table 2. These cells are heavily implicated in determining the outcomes of tissue engineering/regenerative medicine applications. Thus, understanding their roles in the host response towards biomaterials would provide invaluable information and greatly aid in developing immunomodulatory strategies to

Table 2
Cells suggested to focus on in the evaluation of the immune response and regenerative potential of biomaterials. MSCs (mesenchymal stem/stromal cells), neutrophils, macrophages, DCs (dendritic cells), and T cells.

Cell	Response to study	Limitations
MSCs	Regenerative and immunomodulatory potential.	Efficacy decreases over time/after differentiation/*in vivo* and depends on the microenvironment and source of MSCs. Donor viability/MSC heterogeneity [41,44,45,76,86,90,91].
Neutrophils	Recruitment/maturation (Immune response and immunomodulatory potential e.g., tissue regeneration).	If cell line, not representative of *in vivo*. If primary cells, die very rapidly. Donor variability [6,31,90].
Macrophages	Polarisation/activation (Immune response and immunomodulatory potential e.g., tissue regeneration).	If cell line, not representative of *in vivo*. Donor variability [2,6,31,90].
DCs	Maturation/activation (Immune response and immunomodulatory potential e.g., immune tolerance).	If cell line, not representative of *in vivo*. If primary cells, short lifespan. Donor variability [6,11,90].
T cells	Polarisation/activation (Immune response and immunomodulatory potential e.g., immune tolerance).	If cell line, not representative of *in vivo*. Donor variability [6,12,90].

improve the outcomes of biomaterial-based regenerative therapies [10–12,79].

MSCs have been successfully deployed in various tissue engineering/regenerative medicine applications (as previously mentioned) due to their differentiation and regenerative capacity and immunomodulatory properties. However, the responses elicited by MSCs (and EVs), and subsequent therapeutic efficacy, can change significantly depending on the nature of their microenvironment and their source. Over 900 clinical trials to date have investigated the therapeutic use of MSCs, however, after two decades, only 3 approved MSC therapies are on the market, none of which are approved in the United States by the FDA [86]. This lack of clinical success can (at least partly) be attributed to the heterogeneity of MSCs, which arises due to intrinsic biological variability and extrinsically introduced variability. Intrinsic biological variability can result from differences between donors, tissue sources, clonal subsets, and single cells, while extrinsic variability can be introduced because of non-standard methods of isolation, selection, and expansion. Approaches to overcome MSC heterogeneity aim to improve MSC uniformity in order to better clinical results and give insights into possible therapeutic mechanisms, which include the use of biomaterial-MSC constructs, as well as pooled, clonal, cytokine (e.g. IFN-γ)-primed, and pre-treated (e.g. hypoxically preconditioned) MSCs [86–89]. Validated MSC potency assays with defined quality control (QC) criteria would be essential in validating MSC manufacturing consistency. Biomaterial-mediated mechanisms that affect the immunomodulatory functions of MSCs remain largely unknown, and notably, no biomaterial-MSC studies have been performed that have used clonal or increasingly homogenous MSC populations [86]. Thus, investigating the interactions of MSCs with biomaterials, including the potential of biomaterials to reduce MSC heterogeneity, and the interactions of MSCs with host immune cells will allow the use of MSCs to be optimised and significantly improve the outcomes of a variety of applications.

The use of primary human MSCs would be representative of MSCs *in vivo*, thus these MSCs should be the skeletal cell type of choice to be investigated [79,90,91].

In terms of immune cells, macrophages are the obvious cell type to study the response of due to their pivotal role and importance of phenotype in determining the host FBR to biomaterials and thus the success of outcomes of biomaterials [2,10,90]. As described, a higher M2:M1 ratio of macrophages *in vivo* correlates with positive healing outcomes, thus utilising assays to determine macrophage phenotype in the *in vitro* testing of the immune response to biomaterials would provide a clearer indicator of biomaterial acceptance *in vivo* [2]. Standardisation of the markers used to identify macrophage subsets would enhance the reproducibility of these assays [92]. Neutrophils are the most abundant innate immune cell in the blood, are the first cells recruited to an implanted biomaterial, and have been shown to persist around implanted biomaterials. However, the role of neutrophils in the host response to biomaterials is poorly understood. Therefore, investigating the function of neutrophils in the host response to biomaterials may prove to be beneficial in gaining a greater understanding of the FBR, allowing the development of subsequent immunomodulatory strategies to reduce FBR/biomaterial rejection and promote tissue integration/regeneration [6]. DCs are the most potent antigen-presenting cells in the body and play a central role not only in the activation of the adaptive immune response, but also in the induction of immune tolerance. DCs directly interact with biomaterials, and their involvement in mediating the host immune response to biomaterials is poorly studied, although their role in biomaterial interaction is looking increasingly important. Their function in the activation of T cells, particularly T-helper cells that can mediate macrophage phenotype, and their immunomodulatory potential (in mediating immune tolerance to implanted grafts for example), renders DCs attractive options to study to optimise future tissue engineering applications [11]. T cells are increasingly being recognised for playing an important role in modulating the host immune response to biomaterials, for example with Th1 and Th2 cells polarising macrophages towards M1 and M2 phenotypes, respectively. Additionally, Tregs play a vital role in promoting immune tolerance, thus studying the response of T cells towards biomaterials will allow the design and efficacy of biomaterial-based treatments/approaches to be greatly improved through the consideration of long-lasting adaptive immunity [12].

Cell lines of these cell types can be used to study the immune response; however, the phenotype of cell lines is modified, thus they rarely replicate the behaviour of primary cells, and their physiological relevance is limited. Neutrophils, macrophages (through the differentiation of monocytes with macrophage-colony stimulating factor (M-CSF)), DCs, and T cells can all be obtained from human peripheral blood mononuclear cells (PBMCs), and these primary cells replicate the behaviour of cells *in vivo*, thus these should be the immune cell type of choice to study. However, limitations such as the short lifespan of cells and donor variability should be considered when studying primary cells [90]. Cell lines can be utilised as tools to set up experiments, but results must be validated with primary cells.

Table 3
Methods to evaluate the immune response and regenerative potential of biomaterials. Endotoxin content (e.g., LAL assay), imaging (e.g., trypan blue/calcein AM/ethidium homodimer/phalloidin/DAPI/alcian blue), metabolic assays (e.g., PrestoBlue/MTT/G6PD), maturation (flow cytometry/IF), and activation (ELISA, qPCR, and RNA-Seq). LAL = Limulus amoebocyte lysate, calcein AM = calcein acetoxymethyl, DAPI = 4',6-diamino-2-phenylindole, G6PD = glucose-6-phosphate dehydrogenase, IF = immunofluorescence, ELISA = enzyme-linked immunosorbent assay, qPCR = quantitative polymerase chain reaction, RNA-Seq = RNA sequencing [2,90].

Characterisation	Advantages	Disadvantages
Endotoxin content (LAL assay)	Sensitive (can detect low quantities).	Possible unwanted interactions/interference [18,32].
Imaging (trypan blue/calcein AM/ethidium homodimer/phalloidin/DAPI/alcian blue)	Allows visualisation and quantification of cells. Can calculate cell viability and proliferation.	Only allows single time point measurements. Quantification requires multiple fields of view [2,90].
Metabolic assays (PrestoBlue/MTT/G6PD)	Quantitative. Quick and easy detection of metabolic activity of cells.	Only measures viable cells [2,90].
Maturation Flow cytometry/IF	Allows investigation of expression of a variety of cell-surface markers. Flow cytometry allows characterisation and sorting of cells based on physical/chemical characteristics. IF only requires fluorescence microscope and provides images easy to interpret.	Flow cytometry produces large amounts of data to process. Difficult to compare samples from different experiments (although can standardise samples by fluorescence intensity) [2,90].
Activation ELISA	Allows quantification of the concentration of an abundance of proteins (e.g., cytokines, chemokines, growth factors etc.) with high specificity and selectivity.	Relatively long experimental procedure. May require optimisation (e.g., to fit target values into the kit detection range) [2, 90].
qPCR/RNA Seq	Extremely accurate and sensitive. Allows analysis of a variety of markers and their gene expression in real time. RNA Seq provides very high discovery power.	Possible false positives from contamination. Long process with delicate steps critical to produce reliable results [2,90,93,94].

6.2. Methods to evaluate the immune response and regenerative potential of biomaterials

The methods that have been suggested to evaluate the immune response and regenerative potential of biomaterials are described in Table 3.

To date, immunogenicity assays have focussed on cell viability, maturation, and activation, with cell activation being the most studied as a result of its importance in determining functional outcomes [2].

As previously described, determining the levels of endotoxin in biomaterials is vitally important, as even extremely small concentrations (~1 ng/ml) can induce strong pro- inflammatory host responses, affecting the performance and subsequent outcomes of tissue engineering applications. Despite this, the impact of endotoxin contamination in the field of regenerative medicine/tissue engineering has largely been overlooked, therefore, incorporating the assessment of endotoxin levels into the *in vitro* evaluation of the host immune response to biomaterials is of upmost importance [18]. The LAL assay is an effective method that is commonly used for determining the levels of endotoxin in biomaterials and allows the detection of extremely small concentrations of endotoxin. The simplest form is the LAL gel clot assay, although some advanced techniques (such as chromogenic and turbidimetric) have been developed to improve the performance of the gel clot assay. However, potential interference (for example from β-glucans) must be considered with this assay, although commercially available kits have addressed this issue. This assay should be used in conjunction with endotoxin-free products when studying the host immune response to biomaterials and should be the first assay performed (in Phase I, before cytocompatibility and regenerative potential and immunogenicity assays in Phase II) due to the immunostimulatory effects exhibited by endotoxin [18,32]. Endotoxin levels should be found to be within the defined limits for medical devices to proceed with the subsequent immune evaluation. If endotoxin levels are found to be above regulatory limits, endotoxin should be removed and the LAL assay should be performed again, ensuring that endotoxin levels are below regulatory limits before proceeding [18]. Bioassays can also be performed (in Phase I) to elucidate the likelihood of biomaterial acceptance *in vivo* by determining if endotoxin is eliciting a strong pro-inflammatory response, which would be particularly useful for materials that are not compatible with the LAL assay. For example, this can be achieved through the co-culture of a biomaterial with PBMCs and measuring the secretion of TNF-α after 24 hours.

Measuring the viability/cytotoxicity of cells is vital in determining their response to biomaterials. For example, if a biomaterial causes cell viability to reduce, the biomaterial may not provide optimal tissue repair/regeneration *in vivo* as cells will not be able to elicit their effects. Additionally, a large increase in cell viability may also not be desired as this may suggest some level of cellular activation, which may be unwanted [2]. Cell morphology can be assessed by microscopy (fluorescence/confocal/scanning electron microscopy (SEM) for example) accompanied by staining protocols to provide a relatively quick and simple visualisation of the cellular effects in response to biomaterials. Staining with phalloidin allows the cytoskeleton to be visualised, while staining with 4',6-diamino-2-phenylindole (DAPI) allows the visualisation of the cell nucleus. Live/dead staining can be performed with calcein acetoxymethyl (AM)/ethidium homodimer, respectively or through trypan blue staining, allowing the viability of cells to be assessed [2,90]. Cellular viability can also be measured by flow cytometry with the use of Zombie aqua/Annexin V staining, providing information on the percentage of live/dead cells and cells that are in an apoptotic/necrotic state, respectively, allowing the mechanism of cell death to be determined. Additionally, metabolic assays (such as PrestoBlue, MTT, and glucose-6- phosphate dehydrogenase (G6PD)) allow the determination of the metabolic activity of cells in response to biomaterials, elucidating if an unwanted change in metabolic activity has occurred and thus if cellular effects *in vivo* may be affected. However, only the metabolic activity of viable cells can be measured [2,90]. Assessing the proliferation of cells in response to biomaterials is again immensely important, as reduced proliferation may mean that the desired cellular effects will not be elicited *in vivo*, while increased proliferation may again suggest unwanted immune activation. Absolute cell number can be calculated using a haemocytometer when visualising cells using microscopy and can also be quantified using flow cytometry. DNA quantification can also be used to measure cellular proliferation, using assays such as Pico Green and CyQuant [95].

With regards to assessing the regenerative potential of MSCs (if evaluating for possible use in tissue engineering/regenerative

medicine applications), their biofunction in response to biomaterials is a crucial determinant and would be an indicator of the efficacy of their performance in *in vivo* applications. Measuring ECM production is an effective method of determining biofunction, which can be achieved by IF, using staining protocols such as alcian blue to measure the production of GAGs for example and phalloidin to selectively stain for F-actin. Enzyme-linked immunosorbent assay (ELISA) can also be used to quantify the protein concentration of specific ECM components, along with assessing the immunomodulatory potential of MSCs through the quantification of the levels of cytokines that they secrete [1,2,90].

Determining the levels of cell-surface maturation markers expressed by cells that have been exposed to biomaterials would provide vital information about their cellular phenotypes after biomaterial interaction (i.e., if a biomaterial is inducing a cell to mature/differentiate). Thus, the expression of cell-surface markers (of pro/anti-inflammation) would be an effective indicator of the subsequent immune response that would be elicited by the host. For example, determining the expression of CD86/CCR7 and CD163/CD206 for pro-inflammatory (M1) and anti-inflammatory (M2) macrophage subsets, respectively, would reveal the macrophage phenotype induced by a biomaterial and thus the likelihood of host FBR or biomaterial acceptance *in vivo* [2]. Additionally, investigating the expression of the co-stimulatory molecules CD80 and CD86 by DCs would highlight their maturation state and therefore indicate the immunomodulatory effects that they may potentially exhibit *in vivo* (i.e., activating the host immune response through antigen presentation to naïve T cells). Furthermore, assessing the DC expression of tolerogenic molecules (e.g., programmed death-ligand (PD-L)1 and PD-L2) would further reveal the immunomodulatory capacity of DCs following exposure to a biomaterial, for example, their capacity to induce immune tolerance [11]. Thus, this would allow the efficacy of potential biomaterial-based applications *in vivo* to be determined. Although IF is a valid and effective method, flow cytometry provides a highly sophisticated means of measuring cell-surface marker expression, allowing the characterisation and sorting of cells based on their physical and chemical characteristics [2,90].

The most common method of characterising the *in vitro* immune response to biomaterials is assessing cellular activation, which is likely due to the capacity to evaluate a vast selection of pro/anti-inflammatory markers [2]. ELISA allows the quantification of the concentration of various proteins (such as cytokines, chemokines, and growth factors) released from immune cells in response to biomaterial exposure and thus can effectively be used to assess the likelihood of immune rejection or acceptance of biomaterials *in vivo* [2,90]. For example, quantifying the concentration of pro-inflammatory cytokines (such as IL-1α, IL-1β, IL-6, IL-8, and TNF-α) and anti-inflammatory cytokines (such as IL-1RA, IL-4, IL-10, IL-13, and TGF-β) would indicate the likely nature of the host immune response *in vivo*. High levels of pro-inflammatory cytokines would likely be indicative of host immune rejection/FBR, while higher levels of anti-inflammatory cytokines would more likely suggest biomaterial acceptance by the host *in vivo*. Single-marker assays allow the measurement of one protein (i.e., IL-1 or TNF-α for pro-inflammatory cytokines and IL-10 or TGF-β for anti-inflammatory cytokines for example), whereas multi-analyte ELISA kits allow the simultaneous quantification of various cytokines, providing a more comprehensive indicator of the host immune response *in vivo* [2]. Furthermore, measuring the gene expression of inflammatory markers from immune cells following interaction with biomaterials would further reveal the likelihood of *in vivo* acceptance for a biomaterial [2,90]. Quantitative polymerase chain reaction (qPCR) is an extremely sensitive and accurate method of determining gene expression and allows the analysis of a variety of markers (e.g., of pro/anti-inflammation as described) and their gene expression in real-time. Additionally, RNA sequencing (RNA-Seq) allows the use of a hypothesis-free approach to determine gene expression through its ability to detect novel genes and quantify rare variants and transcripts. Despite RNA-Seq being less cost-effective for sequencing a low number of targets ($\leq$ 20) and more time consuming compared to qPCR, it provides a higher discovery power than qPCR, although both methods are highly effective ways of assessing gene expression [93,94].

As previously described, various factors influence the host immune response to biomaterials, and immunomodulation can be achieved using a variety of strategies. Therefore, in phase III, if a biomaterial has been modified in some way for immunomodulatory purposes, for example by seeding MSCs/cytokines onto hydrogels or changing the properties of hydrogels such as surface chemistry/topography, the resultant host immune response should again be evaluated to elucidate the likely effects of this immunomodulation *in vivo,* as the likelihood of biomaterial acceptance may be affected [1,4,5,35, 37].

In terms of interpreting the results from the assays and determining, for example, QC criteria for the biomaterial, the *in vitro* results will need to be validated and linked to what happens *in vivo* (animals and humans) to evaluate which *in vitro* measurements best predict how the biomaterial will interact with the immune system *in vivo*. This will allow the development of a set of QC criteria that can predict the likelihood of biomaterial acceptance *in vivo*, as currently the only *in vitro* QC is for endotoxin levels [18,21]. For example, using human organ-on-a-chip microfluidic culture technology, a recent study developed the first reported *in vitro* model that supported the formation of human lymphoid follicles that recapitulated many features of germinal centres found in secondary and tertiary lymphoid organs. This system allowed the testing of human immunisation responses to vaccines and adjuvants *in vitro* using primary human cells isolated non-invasively from peripheral blood in a patient-specific manner, eliminating donor variability. The continued development of these types of technology may provide a human-relevant, pre-clinical tool for evaluating the immune response to biomaterials and the safety and efficacy of potential biomaterial-based therapies. The immune response to biomaterials could first be assessed *in vitro* and then subsequently by microfluidic technology and *ex vivo* (e.g., tissue explants) before validation *in vivo*. Performing these assays first *in vitro* will inform the design of effective tissue models and facilitate the better design of animal studies, reducing the need to rely on animal models and the amount of animal studies performed [96].

7. Conclusion and future opportunities

7.1. The need for a standard biomaterial characterisation pathway

Despite the central role that the host immune response to biomaterials plays in determining clinical outcomes, current evaluation protocols (ISO-10993) do not incorporate the *in vitro* assessment of the immune response elicited to a biomaterial [2,8]. In addition, the levels of endotoxin in biomaterials significantly affects the host response and subsequent efficacy of tissue engineering applications, however, the impact of endotoxin levels is rarely considered [18]. Thus, the suggested pathway of methods aims to standardise the *in vitro* evaluation of the immune response to biomaterials to predict the likelihood of the acceptance of a biomaterial *in vivo*, with the end goal of incorporation into ISO-10993. The highly desirable physicochemical properties of hydrogels have enabled them to be widely used in tissue engineering/regenerative medicine approaches, therefore the incorporation of their evaluation is necessary. Furthermore, hydrogels can be developed into 3D *in vitro* models, allowing their use as an effective tool to study immunomodulation and pathophysiology [33,37].

7.2. The use of advanced biomaterial processing techniques

The rapidly evolving field of 3D bioprinting is emerging as a promising new approach for the fabrication of complex biological constructs that can mimic native microenvironments. Exploiting 3D bioprinting allows the spatiotemporal control of the micro/nanoscale chemical and topographical features of hydrogels to induce pro/anti-inflammatory immune cell phenotypes and therefore can be utilised to engineer multicellular tissues to model physiological conditions with immune system components. The continuing progression of this field may provide a highly sophisticated means of evaluating the host immune response in a physiologically relevant environment [25,33,95]. In addition, the use of microfluidic-based systems can also provide physiologically relevant conditions, allowing the culturing of cells on biomaterials whilst geometrically confining cells to channels of hundreds of micrometres. Microfluidic technology also enables the controlled perfusion of cells and the adjusting of microenvironmental parameters, including biochemical gradients and environmental cues. Although these fields are still in their infancy and challenges such as a lack of standardisation currently limit their widespread application, these advanced methods offer immense potential to allow the characterisation of biomaterials in a physiologically relevant manner [33,90]. A recent study developed liquefied capsules as immunomodulatory miniaturised 3D platforms for the high-content screening of polymers that have potential uses in scaffolds. The confined, liquefied capsule core enabled a cell-mediated 3D assembly with bioinstructive microplatforms, permitting any potential synergistic effects that cells used in tissue engineering therapies have on the immune microenvironment prior to implantation to be studied. This system enabled the study of the immunomodulatory bioperformance of a wide range of polymers in a scalable and cost-effective manner, while simultaneously evaluating the paracrine signalling between the encapsulated cells and the immune microenvironment [97]. Again, the issue of endotoxin contamination must be considered when manufacturing biomaterials using these advanced processing techniques. Precautions should be taken to minimise endotoxin contamination, including the use of endotoxin-free materials/equipment and a sterile working environment, and the endotoxin levels of manufactured products should be determined to ensure that they are below regulatory limits.

Animal models have been widely utilised to study immune responses, however, animal studies (in addition to being costly and low-throughput) often demonstrate inconsistent and conflicting results and thus provide a poor ability to predict patient responses in humans, which has led to the increasing development of microfluidic-on-chip systems aiming to recapitulate human physiology [98]. Despite the literature suggesting that an individual's specific immunological profile elicits significant levels of inter-individual variation [99], no current approach allows the personalised screening of the FBR to a biomaterial [98]. Thus, there is a need for a high throughput, low-cost method of evaluating an individual's FBR to a given biomaterial. To aim to address this, a FBR-on-a-chip platform was developed that modelled the immune response following biomaterial implantation, which included modelling the implant microenvironment, along with vasculature and circulating immune cells [98]. This platform demonstrated that the release of cytokines (e.g., MCP-1) from hydrogels induced the trans-endothelial migration of immune cells (e.g., monocytes) towards the hydrogels, mimicking biomaterial implant-induced inflammation. Inter-patient variations in FBR were revealed using patient derived PBMCs, highlighting the importance of a personalised system to study inter-patient differences in FBR and the potential of this platform to do this in a physiologically relevant and personalised manner. This platform could be deployed to screen an individual's FBR to a variety of potential biomaterial-based treatments and subsequently use specific immunomodulatory approaches to mitigate a negative host response to a given biomaterial [98].

As described, incorporating MSCs into biomaterials shows immense promise to be utilised in immunomodulatory and regenerative applications, however, the clinical use of MSCs remains limited due to product variability and the inability to predict clinical efficacy and potency, resulting in inconsistent outcomes [100]. To address this, a low cost, high-throughput, scalable, on-chip microfluidic potency assay was developed, which displayed an improved functional predictive power and similarity of human MSC secretory responses *in vivo* when compared to traditional methods. The secretory performance of human MSCs that was achieved in the microfluidic-on-chip system also showed improved similarity in comparison to an *in vivo* model, demonstrating the promise of these novel systems to improve the clinical outcomes of biomaterial-based regenerative therapies [100]. The quantification and dynamic analysis of cytokine release from immune cells allows the precise determination and functional characterisation of the immune phenotype of a patient that can be utilised to aid clinical diagnosis and treatment. A microfluidic device that allowed on-chip isolation, culture, and stimulation, along with the dynamic and highly sensitive cytokine profiling of patient's immune cells was developed, which profiled the secretion of multiple pro-inflammatory cytokines (TNF, IL-6, and IL-8) of PBMCs [101]. This microfluidic immunophenotyping platform can help to reveal the mechanisms of systemic immune responses and thus has the potential to enable the efficient evaluation of the immune response of a patient to a biomaterial and subsequent likelihood of biomaterial acceptance [101].

Various microfluidic organ-on-chip models have been created that can accurately mimic the complex organ microenvironment and thus have the potential to be utilised as valuable tools to evaluate immune responses in an organ-specific manner. As there is a considerable need for the development of physiologically relevant *in vitro* skin models for studying complex skin-specific immune responses (for studying dermatological diseases and biomaterial/drug responses for example), a study developed a microfluidic skin chip that accurately recapitulated the skin microenvironment [102]. Dermal fibroblasts and keratinocytes were co-cultured with vascular endothelial cells (human umbilical vascular endothelial cells), and the formation of a vascular endothelium in the presence of dermal and epidermal layers was verified through analysis of tissue-specific markers. Circulating leukocytes were incorporated to mimic neutrophil migration in response to external stimuli, where an increased cytokine secretion and neutrophil migration was observed following exposure of the skin chip to UV irradiation. This highlights the capability of microfluidic chips to study the immune response of human tissues and thus their potential to be utilised for biomaterial evaluation [102]. Lung-on-chip models have also been established that have enabled the study of the immune response following respiratory viral infection and in several respiratory diseases. One study designed a biomimetic microfluidic device that modelled the alveolar-capillary interface of the lung, which reproduced the complex organ-level responses when challenged with bacteria and pro-inflammatory cytokine exposure in the alveolar space [103]. Another study modified this platform in order to study the small airways [104]. This small airway-on-a-chip microfluidic system mimicked innate immune cell recruitment in the circulation and allowed the investigation of neutrophil recruitment and any potential synergistic effects on cytokine secretion of the lung endothelium and epithelium. This system also enables the identification of potential biomarkers of disease exacerbation, and possesses the ability to measure the immune responses to potential biomaterial-based therapeutics/drugs [104]. To evaluate the immune response to SARS-CoV-2, a bioengineered lung alveolus chip model was developed [105]. This plat-

form demonstrated immune cell recruitment and increased pro-inflammatory cytokine secretion in response to viral infection and allowed the performance of an antiviral therapeutic to be evaluated [105]. Continued research and development of these emerging microfluidic chip systems will potentially enable them to be utilised as efficient tools to accurately evaluate the immune response to biomaterials and subsequent likelihood of biomaterial acceptance and potential biomaterial efficacy, therefore reducing the number of costly (and often inconsistent) animal studies.

7.3. In silico *high-throughput screening techniques to predict immune responses to biomaterials*

As the immunomodulatory effects of biomaterials are not as a result of single factors acting alone, but as a consequence of multiple parameters working synergistically to ultimately define biomaterial performance, high-throughput screening techniques are emerging technologies that can help to identify overall desirable parameters [16]. A recent study used high-throughput microarray screening of polymer libraries to identify materials with the ability to regulate human macrophage function. These immune-instructive materials were validated *in vivo*, demonstrating the ability to modulate the FBR in a murine model. Machine learning approaches were also deployed to develop polymer structure-cell response models, facilitating the prediction of immune-instructive features of potential new biomaterials, thus enabling the rational design of potential immunomodulatory therapeutics [106]. Another study used high-throughput combinatorial screening of biochemical and physical signals of hydrogels for the phenotypic regulation of stem cell-based cartilage tissue regeneration. This study investigated the effects of cross-linking density along with the defined presentation of growth factors, arginine-glycine-aspartic acid (RGD) peptides, and mechanical stimulation on human MSC differentiation into articular or hypertrophic cartilage phenotypes. This technique can thus be utilised as a valuable tool to elucidate critical design parameters of biomaterial-based therapies [107].

7.4. Concluding remarks

To conclude, assessing the immune response to biomaterials *in vitro* cannot fully replace *in vivo* studies. However, the continued evolution, development, and standardisation of *in vitro* techniques and models will provide vital pre-clinical indications of *in vivo* acceptance of biomaterials, thus enabling informed decisions on whether to progress a biomaterial to animal and human studies. Ultimately, this will reduce the amount of resources used (including unnecessary animal use) and decrease the number of inappropriate biomaterials and the time taken for biomaterials to reach the clinic [2,33]. Furthermore, continuing to gain a greater understanding of the interactions of biomaterials with the immune system will enhance the development of effective immunomodulatory biomaterials that can mediate successful tissue regeneration in hosts as opposed to a FBR, enabling the increase of successful translation of therapies into the clinic while reducing development time.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests

Daniel Salthouse reports financial support was provided by Engineering and Physical Sciences Research Council. Ana Marina Ferreira Duarte reports a relationship with Engineering and Physical Sciences Research Council that includes: board membership and funding grants.

The authors declare no conflicts of interest.

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