8 In Vivo Evaluation of the Biocompatibility of Biomaterial Device

L. P. Frazão, J. Vieira de Castro, and Nuno M. Neves

Abstract

Biomaterials are widely used to produce devices for regenerative medicine. After its implantation, an interaction between the host immune system and the implanted biomaterial occurs, leading to biomaterial-specific cellular and tissue responses. These responses may include inflammatory, wound healing responses, immunological and foreign-body reactions, and even fibrous encapsulation of the implanted biomaterial device. In fact, the cellular and molecular events that regulate the success of the implant and tissue regeneration are played at the interface between the foreign body and the host inflammation, determined by innate and adaptive immune responses. This chapter focuses on host responses that must be taken into consideration in determining the biocompatibility of biomaterial devices when implanted in vivo of animal models.

Keywords

Biomaterial device · Biocompatibility · Animal models · Host reaction · Immune response · In vivo · Acute inflammation · Chronic inflammation · Humanized mouse models · Host response

8.1 Introduction

There are a great diversity of biomaterials proposed for regenerative medicine, such as tissue-engineered scaffolds that may contain allogeneic, autologous, or xenogeneic genetic materials, cells, synthetic or modified-natural materials [1]. A tissue-engineered implant can be a combination of biological components and biomaterials that creates a device aiming to restore or modify a tissue or organ function to its functional state. Thus, tissue-engineered devices with a biological component(s) require an expanded perspective and understanding of biocompatibility and biological response evaluation.

Biocompatibility is defined by the ability of a biomaterial or medical device to perform with an appropriate host response in a specific application. Its assessment is a measure of the magnitude and duration of the adverse alterations in homeostatic mechanisms that determine the host response and defines if the biomaterial device presents potential harm to the patient [1]. In fact,

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after biomaterial implantation, an interaction between the host immune system and implanted biomaterial occurs, resulting in a biomaterial-specific tissue response during a complex biological process, which needs to be characterized. The three major responses that must be considered for biocompatibility assessment are inflammation, wound healing, and immunological reactions or immunity. The response to injury may depend on numerous factors, including the extent of the injury, blood–material interactions, the loss of basement membrane structures, the extent or degree of cellular necrosis, provisional matrix formation, and the extent of the inflammatory response [1]. Additionally, the tissue or organ undergoing implantation may contribute significantly to the response. Thus, all these events, in turn, may affect the extent or degree of granulation tissue formation, foreign-body reaction (FBR), and fibrosis or fibrous capsule (scar) development (Fig. 8.1).

The idea of a passive material designed to diminish host response has changed. Currently, an ideal biomaterial is the one that triggers the desired immunological responses, enabling its integration and, consequently, tissue repair [2]. Basically, a balanced interplay between the host immune system and the biomaterial is desired. Invasive implantation methods (e.g., surgery) potentiate adverse host responses, which are determined by the biomaterial [3].

In vitro models have a limited capacity to recreate the complex in vivo environment, such as, the role of angiogenesis in the newly formed tissue, immune reaction to implanted biomaterials, and functional properties of the graft. So, in vivo models offer the whole picture of the host response to a biomaterial and are useful to predict the clinical behavior, safety, and biocompatibility of medical devices in humans. In vivo assays are a midway step between in vitro studies and human clinical trials [4–6].

8.2 The Immune Response

Following in vivo implantation, a host reaction is induced, determining the outcome of the integration and the biological performance of the implant. All implants develop cellular and tissue responses. Also biodegradable biomaterials and its degradation products result in surface changes that activate the immune system [7–9].

The human immune system has two different mechanisms: the innate immune system and the adaptive immune system. So, when a biomaterial is implanted in vivo, a nonspecific inflammatory response is elicited by the innate immune system. After the recognition of the foreign material, the adaptive immune system performs highly specific antigen responses and develops long-term memory. The innate immune system is composed of polymorphonuclear cells, mononuclear phagocyte cells (dendritic cells, monocytes, and macrophages), and lymphocytes (natural killer cells, gamma–delta T cells, and innate lymphoid cells), while the adaptive immune response involves B and T lymphocytes [10, 11] (Fig. 8.2).

The typical host reaction to an implant involves a mechanism that is similar to the early stages of wound healing [12]. However, the presence of an implanted device significantly alters the progression through the subsequent phases of repair [13]. Moreover, the extent of the immune response to a biomaterial is modulated by the characteristics of the material [14].

After the implantation of the biomaterial, an acute inflammatory response begins that in some circumstances can lead to a chronic inflammatory response to a FBR and to the deposition of a collagenous fibrous capsule around the implant (Fig. 8.1). Therefore, the efficacy of the biomaterial is affected by the extent and duration of the inflammatory process [6, 8].

The immune response to biomaterials implanted in vivo can be divided into four major

Fig. 8.1 Sequence of host reactions. (Adapted from Ref. [1])

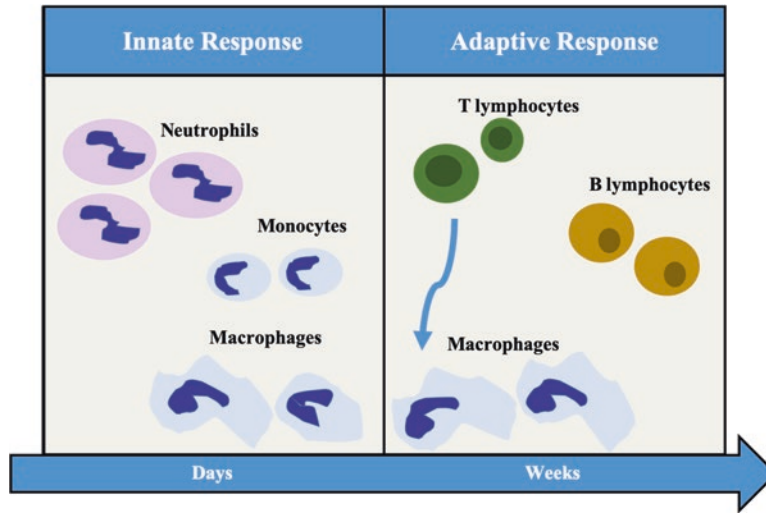

Fig. 8.2 The two components of the human immune system that are involved in the reaction to a biomaterial implant

phases: (1) implantation, (2) blood–biomaterial interaction, (3) inflammation, and (4) tissue remodeling, which start at different times but can overlap with each other. All these four phases are discussed in more detailed below.

8.2.1 Implantation

There are basic responses of the body to implantation even in sham operations. The incision made to introduce the device into the body affects the vasculature, extracellular matrix, and eventually the local nerves [15]. The disruption of the host tissue homeostasis leads to local inflammation and wound healing [13, 15]. The response to injury associated with implantation of the device is essentially dependent on the size, surface area of the injury or implant, and anatomical site. Following injury, a normal wound healing process starts through overlapping phases of blood–biomaterial interactions, inflammation, proliferation, and tissue remodeling [6, 8, 13, 16]. Although the inflammatory response is initiated by the injury, it is mediated by the released chemicals from the plasma, cells, and injured tissue [8.31]. Normally, during the first several days after injury, the predominant cell type is the neutrophils, which are then replaced by monocytes that will differentiate into macrophages (Fig. 8.3).

8.2.2 Blood–Material Interactions: The Formation of the Provisional Matrix

Shortly (within minutes to hours) after biomaterial implantation, changes in the vascular flow and permeability occur [17]. Within a few seconds after the implantation, the blood from the damaged vessels surrounds the biomaterial. Therefore, the blood–material interactions begin spontaneously, and almost immediately, the host plasma components adsorption to the biomaterial's surface occur. These components include lipids, sugars, ions, and proteins, such as albumin, fibrinogen, fibronectin, vitronectin, immune globulins, and a number of coagulation and complement factors [18–20] (Fig. 8.3).

Biomaterial device's characteristics, such as surface energy, chemistry, topography, and roughness, are decisive determinants of the tissue reaction to the implants. It was demonstrated that those implants' characteristics influenced the type, the amount, the composition, and the conformation changes of the adsorbed molecules [18, 21, 22]. Moreover, the composition of the layer of the adsorbed proteins (type of proteins, concentration, and conformation upon adsorption) is associated with the initiation of the coagulation cascade and the complement system, leading to the onset of inflammatory responses [19, 23–25]. From a wound-healing

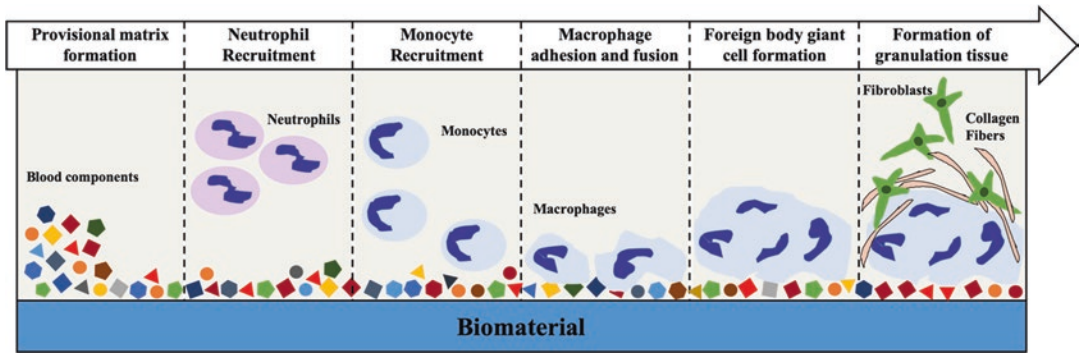

Fig. 8.3 Innate immune response to biomaterial implants: representation of the main cellular players in the interaction biomaterial–immune system. The main events from provisional matrix formation to formation of granulation tissue are represented

perspective, blood molecule deposition on a biomaterial surface is described as provisional matrix formation [1, 25, 26] (Fig. 8.3). However, the clot formation also defines the provisional matrix around the biomaterial implant [27, 28]. Blood coagulation associated with biomaterial implantation is a combination of contact activation, platelet adhesion and activation, and presence of leucocytes [29].

There are two pathways for contact activation of the coagulation cascade: the intrinsic (Hageman factor: factor XII or FXII as initiators) and the extrinsic pathway (tissue factor as initiator). For biomaterials, it has been described an activation of the intrinsic pathway after adsorption of FXII, kallikrein (KK), and high-molecular-weight kininogen (HMWK) as a cofactor [30]. In accordance, complement proteins activated upon contact with the biomaterial, synergistically support platelet adhesion, activation and recruitment of immune cells [7, 31–36]. Regarding the complement system, there are three different pathways that activate this system: the classical pathway, the alternative pathway, and the lectin (mannan-binding) pathway [37]. In biomaterials, it has been described that the complement system is mainly activated by the alternative pathway [36, 38]. However, this activation is related to biomaterials' surface properties, since it is associated with the adsorbed protein layer on biomaterials [31]. Moreover, it was shown that biomaterial surfaces with available OH and NH_2 groups had a greater activation of the complement system than biomaterial surfaces with available COOH [39]. Furthermore, the activation of the complement system leads to the activation of complement factors C3 and C5 that are fragmented into anaphylatoxins C3a and C5a, attracting leucocytes to the site of inflammation, increasing vascular flow and vascular permeability, extravasation of leucocytes, and chemotaxis. Additionally, some complement factors that opsonize bacteria that are also adsorbed to the biomaterial lead to the activation of monocytes and macrophages [7, 40].

In summary, due to the implantation of the biomaterial, there is development of the provisional matrix at the implant site. This provisional matrix is mainly composed of fibrin (produced by activation of the coagulative and thrombosis systems), activated platelets, inflammatory products, and cells, being also rich in cytokines, growth factors, and chemoattractants that are capable of recruiting cells of the innate immune system to the injury site [8, 25] (Fig. 8.3).

8.2.3 Inflammation

The inflammation process acts to contain, neutralize, dilute, or avoid contact with injurious agents or processes. It is defined as the reaction of vascularized living tissue to local injury. These processes initiate a cascade of several events to heal and regenerate the injured site [8].

Biomaterial device is a foreign object and, by definition, elicits an immune response. After the formation of the provisional matrix, an acute inflammatory response occurs followed by a chronic inflammatory response [8, 11, 16] (Fig. 8.3). The intensity and duration of these inflammatory responses are related not only to the extent of tissue damaged but also with the characteristics of the biomaterial device (composition, size, shape, topography, and chemical and physical properties) [1, 8, 16]. Moreover, some proteins that composed the provisional matrix, such as fibronectin and vitronectin, are important in the modulation of the inflammatory response to the biomaterial implant by enhancing cell adhesion. On the other hand, the fibrinogen and complement system are associated with the recruitment of the cellular components of the inflammatory system [41–43].

8.2.3.1 Acute Inflammation

Acute inflammation is the first line of defense of the immune system and a normal and necessary function of the innate immune system. This process is initiated by the presence of pathogens or by tissue damage, for example, through implantation of a biomaterial device [6, 8]. The acute inflammatory response is of relatively short duration, lasting from minutes to a few days. Persistence of the acute inflammation for more than 3 weeks usually indicates the presence of an infection. This response is mainly characterized by an exudation of fluid and plasma proteins (edema) and by the recruitment of polymorphonuclear leucocytes (PMNs), predominantly neutrophils. These cells migrate to the site of injury due to the increase of blood vessels permeability (associated with the activation of the complement system) and also due to the release of chemoattractants associated with the activation of complement factors C3 and C5, activated platelets, and fibrinopeptides (released after blood clotting) [3, 8, 44]. After recruitment, PMNs undergo activation through the release of danger signals by the injured cells at the implant site, such as "alarmins" (which include heat shock proteins, high-mobility group box 1, adenosine triphosphate (ATP), and uric acid) that are an endogenous equivalent of pathogen-associated molecular patterns (PAMPs). Alarmins are recognized by pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), scavenger receptors, and purinergic receptors [3, 45–49].

The main role of neutrophils in acute inflammation is to respond as the first line of cells to defend against invading pathogens (e.g., bacteria and fungi). They initiate a phagocytic response with the secretion of proteolytic enzymes and reactive oxygen species (ROS). PMNs adhere to the biomaterial surface by means of β2 integrins in an attempt to destroy it. However, due to size disparity, phagocytosis does not occur, although, the destructive agents released by neutrophils may corrode the material surface [3, 44, 50–53]. Moreover, neutrophils release neutrophil extracellular traps (NETs) that have, as main functions, to trap pathogens and prevent the spread of infection. These networks are composed of granular proteins, neutrophil elastase, chromatin DNA, and histones. The altered release of NETs restrains the integration between the tissue and the biomaterial, since it leads to excessive production of a dense fibrotic matrix. In addition, it also degrades neutrophil-produced cytokines and chemokines that regulate the healing process**.** Furthermore, NETs released from neutrophils, unable to phagocytose a harmful stimulus, may be considered similar to the formation of foreign-body giant cells (FBGCs) (see subchapter 8.2.3.2) [54, 55].

PMNs have very short life spans (of hours to days) and rapidly disappear from the inflammation site. Usually, PMNs disappear from the implant site in the first 2 days after the biomaterial device implantation [3, 44, 50, 51]. When activated, PMNs secrete several immune-regulatory signals, such as, CX chemokine ligand 8 (CXCL 8) (the most prominent chemokine that primary target neutrophils), monocyte chemoattractant protein (MCP-1/CCL2), and macrophage inflammatory protein (MIP-1β/CCL4), that will activate monocytes, macrophages, immature dendritic cells (DCs), and lymphocytes [3, 44, 56]. The progressive increase of these chemokines leads to monocyte infiltration at the implant site. Moreover, due to the lack of activating signals,

there is a suppression of neutrophils that undergo apoptosis and gradually disappear from the implantation site [8]. So, circulating monocytes that were attracted to the site of injury bind the fibrinogen deposited at the biomaterial provisional matrix. Thus, monocytes differentiate into the classical activated "M1" macrophages. These cells promote the inflammatory response by secreting various inflammatory cytokines and chemokines, such as, interleukin (IL) – 1β, IL-6, tumor necrosis factor (TNF-α), IL-8/CXCL8, and also MCP-1/CCL2 and MIP-1/CCL4, that will promote the invasion of additional inflammatory cells. In an attempt to degrade the biomaterial and in accordance with what happened with leucocytes, macrophages also undergo frustrated phagocytosis by releasing potent oxygen and nitrogen radicals, as well as proteolytic enzymes. This may affect the surrounding tissue since the adjacent healthy cells are also getting damaged and destroyed, which can result in necrosis and present as a threat to patients. However, unlike PMNs, macrophages have longer life spans (from days to months), being the predominant cell type in both acute and chronic inflammation [1, 7, 43, 57–61].

8.2.3.2 Chronic Inflammation

Continuous inflammatory stimulus leads to chronic inflammation that generally does not last more than 2 weeks, and it is confined to the implantation site [6, 7, 59, 62]. This may be associated with the physiochemical characteristics of the biomaterial that leads to continuous opsonization and release of toxic degradation products. Moreover, it can also be associated with insufficient mechanical compliance or movement of the biomaterial at the implantation site [6].

Macrophages are one of the central cell types of the chronic inflammatory response [59, 62, 63]. Due to their large range of plasma membrane receptors, macrophages have a great plasticity and can change their physiology in response to environmental cues, inducing distinct cell populations with different functions [59, 62, 64]. Macrophages are divided into two major phenotypes: M1 and M2. While the M1 phenotype promotes pathogen killing and is related to the classical signs of active inflammation, M2 phenotype supports immunoregulation, tissue repair, and remodeling. These cells secrete anti-inflammatory cytokines, such as IL-10, and induce the migration and proliferation of fibroblasts [59, 62]. So, the adherent macrophages will eventually shift to the M2 phenotype. The overlapping events of the phenotypic M1 to M2 switch, together with the mechanisms of frustrated phagocytosis, result in macrophage membrane fusion to form a foreign-body giant cell (FBGC) [65].

FBGC formation (Fig. 8.3) represents and attempts to increase the cells' phagocytic or degradative capacities. It is also the hallmark of chronic inflammation [6, 65]. As previously described, the M1 macrophages secrete various inflammatory cytokines and chemokines that promote the invasion and activation of additional inflammatory cells, such as mast cells, basophils, and T-helper (Th) cells. These cells secrete IL-4 and IL-13 that have been considered as the main inducers of FBGC on implanted biomaterials, by upregulating mannose receptor on fusing macrophages [8, 65, 66]. Moreover, although the mechanism of macrophage fusion into FBGCs on the biomaterials is not fully understood, it has been proposed that it may depend on adhesion density and migration motility of the cells. Enough cells are needed for fusion to take place, and the attached macrophages have to migrate to meet each other and fuse [67]. So, it has been shown that the adsorbed proteins on the provisional matrix may influence FBGC formation. While all adsorbed proteins support initial monocyte adhesion, only vitronectin strongly promotes macrophage development and FBGC formation. Moreover, fibrinogen, plasma fibronectin, laminin, and collagens (associated with the adhesion of other cell types) do not support IL-4-induced FBGC formation. Additionally, β1 integrins are dominant during monocyte activation to macrophages and during FBGC formation, while β2 integrins are associated with initial monocyte adhesion [41]. Thus, FBGCs adhere to the surface of the biomaterial implant for a long time, forming a barrier between the tissue and the biomaterial. Due to its phagocytic activity, FBGCs secrete reactive oxygen species and other chemical agents, which may result in biomaterial dete-

rioration and eventually in the failure of the implanted devices [7].

Regarding lymphocytes and plasma cells, they are principally involved in immune reactions and are important mediators of antibody production and delayed hypersensitivity responses. Although it is known that T cells attached to the biomaterial surface and become activated through noncanonical pathways, little is known regarding immune responses and cell-mediated immunity directed to biomaterial implants [66]. During chronic inflammation, T lymphocytes, mainly CD4 helper T cells and their subsets (Th1 and Th2), modulate the pro- and/or anti-inflammatory responses, respectively, by producing the majority of cytokines [66]. The change in cytokine expression profile from Th1 to Th2 lymphocytes suggests that T lymphocytes are pivotal (together with the shift from M1 to M2 macrophage phenotype) in promoting the resolution of inflammation leading to tissue regeneration [11].

8.2.4 Tissue Remodulation

The formation of granulation tissue (Fig. 8.3) is the hallmark of healing inflammation, and it is initiated within 1 day after implantation of the biomaterial device. Its name was derived from the pink soft granular appearance on the surface of healing wounds. The granulation tissue is composed of macrophages, fibroblasts, and capillaries. So, the main histological feature of the granulation tissue is the proliferation of new small blood vessels and fibroblasts at the implant site. Depending on the extent of the injury, granulation tissue may be observed from day 3 to 5, after device implantation [1]. During this stage of healing, macrophages polarize toward M2 phenotype through a cross talk with a subpopulation of T cells defined as regulatory (Tregs). Tregs play an important role in tissue immune homeostasis and are able to switch the local immune response from inflammation to a pro-regenerative tissue repair cascade by the secretion of anti-inflammatory cytokines, such as, IL-10 [68].

M2 macrophages and FBGCs produce growth factors, such as, platelet-derived growth factor (PDGF), fibroblast growth factor, transforming growth factor (TGF)-β1, and vascular endothelial growth factor (VEGF), that stimulate fibroblasts, blood vessels formation, and regeneration of epithelial cells. Although there is a lack of information regarding the interaction and synergy between cytokines and growth factors released by activated cells, they are associated with the production of a wide variety of cells, with cell migration, differentiation, and tissue remodeling [1, 11, 43].

Through proliferation, maturation, and organization of endothelial cells into capillary tubes, small blood vessels are formed by budding or sprouting from preexisting vessels (neovascularization) [69]. Moreover, in an attempt to repair the damaged tissue, activated fibroblasts synthesize and deposit collagens and proteoglycans. In the early stages of granulation tissue, there is a predominance of proteoglycans. However, in the later stages, there is a prevalence of collagens (types I and III, being the type I the most abundant) around the biomaterial [1, 13, 70]. However, excessive collagen secretion (due to continuous stimulation form a pro-inflammatory environment) may lead to the formation of a fibrotic capsule around the biomaterial (greater ratio of collagen I/III is associated with a greater fibrotic tissue formation). This fibrous capsule isolates the biomaterial from the host tissues, leading to the failure of many implants, particularly the ones associated with drug release and sensors [71, 72]. Some activated fibroblasts can be differentiated into myofibroblasts, associated with an abundant expression of α-smooth muscle actin (α-SMA). These cells are responsible for wound contraction, promoting wound healing and scar formation [1, 73] (Fig. 8.3).

8.3 Animal Host Response Models and Implantation Site Characterization

Laboratory animal protection legislation assumes that, in specific conditions, it is morally acceptable to use animals for scientific purposes. However, most regulatory systems have the following general objectives: to keep the number of animals used to a minimum, to define legitimate

purposes for which laboratory animals may be used, to ensure the ability of all laboratory personnel and researchers, to avoid animal use when there are practicably available alternatives, to avoid unnecessary pain or distress to animals, to provide for the inspection of facilities and procedures, and to ensure public responsibility [74].

The choice of an appropriate animal model is intrinsically related to the specific goals of the experiment. Moreover, when choosing an animal model, it is important to take into account various aspects, such as the cost (e.g., the maintenance of smaller animal models is less costly than the maintenance of larger animals), the number of variables (e.g., a well-defined and well-described model may reduce random effects), the methodologies used to assess the sample collection and characterization, and the controls that should contain the clinical standard (or a material already in clinical use), an empty defect (to prove that the obtained results are related with the implantation of biomaterial), and if using cells, the material without cells [5].

Regardless of the effort of researchers to use the most adequate models for their experiments, it is difficult to draw valuable information due to the differences in model and reaction mechanisms in the implanted material surface properties. Thus, it is important to take into account the different types of host reactions that can be elicited after the implantation of biomaterials. After the initial primary acute inflammatory reaction, different scenarios can happen: (1) the implanted biomaterial does not degrade in the course of the inflammatory reaction and is surrounded by a fibrotic capsule and a foreign-body reaction is observed; (2) the biomaterial degrades in a relatively short time frame (while the inflammatory response is still being observed), and the degradation products are metabolically excreted by the host or may cause inflammation themselves; (3) the host does not surround the biomaterial with the fibrotic capsule, but is not able to degrade it, and thus two situations may occur: (i) the host immune system is activated into setting up a chronic reaction or (ii) the acute inflammation persists and a nonhealing wound appears at the implantation site [5].

The first approach to test a biomaterial in vivo is the ectopic model in small animals, commonly mice or rats. Ectopic models refer to studies where the implantation is done out of the intended final tissue. In contrast, orthotopic models refer to the implantations done in the tissue of interest. In the early phases of the research, ectopic models are preferred to orthotopic models due to the easier identification of the response and also of the effects, the technical abilities needed to perform them, and also because it is easier to compare results between a wealth of experiments reported in the literature. The most common ectopic models are the implantation in the subcutaneous, intramuscular and intraperitoneal sites. These locations are able to provide information about chronic or persistent acute inflammatory responses, particularly the chronic inflammatory response and regarding the integration of the biomaterial within the host tissue after long periods of implantation. Subcutaneous implantation is normally done in the dorsum of animals to prevent them from having access to the sutures, in order to maintain the biomaterials or cells in place. Intramuscular implantation, in small animals, is normally done in the hind limb, and the intraperitoneal implantation is performed in peritoneum (a body cavity) [4, 5]. Despite the intended final application of the biomaterials, the subcutaneous and intramuscular implantation models offer information about the direct effect of the biomaterial at the implant site, while intraperitoneal implantation provides data on the effect in the abdominal organs of the host, such as liver, kidney, spleen, mesenteric lymph nodes, and related adipose tissue [75–77], as an indication of the systemic influence of the biomaterials on the host. Moreover, intraperitoneal models are the most suitable for evaluating cell recruitment and activation status [77, 78] at short [19, 77, 79, 80] and long periods [77, 81] of reaction.

There are some differences in foreign-body response (FBR) related with the implantation site. For example, it was shown that the intraperitoneal site had higher levels of proangiogenic factors and lower levels of pro-inflammatory cytokines during the initial stage of the FBR, when compared with the subcutaneous

site. However, both sites led to fibrous encapsulation. This is associated with faster healing response that occurs in the peritoneal cavity compared to the dermis [82–84]. Moreover, the mouse strain also affects the FBR. It was shown that C57BL/6 strain is associated with a more robust FBR and with a fibrous encapsulation more similar to that of humans, when compared to BABL/c strain [85].

To study cell types and/or pathways that mediate FBR, genetically modified mouse models are frequently used. For example, it was shown that after biomaterial implantation in mice deficient in T cells [86], natural killer cells [87], or mast cells [87, 88], there was a normal formation of FBGCs and/or fibrous capsule formation, suggesting that those inflammatory cells are not essential for FBR [85]. Additionally, genetically modified mice/rats to not develop thymus must be used when it is necessary to conduct studies with allogeneic and xenogeneic cells with or without biomaterials. These immunocompromised animals are unable to produce mature T lymphocytes, key immune cells in graft or implant rejection [5].

Large animal models are generally used in orthotopic models due to easier comparison to man implantation. For example, sheep are usually used for the evaluation of heart valves. This is based on the rapid growth of these animals and also on the accelerated calcification, which has its clinical correlation in young and adolescent humans [6]. Moreover, pigs may be used to mimic pediatric and neonatal conditions due to its fast-growing ability [89, 90]. And due to its size and similar biological complexity with humans, pigs are largely used in wound healing studies, decreasing the number of animals needed [91].

Additionally, models as air pouches [92–94], cage implants [95, 96], or dorsal skinfold chambers [97] have also demonstrated consistent results concerning the interplay between direct and indirect material surface reactions. In fact, in the dorsal skinfold chamber, recruitment and accumulation of leukocytes were observed using intra-vital fluorescence and avoiding the killing of animals at different timepoints [97]. Moreover, the cage implant models are demonstrated to be useful for identifying recruited and adherent cell types [96], macrophage fusion into FBGCs [95], and cytokine release [98] in response to implanted materials either in mice [98] or rats [95, 96]. On the contrary, host reaction evaluation should not be limited to the assessment of inflammatory reaction, particularly if the biomaterial is aimed to remain in the host for long periods and/or if it degrades during the implantation time. Therefore, the evaluation of the immune response should also be done before the implantation. An adequate method to assess in vivo immune stimulation by biomaterials is to do repetitive implants (rat subcutaneous [69] or intraperitoneal [54] model) and analyze the host–tissue response, immune cells, and antibody production [54, 69].

After the in vivo implantation of a biomaterial, it is important to characterize not only the associated biological processes, such as the adsorption of plasma proteins and immune cells, but also the mechanical, physical, and chemical properties of the device that may change overtime [15].

Generally, the characterization of biological processes associated with in vivo implantation starts with a histological analysis in order to visualize and/or differentiate microscopic structures. This analysis is enhanced by the use of different staining that dye specific tissue components, such as connective tissue, elastic fibers, blood cells, and basement membrane. However, when the biological material is collected, it is necessary to assess some histopathological outcomes, such as herniations and/or adhesions.

The preparation of samples for histological analysis comprises five steps. The first step is the fixation of samples using chemical fixatives (the most used is 10% neutral buffered formalin). Sample fixation will avoid tissue degradation and maintain cellular structures and subcellular components. In the second step, the sample must be dehydrated, normally using a sequence of increasing concentrations of ethanol. The aim of this step is to remove the water from the tissue, replacing it with a hard matrix (generally molten paraffin wax), allowing the cut of thin sections (typically of 5 μm). So, xylene is used to remove ethanol from the sample, and then molten paraffin

wax infiltrates in the tissue to replace xylene. The third step consists in the external embedding of the material. Samples are placed in molds with an embedding liquid, such as agar, gelatin, or wax. These liquids are then hardened. The most common method used for biological tissues is the formalin-fixed paraffin-embedded (PPFE) tissues because it allows samples to be stored indefinitely at room temperature and also because of the recovery of nucleic acids (DNA and RNA) in the samples. Sample sectioning and mounting on a glass microscope slide is the fourth step. Sample sections can be cut in different directions, such as vertical (longitudinal sections, by cutting perpendicularly to the surface of the tissue), horizontal (transverse sections), or transversely. The last step deals with section staining, since biological tissues have little inherent contrast in light or electron microscope [99].

Eosin and hematoxylin (E&H) is the most frequently used histological staining. Eosin is an acid dye that stains the cytoplasm pink, and hematoxylin is a basic dye that stains the nuclei dark blue/black due to the affinity to nucleic acids. Moreover, muscle fibers appear in deep red, red blood cells (RBCs) in orange red, and fibrin in deep pink. This staining is widely used to analyze foreign-body response, allowing the identification of capsule formation, FBGCs, and macrophages [13]. Additionally, it can also be used to identify the formation of new tissue. Other histological stainings may be used depending on the goals of the study. For example, Masson's trichrome staining is commonly used for connective tissue: staining cartilage and collagen fibers in blue or green, muscle fibers in red, nuclei in black, cytoplasm in red or pink, and RBCs in red [100].

In accordance with the purpose of the study, immunostaining may be necessary to be performed. Immunostaining is an antibody-based method enabling to detect a specific protein in a sample and may be performed in fresh tissue or in histological sections mounted in glass microscope slides after antigen retrieval. In each histology section, different parameters must be evaluated, such as the number (expression) of inflammatory cells (neutrophils, plasma cells, lymphocytes, and macrophages), FBGCs, the severity of necrosis, the extent of neovascularization, fibrosis, and fatty infiltration [101–103].

8.4 Humanized Mouse Models

Currently, there are no set standards that recapitulate the human immune response for preclinical evaluations, often leading to poor or unexpected outcomes in human recipients. One major challenge is that the commonly used animal models in these preclinical studies, such as the wild-type or immunodeficient murine models, provide a limited representation of the human immune response. In fact, it is known that human immune cells have several unique characteristics and interactions that are not observed in murine cells [104–106]. One method that is used to address this challenge is the use of humanized immune system mouse model. Humanized mouse model are created by the implantation of human tissue (e.g., cells or genes) into mouse, which will allow these animals to produce functional human cells and gene products in vivo. These types of mice have been advantageous in the studies of human diseases involving human–immune cell interactions, such as, infectious diseases [107], oncology [108], testing for potential graft rejection [109], and therapeutic toxicity [110]. Regarding the biomaterial field, the humanized mice can be a powerful tool that provides a potential platform for improving our understanding and tailoring our biomaterials for safer and more effective interactions with the human immune response. Humanized mice are classified as chimeric mice that were transgenically or surgically modified by the integration of human cells, tissue, and/or genes to generate models of human biological responses. The generation of humanized mice producing human immune cells stems from the discovery of genetic mutations creating immunodeficient mouse lines that allow for the engraftment of multiple human-derived tissues and cells. In fact, particular practices and selections have a greater reconstitution of the human immune response, which leads to the numerous versions of humanized mice [111].

The first model of humanized mice involved the engraftment of human peripheral blood

lymphocyte in SCID mice (Hu-PBL-SCID) by the injection of mature human peripheral lymphocytes [112]. Additionally, a human SCID-repopulating cell NOD-SCID (Hu-SRC-NOD-SCID) mice was also developed by intravenous injection of human CD34+ cells derived from fetal liver, cord blood, bone marrow, or G-CSF cytokine-mobilized peripheral blood mononuclear cells [113]. In order to overcome some limitations observed in the first model, including (1) the engrafted human T and B cells, which are present only for a limited period of time; (2) the human T cells that interacted with the host MHC molecules, inducing a xenogeneic graft-versus-host disease response; and (3) the development of T cell, which is limited due to the lack of human thymic tissue in the host animal [114], the SCID-Human (SCID-Hu) model was developed. In this humanized mice model, there is a co-implantation of human fetal thymus and liver under the kidney capsule of SCID mice [115]. These mice developed autologous thymic educated and human leukocyte antigen- (HLA-) restricted T cells in vivo, making it an excellent model for HIV studies [116]. However, the level of hematopoietic lineage cells was low, and the functionality of the human immune system was poor [117]. Thus, the bone marrow, liver, thymus (BLT) humanized mouse was developed being a modification of the SCID-Hu model. This model is generated by the co-implantation of human fetal liver and thymus under the kidney capsule along with the intravenous injection of the autologous human CD34+ cells, with the maturation occurring in the implanted autologous thymic tissues, resulting in a long-term and systematic repopulation of multiple lineages of hematopoietic cells in the mouse [118]. Additionally, human T cells are developed with a human HLA-restricted T cell. However, it was demonstrated in the long-term studies that BLT humanized mice are susceptible to thymic lymphoma, giving them a short life span and limiting long-term studies [119]. Thus, issues with thymic lymphoma development can be mitigated by the use of NOD-SCID IL2Rgamma(null) (NSG) host mouse [120].

Regarding in vivo biomaterial assessment, the humanized mouse model generated by human thymus implantation and intravenous transfusion of human CD34+ fetal liver cells were used to evaluate the immune response of decellularized cardiac extracellular matrix (ECM) hydrogels [121]. Humanized mice have also been used for the biocompatibility assessment of cellular grafts (human embryonic stem cell allografts) that can also be useful for biomaterial assessment as a delivery vehicle for cellular therapies [122]. In addition, recent studies have demonstrated the power of humanized mice in evaluating the immunogenicity of cells derived from human-induced pluripotent stem cells [123]. Thus, the humanized mouse model could be further used to assess the potential benefits or complications that can occur when stem cells are used in synergy with the biomaterials; therefore, additional modifications to the standard models can also provide insight into specific patient populations of interest. In fact, these mice models allow for more personalized immune assessments and therapy developments to be validated in patient populations susceptible to unexpected immune responses.

In summary, although humanized mouse models do provide an improved representation of the human immune response, there are some limitations that should be considered, demonstrating that further research is needed to improve and expand the capabilities of humanized mice as representative models of the human immune response. Nevertheless, humanized mice models can provide a critical tool for a more effective and safe translation of novel therapies in the rapidly developing biomaterial field.

8.5 Conclusions and Future Perspectives

Tissue-engineered devices are combinations of biological–biomaterial in which some components of tissue have been combined with a biomaterial to create a device for the regeneration of tissue or organ function. The development of new biomaterials requires an in-depth understanding of the biological responses to implanted biomaterials.

The inflammatory response is the first step of wound healing but is also the underlying reason for the failure of many implanted scaffolds. However, the immune system remains the most significant critical issue for the development of tissue engineering. Once a biomaterial device is implanted, a sequence of events takes place leading to the formation of FBGCs at the biomaterial–tissue interface. However, the type of cellular and tissue response to the implant is dependent on the nature of the implanted biomaterials. It is known that several immune cell subpopulations and immune-modulating factors are involved in the different phases of healing; however, the impact of material properties on immune activation and through which mechanisms this activation occurs still need to be fully elucidated. In fact, it is of great importance to understand the process of the innate immune inflammation by which neutrophils and monocytes or macrophages can be activated by biomaterials devices. Additionally, it is important to clarify why some macrophages shift from an inflammatory to an anti-inflammatory phenotype in certain types of tissues, while a distinct population of anti-inflammatory macrophages is mobilized in others.

The increasing knowledge and awareness deriving from biological systems and new structural, chemical, and physical understandings of human-derived biomaterials, together with advances in the synthesis of new biomaterials, will open the way to a new and more sophisticated device designs and scaffolding technology. The future of this field will continue to grow and evolve with the collaborative development of tissue-engineered products that offer simple solutions to complex problems. Nevertheless, we should also be interested in knowing what the safety of immune-engineered biomaterials and their long-term efficacy will be.

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