Chapter 8

# The Immune Response in *In Situ* Tissue Engineering of Aortic Heart Valves

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## 1. Introduction

The gold standard for treatment of advanced heart valve disease is surgical heart valve replacement. None of the currently available mechanical and bioprosthetic heart valve substitutes resembles normal heart valve function. While mechanical heart valves offer excellent durability, they require life-long anticoagulation to control thromboembolism, which inherently leads to an increased risk of hemorrhage complications. Bioprosthetic valves on the other hand, retain a more physiological blood flow pattern, but these valves are prone to calcification and structural deterioration, limiting their lifespan. For both types of replacement valves, the main limitation is that they are non-living prostheses, incapable of adapting to changes in the hemodynamic environment. It was shown that a living autograft implanted in the aortic position (the Ross procedure) improves long-term clinical outcome compared to a non-living homograft [1]. This illustrates the importance of the regulatory and adaptive properties of a living valve substitute. Tissue engineered aortic valves can provide such an autologous, viable valve with the potential to grow, adapt, and regenerate within the hemodynamic environment. Evidently, the pediatric and young adult population would benefit most from such a tissue engineered aortic valve. The valve's ability to grow as the recipient grows and matures, eliminates the need for repetitive surgeries. [2-7].

Foundational principle of regenerative medicine is restoring the native tissue structure and function by providing a microenvironment necessary to promote tissue regeneration. Tissue engineering scaffolds are biomaterials designed to create this microenvironment and to promote tissue regeneration [8]. The traditional tissue engineering paradigm for creating trileaflet heart valves consists of harvesting autologous cells from the patient, expanding the cells *in vitro*, and subsequently seeding the cells into a biodegradable scaffold. The cell-scaffold constructs are conditioned in a bioreactor to promote extracellular matrix formation (ECM), while

the scaffold is degraded [9]. Although this approach leads to an autologous, living heart valve, the *in vitro* process is a very costly and time consuming procedure. Therefore, a novel approach emerged from this, in which the *in vitro* phase is completely omitted, the so-called *in situ* tissue engineering, or 'guided tissue regeneration' (figure 1). This approach relies on the body's natural regenerative potential and uses the human body as a bioreactor [4]. Key to this process is the use of a functional scaffold, capable of host cell repopulation and subsequent *in situ* tissue remodeling. After implantation, cells will colonize the scaffold to form tissue, while the scaffold withstands physiological stresses and strains from its hemodynamic environment and may gradually degrade [2,4]. Clearly, these characteristics put more stringent demands on the biomaterial used, as it should provide both mechanical strength and the cellular niche, balancing between material degradation and tissue formation. The scaffold can be biological or synthetic or a combination of both (a hybrid scaffold), loaded with bioactive components and/or cells to provide stimuli for favorable host cell repopulation, differentiation and tissue formation. The *in situ* approach allows for the minimization of risks and costs associated with cell and tissue culture, while providing off-the-shelf availability.

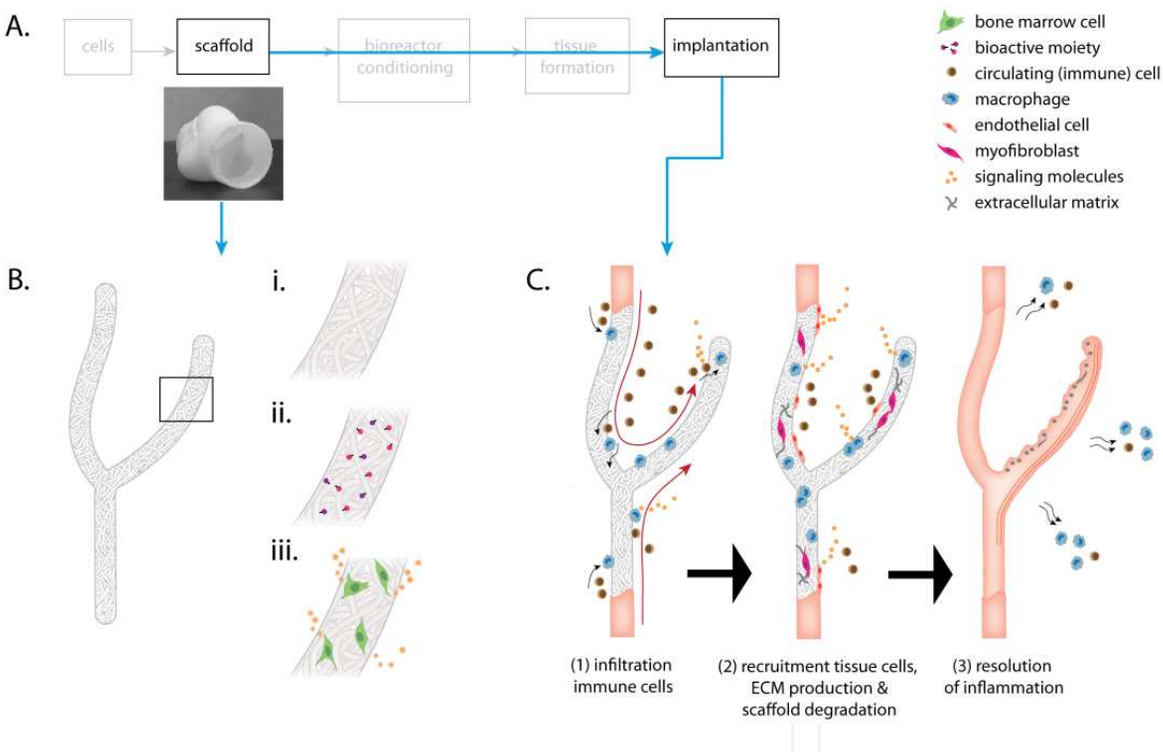


**Figure 1.** (A) The *in situ* tissue engineering paradigm in which the scaffold is directly implanted, omitting lengthy *in vitro* conditioning phases. (B) The scaffold consists of the bare biomaterial (*i*), which may harbor incorporated bioactive moieties (*ii*) and/or cells (e.g. bone marrow stromal cells) seeded directly prior to implantation (*iii*). (C) Hypothesized mechanism of *in situ* heart valve tissue engineering: after implantation, the scaffold will trigger the host immune response, leading to recruitment of various immune cells and macrophage infiltration. The infiltrated cells secrete cytokines and growth factors to attract additional immune cells, as well as tissue cells, originating from surrounding tissue and/or circulating (progenitor) cells. Endothelial cells cover the blood-scaffold interface and activated myofibroblasts migrate into the scaffold to produce extracellular matrix. Scaffold degradation correlates to a decrease in pro-inflammatory stimuli, eventually leading to resolution of inflammation. Ideally, the valve remodels into the physiological three-layered structure with endothelial cells covering the blood-contact area and quiescent myofibroblasts as valve interstitial-like cells populating the spongiosa layer. Illustration by Anthal Smits.

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The *in situ* tissue engineering approach is heavily reliable on the wound healing response. Both the injury incurred during the implantation process and the host inflammatory response to the implanted biomaterial and its degradation products, influence the local microenvironment created by the scaffold. The biomaterial intensifies the inflammatory response by inducing a foreign body response (FBR), propagated by infiltrating immune cells [10,11]. This response is characterized by the presence of macrophages and their fusion into multinucleated giant cells associated with chronic inflammation arising from the persistent presence of a foreign body. The FBR, especially inflammation, may drive valve calcification. However, inflammation is not merely a detrimental response to biomaterial scaffolds. Rather, it can be considered as a natural agent of tissue remodeling, orchestrated by various cell types and potent signaling molecules. By unraveling the inflammatory response towards the foreign biomaterial and the triggers for pathological outcome, targets may be identified to control the inflammatory response through modifications of the biomaterial. The goal is to develop strategies that harness the beneficial aspects of the inflammatory response, while limiting its potential deleterious effects by modulating the inflammatory response towards regeneration.

This chapter deals with the use of biomaterials for *in situ* heart valve tissue engineering and the immune response to the implanted biomaterial. The FBR to biomaterials is discussed, leading to biomaterial design approaches directed to immunomodulation towards tissue regeneration, identifying pitfalls as well as current research challenges for this innovative technology.

## 2. Biomaterial scaffolds

Biomaterials are materials that interact with the body and its cells. As such, they are central to many strategies for regenerative medicine. They are employed as vehicles for transplanting (progenitor) cells, timed and localized delivery of bioactive moieties, and/or as 3D scaffolds for tissue engineering. Scaffolds are biomaterials designed to create a microenvironment that promotes regeneration. Besides creating and maintaining a defined space for tissue growth, biomaterial scaffolds also provide mechanical stability, and support cell adhesion and migration. Ideally, a scaffold for tissue engineering should be bioresorbable, biocompatible and have a highly porous macrostructure necessary for cell growth, nutrient supply, and waste removal [2,3,6,8,12]. By engineering the proper cellular niche, such scaffolds can provide an environment suitable to modify host responses and direct cell survival, migration, proliferation, differentiation, as well as matrix formation and remodeling. The premise is that in order to unlock the full potential of the cells, at least some aspects of the native 3D tissue environment associated with their renewal, differentiation and organization needs to be mimicked in the applied scaffold materials [3].

### 2.1. Biomaterial scaffold use in *in situ* heart valve tissue engineering

Trileaflet heart valves are sophisticated tissues with an anisotropic three-layered structure, optimized to withstand the repetitive hemodynamic loads it is subjected to. A human heart

valve opens and closes approximately 100.000 times per day, resulting in cyclic changes in the shape, dimensions, and stress of its leaflets and supporting structures. Furthermore, rather than being a purely passive structure, heart valves consist of active components that allow them to adapt to changes in the hemodynamic environment to a certain degree. This puts stringent demands on biomaterial scaffolds used for heart valve tissue engineering, in particular on the mechanical properties. The hemodynamic environment requires a strong biomaterial bearing the repetitive and substantial mechanical stresses applied, especially in the aortic position. This calls for excellent elastic and fatigue properties of the scaffold. A successful tissue engineered heart valve must not only accommodate the resulting deformations, but also have ongoing strength, flexibility, and durability, beginning at the instant of implantation and continuing throughout the lifetime of the recipient [2]. For the *in situ* tissue engineering approach, this means the scaffold has to maintain valve functionality while ECM is formed and remodeled and the biomaterial is degraded *in situ*. In contrast, for the traditional *in vitro* tissue engineering approach the load-bearing function of the biomaterial is overtaken by ECM *in vitro*, prior to implantation. This balance between scaffold resorption and synthesis of new matrix by the host's cells is one of the main challenges in designing scaffolds for *in situ* tissue engineering [4].

### *2.1.1. Biological scaffolds*

The ECM is the natural scaffold for tissue and organ morphogenesis, maintenance, and reconstruction following injury, and is associated with constructive tissue remodeling [12]. The ECM proteins are potent regulators of cell adhesion and activation, and provide a 3D scaffold for cellular organization and migration. They provide mechanical support and store and mobilize signaling molecules. The fibrous ECM structure is provided by collagen, elastin, and fibrin, while non-fibrous proteins as fibronectin and laminin are domains for cell-matrix interactions. This protein scaffold is embedded in a gelatinous matrix composed of glycosaminoglycans (GAGs) and proteoglycans. It serves as a lubricant and as a reservoir for signaling molecules, regulating their distribution and mode of action serving cell-matrix interactions, and activation of enzymes and mediators [11]. Hence, ECM serves as a native modulator of cell activity, also in immune responses and tissue repair. The 3D organization of its components and the complexity of the composition distinguish the native ECM from synthetic scaffolds [4,12,13]. Consequently, the use of a decellularized valve is currently the predominant choice as scaffold material for application in *in situ* engineering of heart valves.

In contrast to cross-linked bioprostheses, decellularized xenografts or homografts allow for infiltration of host cells and matrix remodeling, which may render an autologous, living replacement valve in time. Upon decellularization, the xenograft or homograft is depleted of cells and cellular components. The decellularized matrix possesses a native-like geometry and structure with mechanical properties and physiological hemodynamics similar to its native counterpart [3,4,14]. Signaling components present in the matrix provide natural cues to dictate cell adhesion, proliferation, and growth. With respect to biocompatibility, it is crucial to remove all cellular components, without harming or altering the matrix properties by the decellularization treatment [15]. The method of decellularization strongly determines the

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degree of preservation of matrix integrity, as well as the efficiency of cell removal. Various decellularization techniques are being studied in an effort to suppress the immunogenic potential of such biological matrices while retaining matrix integrity [2,4,5,7,14]. Results from studies on recellularization of decellularized homografts and xenografts in animal models are controversial, as reviewed elsewhere [2-4,7,16]. In decellularized aortic valves, residual devitalized cells and their epitopes are primary initiators of valve calcification leading to failure of this bioprosthetic valve [7,17]. It is suggested that inflammation inhibitory factors, naturally present in the ECM, are lost due to the decellularization treatment, accounting for the activation of granulocytes and the initiation of the immune response [4]. Furthermore, xenografts are associated with the risk of immunogenic reactions or disease transmission and availability of homografts is limited. To overcome these issues, recent studies have suggested the use of homologous decellularized tissue engineered heart valves. For this, heart valves were engineered *in vitro* using adult saphenous vein cells seeded onto a synthetic polyglycolic acid (PGA)/poly-4-hydroxybutyrate (P4HB) scaffold. After conditioning the cell-scaffold construct in an *in vitro* bioreactor, the tissue engineered valve was decellularized and used as a starter matrix for subsequent recellularization and remodeling *in situ* [18].

An alternative, natural resorbable scaffold material suitable for *in situ* tissue engineering and studied extensively is small intestine submucosa (SIS) [2,3,13]. SIS consists almost entirely of acellular collagen so there is no need for these substrates to undergo extensive decellularization procedures, making it an attractive alternative to decellularized matrices [3]. The success of SIS has been attributed to its intrinsic ECM proteins, cytokines, and growth factors, showing rapid remodeling by the host tissue and exhibiting good vascularization and tissue growth without excessive inflammation and FBR [2]. With respect to heart valve tissue engineering, complete valvular replacements from SIS have been produced demonstrating remodeling potential *in vivo* [19]. As for all animal derived materials, a disadvantage of using SIS is the risk of transferring zoonoses and its availability may be a limiting factor when homograft material is used [3,14]. Further studies should clarify the underlying mechanisms involved before translating the use of decellularized matrices as heart valve scaffolds to human clinic.

### *2.1.2. Synthetic scaffolds*

Synthetic scaffolds have the advantage that they can be tailored to demands, offering precise control of various aspects, such as mechanical properties, chemical properties, degradation rate, as well as the immunogenic potential [7,8]. However, this level of control comes at a price in the sense that multi-disciplinary in-depth knowledge is required to engineer a scaffold appropriate for *in situ* tissue engineering. Engineering of synthetic scaffolds is a discipline that spans multiple length-scales. On the macroscale, a scaffold should exhibit mechanical properties appropriate to fulfill its function. The 3D architecture affects the global mechanical properties of the scaffold, but additionally, on the microscale, it influences cell infiltration and organization (e.g. by contact guidance). Apart from the global mechanical properties of a scaffold, the local mechanical properties, such as material surface stiffness, determine the stimuli experienced by the cells. Surface chemistry (e.g. hydrophobicity/hydrophilicity) has a major effect on cell- and protein-biomaterial interactions and with state-of-the art incorpora-

tion of bioactive or even bioresponsive molecules, scaffold engineering has advanced down to the nanoscale [3]. This emphasizes that not only the choice of biomaterial but also the method of processing is of key importance in scaffold development.

Synthetic biodegradable materials, such as PGA, P4HB, polylactic acid (PLA), polycaprolactone (PCL) and copolymers, are the main biocompatible materials of choice, varying in their rates of degradation and manufacturing possibilities [2-4,6,7,14]. Their degradation rate can be tailored by varying their copolymer ratio [3]. Fast-degrading scaffolds such as PGA/P4HB have been used extensively as scaffolds for *in vitro* tissue engineering procedures of heart valves [9,20,21]. Whereas the use of synthetic scaffolds in traditional *in vitro* tissue engineering is abundant, experience with the use of synthetic scaffolds for *in situ* tissue engineering of heart valves is rather limited. However, recent studies applying synthetic scaffolds for small-caliber blood vessels demonstrate the ground-breaking potential of such scaffolds for endogenous regeneration. Small-diameter nanofibrous PCL grafts showed fast endothelialization and ECM formation in the systemic circulation of a rat model [22]. Vascular grafts composed of a nonwoven PGA mesh with a PCL/Poly-L-lactic acid (PLLA) copolymer, seeded with bone marrow stromal cells, demonstrated regeneration of mature blood vessels *in situ* via an inflammation-mediated response in a mouse model [23], as well as in clinical trials [24]. To improve mechanotransduction from the biomaterial to the cells, Wu *et al.* employed a fast degrading elastomeric graft, consisting of poly(glycerol sebacate) (PGS), resulting in fast *in situ* regeneration of neoarteries with mechanical properties and functionality similar to the native vascular tissue [25]. Alternatively, Yokota *et al.* developed a hybrid scaffold consisting of a collagen sponge with layered PGA and PLLA, resulting in regeneration of the canine carotid artery [26]. Although these results demonstrate the great potential of synthetic or hybrid scaffolds for *in situ* tissue engineering, translating these results to heart valves is not trivial due to the complexity of mechanical loads and high-demanding function of the heart valve. Furthermore, slow and/or incomplete polymer degradation may result in excessive chronic inflammation, possibly leading to fibrosis and hampered valve function.

Despite these challenges to overcome, synthetic scaffolds have the potential to offer a strong cost-effective off-the-shelf alternative for heart valve replacements, yielding them very interesting for future clinical application.

### 2.2. Modulating the immune response

Independent of the biomaterial, the injury incurred during the implantation process will trigger an immune response, due to the disruption of host tissue and induction of cell damage. However, the extent of the inflammatory response evoked is dependent on location, implantation procedure, and biocompatibility of the biomaterial [14,27]. The natural human host response to the scaffold is an excellent target to modulate and control cell and tissue fate. Valvular regeneration is hypothesized to start with a rapid infiltration of the scaffold by monocytes. These monocytes differentiate into macrophages and attract progenitor cells that differentiate into tissue-producing cells. In addition, the macrophages themselves may differentiate into tissue-producing cells. Next, clearance of the macrophages occurs and extracellular matrix is formed and remodeled toward the natural heart valve matrix

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architecture with quiescent cells. Detailed understanding of this response will provide guidelines to achieve cell and tissue homeostasis, while preventing adverse tissue development (e.g. fibrosis) by mitigating early cellular responses. As the nature of the infiltrating cells in the scaffold and their differentiation is believed to tune the balance of later stage tissue formation towards regeneration or fibrosis, controlling the endogenous production or presentation to the cells of key regulating cytokines in these early processes is essential. Thus, insights in the sequential cell influx and cytokine production and their role in cell differentiation/polarization and tissue formation, will allow the development of optimized scaffolds for *in situ* heart valve tissue engineering applications.

## 3. The immune response to biomaterials

The defensive response of the human body to invasion by disease-causing entities is referred to as the immune response. In general, its main function is to resolve the infection, restore the tissue damage and reestablish a state of homeostasis. The ideal response is rapid and destructive when necessary, yet specific and self limiting [28]. The immune response consists of two stages: the innate response and the adaptive response (figure 2). The innate immune response refers to the antigen-nonspecific defense mechanism that a host uses immediately or within several hours after exposure to a pathogen or other foreign entity, e.g. a biomaterial. The response is aimed at the recognition and removal of the entity, inhibiting infection and inducing a state of inflammation. When the innate immune response is outrun by a continuing infection and antigen is drained to regional lymph nodes, the adaptive immune response is triggered. Adaptive immunity is antigen-specific, generating responses that are tailored to maximally eliminate pathogens and cells displaying non-self antigens. A key feature of adaptive immunity is the development of immunological memory, in which specific antibodies are generated [29,30]. Synthetic biomaterials are thought not to initiate an adaptive response because they are typically not immunogenic. However, cells and mechanisms involved in the initiation of an adaptive immune response have been found at sites of synthetic implants, suggesting the involvement of an adaptive response in the immune response to biomaterials [11]. In *in situ* tissue engineering, an immune response is inevitable and its beneficial aspects, i.e. dead cell removal and initiation of wound healing, must be harnessed while the potential deleterious effects, i.e. excessive inflammation and fibrosis, must be limited.

### 3.1. The acute and chronic inflammatory response

The classification 'acute' or 'chronic' is primarily defined by the duration of the inflammatory response and the type of cells infiltrating in response to pro-inflammatory signals [27]. As part of the innate immune response, the acute inflammatory response occurs in the first days after implantation and is characterized by the presence of blood-derived polymorphonuclear leukocytes (PMNs, or granulocytes), predominantly neutrophils. The infiltrating cells cause a state of inflammation to develop within the tissue, generally described by the local accumulation of fluid accompanied by warming (*calor*), pain (*dolor*), reddening (*rubor*), swelling (*tumor*), and functional changes (*functio laesa*). These cells secrete reactive oxygen

intermediates (ROIs) and inflammatory cytokines, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), which orchestrate the character and degree of the subsequent immune response [31]. Their actions can, besides eliminating the pathogen or other foreign entity, also cause secondary damage to the surrounding tissue. Controlling the numbers and types of immune cells at the implant site has the potential to reduce secondary tissue damage and promote regeneration [8].

The inflammatory response prolonging within subsequent weeks, months or even years after implantation is referred to as the chronic inflammatory response. Ideally, the chronic phase of the inflammatory response is avoided through adequate and quick elimination of the disease-causing entity during the acute phase. Chronic inflammation develops as inflammatory stimuli persist at the implant site, with macrophages representing the driving force in continuing the inflammatory response, mediating prolonged expression of cytokines, i.e. IL-1β and TNF-α [11]. Implantation of a biomaterial can intensify the inflammatory response by inducing a FBR, propagated by the infiltrating macrophages, which influences subsequent wound healing.

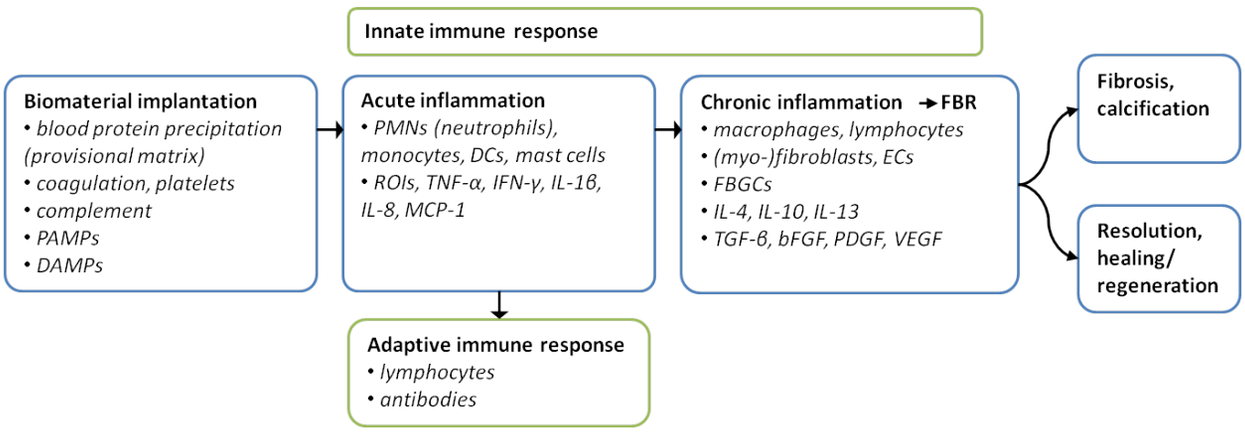


**Figure 2.** Overview of the immune response to a biomaterial; pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), polymorphonuclear leukocytes (PMNs), dendritic cells (DCs), reactive oxygen intermediates (ROIs), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1β (IL-1β), monocyte chemotactic protein-1 (MCP-1], foreign body response (FBR), endothelial cells (ECs), foreign body giant cells (FBGCs), transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF).

### 3.2. Mediators of the immune response

The complete process from innate immune response to wound healing is tuned by a broad spectrum of cytokines and growth factors. Cytokines are a large family of proteins, peptides and glycoproteins, recognized as intercellular signaling immunomodulators [27]. Cytokines are produced by cells and affect the behavior of other cells by binding to specific receptors on their target cells. The term chemokine refers to a specific class of cytokines that is involved in guiding leukocytes to sites where their functions are needed, and as such, have a central role in inflammatory processes. This migration of cells to the site of interest via unidirectional movement towards an increasing gradient of a chemical signal is called chemotaxis [27,30].

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Chemokines are not only involved in orchestrating cellular migration in inflammation and wound healing, but also play roles in hematopoiesis, angiogenesis, and tumor metastasis [10].

The secreted chemical mediators usually have a very short half-life due to their high susceptibility to proteolytic degradation. Local linkage to the ECM protects them from enzymatic cleavage, while others may become inactive when bound and can only act when released by matrix proteolysis [11]. Hereby, chemical mediators and ECM proteins collaborate in creating a distinct cellular niche that regulates tissue regeneration.

### 3.3. Initiation of the innate immune response

Implantation of a biomaterial scaffold typically leads to thrombus formation and initiation of an acute immune response by activation of the coagulation system, complement system, fibrinolytic system, and platelets. Interaction of the biomaterial with blood leads to protein deposition on the biomaterial surface forming a provisional matrix, which affects subsequent leukocyte adhesion interactions [8]. Synthetic polymers, their degradation products and/or the associated provisional matrix activate the complement cascade, marking the biomaterial as being foreign. Phagocytic cells are recruited to the implant by the chemokines released from the provisional matrix and surrounding cells. These phagocytic cells adhere to the matrix surface and further enhance secretion of inflammatory products.

#### *3.3.1. Blood protein precipitation*

Upon contact with blood, the blood-biomaterial interaction leads to adsorption of blood proteins, dependent on the biomaterial surface properties [10,11,29,31,32]. The adsorption of endogenous proteins from blood or interstitial fluid onto the surface of the biomaterial, rather than the biomaterial itself, dictates the immune cell response to implanted biomaterials [27]. All other host components, including leukocytes, encounter and/or interact with this surface as an adhesion substrate. It serves as a provisional matrix, which may also contain a milieu of cytokines, chemokines, or other bioactive agents. This provisional matrix furnishes structural, biochemical, and cellular components to the processes of wound healing and FBR. It can be seen as a naturally derived, biodegradable sustained release system in which bioactive moieties are released to control subsequent phases of wound healing [10].

The precipitation of proteins from blood and tissue occurring immediately after implantation determines the activation of the coagulation cascade, the complement system, platelets and immune cells. The proteins guide their interplay, leading to the formation of the provisional matrix and to the onset of the immune response [11,29]. The adsorbed protein layer includes complement activation fragments, immunoglobulin G (IgG), fibrinogen, fibronectin, and vitronectin. Fibrinogen and fibronectin bind a large number of extracellular macromolecules as well as cell surface proteins, providing a matrix for cell proliferation and organization [33]. Whereas complement and fibrinogen mainly contribute to the activation of inflammatory cells, fibronectin and vitronectin are critical in regulating the inflammatory response to biomaterials [11]. The composition of the protein layer changes over time, described as the Vroman effect [10,29]. Adsorbed proteins may desorb rapidly and, therefore, present time-dependent

variations in the type and level of proteins which cells encounter. The highest mobility proteins of the blood serum generally arrive first, e.g. albumin and globulin, and are later replaced by less motile proteins that have a higher affinity for the biomaterial surface, e.g. fibronectin and factor XII.

Complement receptors function as a non-specific aid in detection and removal of foreign materials. Activation of the complement system leads to subsequent reactions in host defense and functions as one of the players in the tight cross-talk between the different cascade systems, platelets, and leukocytes, inducing clotting and inflammation. The complement system is activated by the coating of the implant with complement activation fragments within the provisional matrix, and through release of anaphylatoxins, i.e. C3a and C5a, which are chemo-attractants for leukocyte infiltration and cause leukocyte activation [32,34]. Upon complement activation, proteases in the system cleave specific proteins to release cytokines, e.g. TNF-$\alpha$, IL-1$\beta$, IL-6, and IL-8, and initiate an amplifying cascade of further cleavages. Furthermore, it contributes to the onset of the inflammatory response by triggering degranulation of mast cells, attraction and activation of PMNs and monocytes, induction of ROI-release by PMNs, supporting platelet adhesion and activation, and promotion of tissue factor expression by monocytes and PMNs on biomaterial surfaces [11]. Destruction of host cells is prevented by the presence of membrane-bound complement regulatory proteins, e.g. CD46, CD55, and CD59 [29].

Blood coagulation on biomaterials requires the combination of contact activation by factor XII, platelet adhesion and their activation by thrombin. This leads to the cleavage of fibrinogen to fibrin and subsequent clot formation. Platelet adhesion and activation, through adsorbed IgG and fibrinogen, mediates neutrophil reactive oxygen generation and monocyte tissue factor expression, leading to neutrophil and monocyte adhesion [29]. Platelets trapped in the fibrin clot, as well as fibroblasts and leukocytes themselves are major resources of chemo-attractants at the site of implantation, initiating and modulating inflammatory reactions and immune responses [35,36].

Cell adhesion and activation on biomaterials primarily occurs through interaction of adhesion receptors with the adsorbed proteins. The major adhesion receptors of leukocytes are represented by integrins, which regulate aggregation, immune functions, cell migration, matrix deposition, and wound contraction [33]. Surface integrin molecules allow cells to migrate through the ECM and mediate signal transduction between the cell and its environment, enabling the cell to respond to its environment. Integrin molecule engagement on leukocytes promotes leukocyte survival, activation, and differentiation [29]. Ligands for integrin receptor binding and cellular adhesion are provided by the adsorbed proteins, including fibrinogen, IgG, iC3b, fibronectin, and vitronectin [10].

### *3.3.2. Pattern recognition*

Besides recognition of biomaterials through adhesion receptors, i.e. integrins, immune cells are activated by another type of receptor-ligand interaction that is based on pattern recognition. A class of molecules classically defined as pathogen-associated molecular pattern (PAMP) molecules, alerts the innate immune system and triggers defensive immune

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responses. PAMPs include lipopolysaccharide (LPS), viral RNA and bacterial peptidoglycans, which interact with dedicated receptors on immune cells, the pattern recognition receptors (PRRs) [8,11,28,29,35,37,38]. These receptors are specialized in the recognition of microbial components that are chemically distinct from the host's endogenous molecules [39]. PRRs include transmembrane Toll-like receptors (TLRs), cytoplasmic NOD-like receptors (NLRs), and cytoplasmic C-type lectin receptors (CLRs) [28,37]. The TLR family is a well-known family of PRRs, in which each member recognizes a specific set of molecular patterns. For example, TLR2 and TLR4 recognize damaged ECM by binding breakdown products of hyaluronan cleaved in tissue damage, while TLR7, TLR8, and TLR9 recognize host RNA and DNA [28]. TLRs are expressed on e.g. platelets, macrophages, dendritic cells (DCs), neutrophils, and endothelial cells [36,37]. Tissue-resident macrophages and DCs, both functioning as antigen-presenting cells (APCs), are most influential in early PRR-signaling and are the primary inducers of an inflammatory reaction [28,39].

An inflammatory response can, besides initiation by PAMPs, also be initiated by several endogenous molecules interacting with signaling receptors. These innate danger signals are described as endokines or alarmins, but are also known as damage-associated molecular patterns (DAMPs) [11,35,38,40]. These signals have immunostimulatory effects and include an array of structurally diverse, multifunctional host proteins that are rapidly released during infection or tissue damage, e.g. after biomaterial implantation. The resulting necrotic cell death leads to the release of cytoplasmic and nuclear components that contain DAMPs, recognized by PRRs expressed by leukocytes. In addition, proteases and hydrolases released from dead cells modify extracellular components to generate mediators (e.g. complement fragments) or other DAMPs (e.g. ECM fragments), which can activate leukocytes [39]. DAMPs have intranuclear, intracellular and/or extracellular functions in mobilizing and activating receptor-expressing cells engaged in host defense and tissue repair, e.g. macrophages and DCs. One of the members of the DAMP family is the group of high-mobility-group box (HMGB) proteins, which are chromosomal proteins helping in transcription, replication, recombination, and DNA repair. HMGB-1 is one of the best known proteins within this family and is released by necrotic cells, cytolytic cells, and cells stimulated by pro-inflammatory stimuli. It was shown to have extracellular activity as a chemokine, attracting neutrophils and mononuclear inflammatory cells [28,39,40]. Other members of the DAMP family include interleukins, heat-shock proteins, defensins, eosinophil-derived neurotoxin, macrophage-/PMN-derived cathelicidins, and nucleosomes. Injury-related TLR-ligands are small hyaluronan fragments, fibrinogen, and fibronectin, of which the latter two are present in the adsorbed protein layer on a biomaterial surface [28].

Induction of inflammation through pattern recognition leads to activation of the receptor expressing cell. When a ligand has bound to a PRR, activation signals are sent out, which initiate signaling pathways leading to the activation of transcription factors, notably nuclear factor κB (NF-κB). This factor migrates into the nucleus and mediates gene transcription and production of inflammatory mediators, such as chemokines, adhesion molecules, growth factors, and pro-inflammatory cytokines, especially TNF-$\alpha$, and IL-1, which themselves also mediate activation of NF-κB [37]. Many molecules with important functions in immunity and

repair mediate their effects through activation of the NF-κB pathway. Transcriptional control of inflammation by NF-κB during the immune response has emerged as one of the most important signaling cascades in the regulation of the inflammatory response [35].

### 3.4. Cell recruitment in the acute inflammatory response

The acute inflammatory response is driven by fast acting leukocytes, mostly neutrophils and macrophages, as the primary defense against nonspecific infecting entities [11,27]. After implantation of a biomaterial, inherently causing cell damage, these immune cells are activated through the engagement of their integrins and PRRs with the protein-coated biomaterial surface. The activation of immune cells leads to the initiation of inflammatory cytokine production and subsequent chemokine recruitment of more immune cells, i.e. PMNs, monocytes, and macrophages, but also endothelial cells and fibroblasts to the site of implantation [28,35]. Activation of PMNs includes a phagocytic response and degranulation, which subsequently leads to biomaterial degradation and potential damaging of the surrounding tissue, prolonging the inflammatory response [11].

During the inflammatory response, macrophages and lymphocytes predominantly synthesize and release immunoregulatory cytokines, e.g. IL-1β, IL-6, and TNF-$\alpha$, and chemokines, e.g. IL-8, monocyte chemotactic protein-1 (MCP-1), and macrophage inflammatory protein-1β (MIP-1β). These are potent chemo-attractants and activation factors for inflammatory effector cells such as PMNs, monocytes, macrophages, immature DCs, natural killer (NK) cells, and lymphocytes. Changes in cellularly released chemical factors mediate additional cell recruitment and activity [27]. The increasing influx of mononuclear cells over time is balanced by a decreased infiltration of PMNs, leading to a decrease in PMN activation signals followed by their apoptosis and engulfment by macrophages. Within two days after implantation PMNs typically disappear from the site [11].

#### *3.4.1. Neutrophils*

Neutrophils are the most dominant cell type among the PMNs present in the acute inflammatory response. They are phagocytic leukocytes containing granules and are activated by pro-inflammatory cytokines, such as IL-1β, TNF-$\alpha$, and IFN-$\gamma$ [35]. The life span of a neutrophil inside the blood stream is 12 hours, but increases to 24-48 hours upon activation outside the vasculature [27,39]. Crucial mediators for neutrophil recruitment in acute inflammation are chemokines and their receptors, e.g. IL-8 [35]. The primary function of IL-8 is induction of the chemotaxis of neutrophils, with their arrival at the site within hours after injury, followed by a later influx of monocytes [38].

Neutrophils eradicate foreign entities by immediate phagocytosis, a process by which solid particles are uptaken by the cell. After phagocytosis of the biomaterial, neutrophils die, and are, together with other material debris, cleared by resident macrophages [27]. This uptake promotes anti-inflammatory lipoxin production by the macrophage, which down-regulates further neutrophil recruitment and activity, while promoting monocyte migration [39]. When neutrophils detect TNF-$\alpha$, but do not directly encounter any exogeneous particles, they

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mobilize and release their granules into the extracellular space, a process called degranulation, to create an inhospitable environment for nearby foreign entities [28]. The granules of neutrophils are loaded with proteases, which, together with the production of ROIs and reactive nitrogen intermediates (RNIs), leads to the denaturation of proteins, disruption of lipids, and damaging of DNA [28,39]. Upon degranulation, the neutrophil reorganizes its surrounding microenvironment and promotes the recruitment of additional immune responsive cells, mainly monocytes, but also generates secondary damage to the host tissue and cells [8,28]. Therefore, neutrophil activation has to be tightly controlled to avoid excessive tissue damage while enabling the rapid recruitment of monocytes [39].

*3.4.2. Monocytes*

After neutrophils, monocytes enter the site of implantation and subsequently mature into tissue macrophages or DCs. Bone marrow precursors give rise to the monocytes in the blood, which circulate for a few days before they migrate into the tissue and mature [41]. Monocytes are recruited by cytokines and chemotactic factors, released by resident macrophages and neutrophils [27]. In general, monocytes reach maximum numbers 24-36 hours after injury [37]. There is a guided movement of monocytes in response to chemokines and other chemoattractants [10]. MCP-1, also known as C-C chemokine ligand 2 (CCL2), binds to C-C chemokine receptor 2 (CCR2) and mediates monocyte recruitment [42]. Although expression of CCR2 is restricted to only a few cell types including monocytes, most (if not all) nucleated cells express MCP-1 in response to activation by pro-inflammatory cytokines or stimulation of innate immune receptors. A hypothesis on the mechanism of action of MCP-1 in monocyte recruitment from the bone marrow is that MCP-1 dimerizes and associates with tissue GAGs, creating a gradient which guides monocytes toward the site of infection or inflammation [42].

In the blood, monocytes are not a homogeneous population of cells. Human monocytes are divided into subsets according to their surface expression of CD14 and CD16 [39,42]. CD14 is a PRR that can recognize and bind various structures from invading microbes (e.g. LPS), while CD16 is a receptor binding IgG antibodies [41]. $CD14^{++}CD16^{-}$ monocytes are the most prevalent monocyte subset present in the blood (~85% of total monocytes) and express CCR2 [43]. The $CD16^{+}$ monocyte population comprises two subsets, $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ monocytes [41,42]. There appears to be a developmental relationship between these different subtypes in that, during the course of an inflammatory reaction, the $CD14^{++}CD16^{-}$ monocytes first develop into $CD14^{++}CD16^{+}$ monocytes to then become $CD14^{+}CD16^{++}$ monocytes. Hence, $CD14^{+}CD16^{++}$ monocytes may represent a more mature subset [41]. The precise role of the different monocyte subsets in initiating immune responses remains unclear, although $CD14^{++}CD16^{-}$ monocytes are believed to contribute more effectively to pathogen clearance while $CD14^{+}CD16^{++}$ monocytes show a patrolling role and account for more vigorous production of pro-inflammatory cytokines [39,42].

At the site of implantation, monocytes become activated and develop into DCs or mature tissue macrophages, undergoing a phenotypic change. This process is directed by mediators present in the microenvironment, such as cytokine receptors, TLRs, and complement receptors, which are crucial for the proper adaptation of cell function to the specific requirements at the site.

[11,35]. For example, TLR-activated monocytes produce IL-10, which has a central role in preventing excessive inflammation [38]. Additionally, there is substantial debate about whether specific monocyte populations give rise to specific tissue macrophages [39,44]. It has been suggested that monocytes continue maturing in the blood and can be recruited to the tissue at various points during this maturation continuum. The point at which they leave the blood may define their function [42].

*3.4.3. Dendritic cells*

Dendritic cells (DCs), monocytes, and macrophages are closely related, as blood monocytes can differentiate into macrophages and DCs and, in their turn, DCs can differentiate into macrophages [41]. The main function of DCs is to function as APCs, processing foreign material and presenting it on its surface to other immune cells, e.g. T-lymphocytes (T cells) [45]. They act as messengers between the innate and adaptive immune response, initiating the T-cell response [29]. Besides their presence as resident cells in tissues, they are also found in the blood, circulating in an immature state. Upon activation, DCs migrate to the lymph nodes and interact with lymphocytes to initiate and modulate the adaptive immune response [30].

By triggering receptors and signaling cascades of the pathogen recognition system, biomaterials activate DCs through the adherent protein layer [11,29]. DC maturation is promoted or inhibited depending on which PRR is engaged, leading to immunity or tolerance, respectively. Immunogenic DCs may prolong the immune response to biomaterials and delay wound healing, while tolerogenic DCs are capable of down-regulating the immune cells and resolve inflammation [11]. Activated immunogenic DCs promote T-cell proliferation and secrete pro-inflammatory cytokines, e.g. IL-1$\beta$, IL-6, IL-12, and TNF-$\alpha$, which further amplify DC maturation by autocrine stimulation. The immature and semi-mature tolerogenic DCs are promoters of tolerance and secrete e.g. IL-10 and TGF-$\beta$ [45]. Besides PRR engagement, integrin signaling due to binding of DCs to ECM proteins on the biomaterial, may act as an alternative mechanism of DC maturation and activation and should be taken into account in the strategy of modulating immune responses to biomaterials.

*3.4.4. Mast cells*

Mast cells are a leukocyte subset represented in most tissues and are best known for their role in allergy. However, they play an important protective role as well, being intimately involved in host defense and wound healing. In the innate immune response, they are an important source of pro-inflammatory mediators and cytokines, containing many granules that are rich in histamine, and producing prostaglandins and cytokines that promote inflammation [35]. Together with tissue-resident macrophages and DCs, mast cells are responsible for the recruitment of inflammatory cells in the innate immune response, i.e. chemotaxis of PMNs and monocytes through secretion of e.g. IL-1$\beta$, TNF-$\alpha$, and MCP-1 [11,28,29]. Besides functioning in host defense mechanisms, mast cells participate more generally in the orchestration of inflammatory responses, e.g. through IL-10 secretion, and tissue remodeling, through secretion of proteases and anti-inflammatory cytokines, such as IL-4 [46]. They express a large set of receptors allowing them to respond to a large variety of stimuli, with activation of specific

receptors leading to specific actions. Therefore, mast cell functions are highly dependent on the physiological context, as small differences in the mast cell environment may yield variant or even opposite actions [46].

### 3.5. The chronic inflammatory response

Once neutrophils, monocytes and macrophages have entered the site of injury or infection in the acute phase of the response, they collaborate to remove the foreign entity [39]. The transition of the acute inflammatory response to the chronic inflammatory response is signified by the departure of the PMNs and infiltration of more macrophages and lymphocytes, which give rise to new tissue formation [8,10,31].

#### *3.5.1. Macrophages*

Generally, when the acute phase of inflammation has not sufficed to clear the source of infection within the first 2 days after implantation, macrophages become the dominating force in the persisting inflammatory response. Macrophages are recruited by many of the same signals as neutrophils but have a longer life span. Tissue-resident macrophages have a life span up to months [27,28]. The primary role of macrophages is to function as a common guardian cell of which the main function in homeostasis is to clear the interstitial environment of extraneous cellular material through phagocytosis [44]. Macrophages are professional phagocytes with extraordinary synthetic and secretory capacities and exert key controlling influences on wound healing and fibrosis responses [31].

A resting macrophage is activated by microbial products, immune complexes, chemical mediators, certain ECM proteins, and T-cell-derived cytokines. An adherent macrophage on a biomaterial is activated to initiate phagocytosis and cytokine secretion, hereby directing the inflammatory and wound healing response to the biomaterial [10]. Several of the key biomaterial-dependent chemokines and cytokines (e.g. IL-1β, IL-6, IL-8, and TNF-$\alpha$) have the potential to induce multiple autocrine and paracrine effects in the chronic inflammatory and wound healing phases, as well as a time-dependent switch in cytokine secretion from acute to chronic phase phenotype [31]. Uptake of apoptotic neutrophils can stimulate macrophages to release mediators that suppress the inflammatory response, e.g. TGF-β, IL-10 and prostaglandin $E_2$ ($PGE_2$) [39]. In bridging the innate and adaptive immune response, macrophages can fuse to become multinucleated giant cells and act as APCs to activate leukocytes which are responsible for the adaptive immune response, e.g. through expression of co-stimulatory molecules that are essential for T-cell activation [27,37].

Beside their function as phagocytes and APCs, it is assumed that macrophages play a prominent role in a successful wound healing response through the synthesis of growth factors such as TGF-β, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), which promote cell proliferation and synthesis of ECM molecules by resident cells [10,35]. TGF-β is a potential stimulator of ECM production, promoting both fibronectin and collagen synthesis in fibroblasts, and decreasing collagen breakdown. With respect to angiogenesis, bFGF is probably one of the major growth factor

families involved, being strongly mitogenic for endothelial cells, directing their migration and proliferation. PDGF recruits neutrophils and monocytes, stimulates the activation of macrophages, and induces expression of TGF-β [33]. Production of VEGF by cells present in the damaged microenvironment is induced by both IL-1β and IL-10, stimulating vasculogenesis and angiogenesis [38].

### *3.5.2. Macrophage phenotypes*

Macrophages show remarkable plasticity, which allows them to efficiently respond to environmental signals and change their phenotype concordantly. The different macrophage phenotypes are identified and distinguished according to markers present at the cell surface and profiles of cytokine and gene expression. Both the acute and the chronic inflammatory response can markedly alter the physiology of macrophages [44]. Furthermore, surface topology and molecular organization of biomaterials affects macrophages, and the cell-surface interaction can change quantity and identity of secreted pro-inflammatory cytokines and chemokines, gene expression patterns, and downstream remodeling events [47].

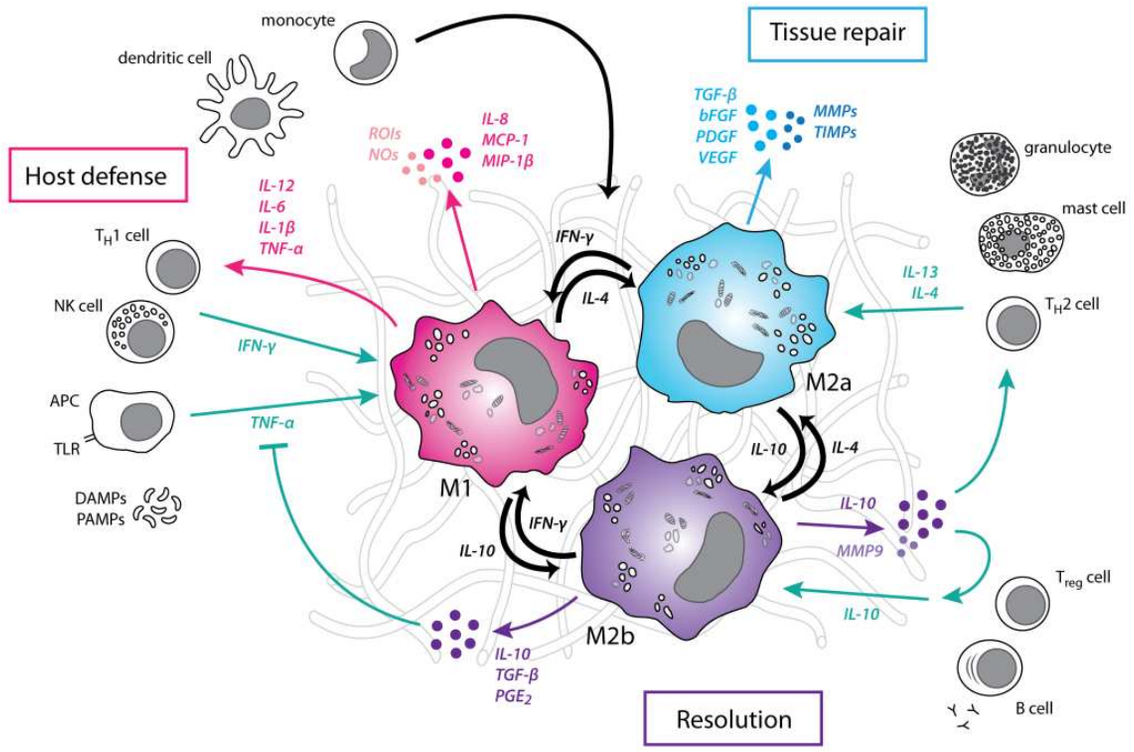


**Figure 3.** Schematic representation of macrophage plasticity. Macrophages can adapt their phenotype in response to environmental cues provided via paracrine or autocrine signaling. Illustrated are extremities within the continuous spectrum of macrophage polarization states ('M1', 'M2a', 'M2b') (adapted from [11,44]). Illustration by Anthal Smits.

Two main macrophage phenotypes have been suggested, classified as "M1" and "M2", mirroring the T helper 1 ($T_H1$) and T helper 2 ($T_H2$) cell polarization [48,49] (figure 3). The pro-inflammatory, cytotoxic macrophage phenotype, signified as M1, is characterized by the

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promotion of pathogen killing and is associated with classic signs of inflammation. These classically activated macrophages are involved in killing intracellular pathogens, up-regulation of pro-inflammatory cytokines, inhibition of anti-inflammatory cytokines, and synthesis of oxygen and nitrogen radicals, making them a crucial part of host defense [10,11,44,45,47]. Their activation is stimulated by pro-inflammatory cytokines, e.g. IFN-$\gamma$ (released by $T_H1$ cells or NK cells), TNF-$\alpha$ (released by APCs), IL-1$\beta$, IL-6, and IL-12, but also by PAMPs, DAMPs, hypoxia, and abnormal matrix, such as pathological collagen deposition [11,29]. Activated M1 macrophages also secrete pro-inflammatory cytokines themselves, i.e. TNF-$\alpha$, IL-1, IL-6, IL-12, and IL-23, inducing $T_H1$ cell responses [45]. Furthermore, they produce low levels of anti-inflammatory IL-10 [29,48]. Macrophages activated by a biomaterial are typically of the M1 phenotype and can promote the invasion of additional inflammatory cells by secreting chemokines such as IL-8, MCP-1, and MIP-1$\beta$. They also secrete degradative enzymes and display high phagocytic activity [11]. Via the production of a variety of enzymes that degrade ECM components, such as matrix metalloproteinases (MMPs), collagenase, and elastase, M1 macrophages are crucial in matrix destruction and tissue reorganization, allowing them to quickly migrate through injured tissues [45]. However, prolonged activation of M1 macrophages can lead to tissue damage.

Immuno-regulation, tissue repair, and constructive tissue regeneration are promoted by the anti-inflammatory macrophage phenotype, signified as M2. These macrophages inhibit pro-inflammatory cytokine secretion, promote anti-inflammatory cytokine secretion, and up-regulate mannose receptors which are necessary for FBGC-formation and play a role in matrix remodeling [10,47]. This alternative macrophage activation is stimulated by the release of IL-4 and IL-13 by $T_H2$ cells, cytokines (e.g. IL-10, TGF-$\beta$), glucocorticoids, and apoptotic cells [29,35,50]. In contrast to M1 macrophages, M2 macrophages typically produce high levels of IL-10 and low levels of IL-12, leading to $T_H2$ cell responses [45,48]. These macrophages show reparative actions by promoting angiogenesis, production of pro-fibrogenic factors resulting in enhanced fibrinogenic activity of fibroblasts, over-expression of certain ECM proteins, and differential secretion of MMPs and tissue inhibitors of metalloproteinases (TIMPs) [10,49].

The M2 phenotype can be divided into two subsets, i.e. wound healing (M2a) and regulatory (M2b) macrophages (figure 3) [11,44]. Wound healing macrophages are mainly triggered by IL-4, released by mast cells, granulocytes, or $T_H2$ cells, which down-regulates pro-inflammatory cytokine secretion by the macrophage. These macrophages promote wound healing processes by contributing to the production of ECM proteins, such as fibronectin, and by the activation of fibroblasts [11]. Although M2a macrophages exert anti-inflammatory activities, they are not capable of down-regulating immune responses. Regulatory macrophages are triggered by a variety of signals, e.g. IL-10, apoptotic cells, immune complexes, and glucocorticoids. Their main task is to limit inflammation and to dampen the immune response, restoring homeostasis while limiting the development of fibrosis [11,49]. They achieve this by releasing high levels of IL-10, which is a very potent immune-suppressive cytokine acting through inhibition of IL-6 signaling and NF-$\kappa$B activation [38].

Macrophages seem to retain their plasticity and respond to environmental signals. The activation of stimulus-specific transcription factors is likely to dictate the functionalized

polarization of macrophages through effects on inducible gene promoters with specific features, translating signals in the microenvironment of the macrophage into a polarized phenotype [51]. The progression from an inflammatory macrophage phenotype (M1) toward a more regenerative/anti-inflammatory macrophage phenotype (M2a/b) correlates with a change in cytokine secretion profile by T helper cells changing from type 1 ($T_H1$) to type 2 ($T_H2$), promoting resolution of the inflammation [8]. The phenotype of a macrophage population can change over time but a single biochemical marker to distinguish between populations has not been identified [44]. It is suggested that macrophages possess a continuum of phenotypes for distinct biological functions, showing overlap of biomarkers and functions for M1 and M2 macrophages [45]. The primary three macrophage phenotypes suggested here, i.e. pro-inflammatory, wound healing, and regulatory, can blend into a continuum of secondary phenotypes that serve a wide variety of functions [44]. It is also unknown whether uncommitted macrophages are recruited to the site of scaffold remodeling and subsequently stimulated to differentiate locally or whether phenotype-committed macrophages are selectively recruited to sites of remodeling, depending on the antigens or substrates that are present [47]. The molecular determinants that precisely control macrophage plasticity, e.g. switching between polarization states, are to a large extend unknown, which makes targeting transcription factors for modulatory aims a challenge [51].

*3.5.3. Lymphocytes*

In the chronic phase of the inflammatory response, lymphocytes appear at the site of inflammation together with macrophages [31]. Lymphocytes play a role in the adaptive immune response, involving major histocompatibility complex (MHC) class I and class II molecules, expressed on the surface of APCs, and recognized by receptors and co-receptors on T cells. In general, MHC class I molecules present peptide antigens derived from pathogens that replicate intracellularly and whose proteins are present in the cytosol of the cell, to cytotoxic $CD8^+$ T cells. MHC class II molecules present peptides obtained from pathogens and their products that are present in the extracellular milieu and have been taken up into the endocytic vesicles of phagocytic cells, to helper $CD4^+$ T cells. The $CD4^+$ $T_H1$ cells and cytolytic $CD8^+$ T cells migrate to the infected tissue, where they activate macrophages to kill antigen-bearing pathogens. This response is referred to as the cell-mediated immune response. On the other hand, $CD4^+$ $T_H2$ cells and B-lymphocytes (B cells) perform their functions in the lymphoid tissues, where $T_H2$ cells activate B cells to produce antibodies against target antigens, called the humoral immune response [29,30].

Accumulation of T cells is associated with the expression of MCP-1 few days after injury, with production of the chemokines interferon-γ-inducible protein-10 (IP-10), and monokine induced by interferon-γ (MIG), of which macrophages appear to be a major source [35]. T cells become activated via interactions with APCs, i.e. macrophages, DCs, and B cells, which present processed antigens bound to MHC molecules on their cell surfaces. Additional co-stimulatory interactions with specific molecules on APCs are required upon lymphocyte activation, i.e. interaction between CD80 or CD86 on the APC and CD28 on the T-cell surface [30]. Characteristics of activation include expression of specific cell surface markers and production

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of the classic activation cytokines IL-2 and IFN-γ [31]. T cells will undergo clonal expansion by proliferation and up- or down-regulation of their effector function. When T cells are activated but not co-stimulated, they become anergic, a mechanism for suppression of inappropriate immune reactivity. Via this mechanism, cells that may have been inappropriately activated, undergo apoptosis, and are removed by macrophages. For example, anti-inflammatory IL-10 induces antigen specific anergy of T helper cells, helping in the prevention of excessive inflammation [31].

Macrophages and lymphocytes are capable of activating each other through direct and indirect mechanisms [31]. Activated T cells induce production of pro-inflammatory cytokines IL-1β, TNF-α, and IL-6, and chemokines IL-8, MCP-1, and MIP-1β by macrophages in a contact-dependent manner. T cells promote the adhesion of macrophages to biomaterials and their subsequent fusion, as well as biomaterial-dependent cytokine production, having consequences for the biocompatibility of the biomaterial [31]. NK cells, a lymphocyte subset next to T and B cells, are potential sources of IL-4 and IL-13 and may promote the FBR by inducing macrophage fusion into FBGCs [31].

### 3.6. Inflammatory resolution and wound healing

After the inflammatory stimulus has been eliminated, the ongoing inflammatory response must be resolved to avoid excessive tissue damage and to initiate the healing process. During the resolution of inflammation, further infiltration of leukocytes is prevented and removal of debris from the inflamed site is promoted, thereby restoring tissue homeostasis [39]. The process of resolution is an active process requiring signals that turn off neutrophil infiltration and, at the same time, promote the uptake and clearance of apoptotic cells and debris. Lipid mediators, e.g. lipoxins and resolvins, seem to have a key role in this process, and the resolution of inflammation is accompanied by an active switch in the types of lipid mediator found at the inflamed site [28,39]. During the inflammatory response, prostaglandins and cytokines that amplify inflammation are generated by various cell types, including neutrophils, monocytes, and macrophages. Following this, $PGE_2$ and prostaglandin D2 ($PGD_2$) gradually promote the synthesis of anti-inflammatory and pro-resolving mediators, such as lipoxins. Another mechanism of inflammatory resolution is inactivation of chemokines through cleavage by MMPs, terminating inflammatory cell influx [39].

The initiation of wound healing is generally marked by the arrival of fibroblasts for the production of ECM proteins, and of endothelial cells for angiogenesis. They occur within the 3 to 5 days of monocyte invasion and activation of resident macrophages, resulting in the formation of granulation tissue [27]. Granulation tissue formation is a wound healing response in which fibroblasts and endothelial cells recruited by macrophages, invade and proliferate within the inflamed tissue in an attempt to establish structure and homeostasis at the local inflammation site [11,27]. Granulation tissue consists of a dense population of macrophages, fibroblasts, and neovasculature embedded within a loose matrix of fibronectin, collagen, and hyaluronic acid, serving as an intermediary substrate [31,33]. Fibroblasts are mesenchyme-derived cells with their primary function being to produce and remodel the local ECM, providing scaffolding and framework to repair the wound [3]. The persistent presence of

macrophages within the granulation tissue ensures constant remodeling of the tissue matrix and constant recruitment of fibroblasts and endothelial cells [27].

The outcome of tissue regeneration or scar formation, i.e. fibrosis, is dependent on the duration of the chronic response that contributes to cytokine production and formation of granulation tissue [8]. Fibrosis is the excessive deposition of matrix components that results in destruction of normal tissue architecture and compromised tissue function and arises from a continuous injuring stimulus, excessive synthesis or decreased degradation [33]. Synthetic and degradative functions of fibroblasts are controlled and regulated by signals from the matrix, as well as leukocyte cytokines and growth factors, wherein macrophages and their phenotype play an important role [27,52].

## 4. The foreign body response to biomaterials

The implantation of a biomaterial can intensify the inflammatory response by inducing a foreign body response (FBR). The FBR at the tissue-material interface is composed of macrophages and foreign body giant cells (FBGCs) and forms the end-stage response of the inflammatory and wound healing responses following implantation of a medical device, prosthesis, or biomaterial [10,29]. Typically, within 2-4 weeks the foreign material is encapsulated within an almost avascular, fibrous connective tissue, depending on the porosity of the biomaterial [52]. The FBR is characterized by the presence of macrophages and FBGCs together with the components of granulation tissue. Macrophages and FBGCs are believed to exert critical effects on both tissue and implanted material, e.g. degradation, and chemokine and cytokine production [31].

### 4.1. Macrophage fusion

Macrophages develop integrins, which play a major role in the adhesion of macrophages to a biomaterial and in the IL-4-induced macrophage fusion to form FBGCs [10]. Macrophage-integrin binding to the protein layer on the biomaterial surface provides intracellular signals that can modulate macrophage behavior, such as cytoskeletal rearrangements and the formation of adhesion structures, called podosomes. There is extensive interplay between intracellular signaling molecules activated by integrin binding and cytoskeletal proteins. Disruption of the adhesion signals promotes anoikis, i.e. apoptosis induced by cell detachment from its supportive matrix. A hypothesis is that macrophage fusion to form FBGCs is an escape mechanism to avoid apoptosis [10].

Macrophages adhere to the surface of an implanted biomaterial when they are unable to phagocytose the material due to a large material-to-cell size ratio. Phagocytosis of large, non-degradable implanted materials usually does not occur due to the size disparity. When the particle size in phagocytosis >5 μm, frustrated phagocytosis may occur instead, a process in which ROIs are secreted aimed to degrade the biomaterial [10,29]. Macrophages fuse with other macrophages to form multinucleated FBGCs, associated with chronic inflammation arising from the persistent presence of a foreign body. These multinucleated cells are

characteristic of granulomatous inflammation and show abundant chromatin with scattered nuclei in an irregular pattern [31]. The fusion of macrophages to form FBGCs serves to prolong the life span of these frustrated macrophages, allowing continued release of cytokines and growth factors [27].

Lymphocytes also seem to play a critical role in the FBR. They have been observed to associate with adherent macrophages and FBGCs, and enhance macrophage adhesion and fusion, while the presence of macrophages stimulates lymphocytes to proliferate [31]. Dependent on the biomaterial, next to macrophages, lymphocytes themselves also produce inflammatory mediators [10,31]. Lymphocytes enhance adherent macrophage and FBGC activation in terms of inflammatory cytokine production via paracrine (indirect) and juxtacrine (direct) means [10]. T cells have been demonstrated to promote macrophage adhesion and fusion via paracrine effects, however, close association of lymphocytes and macrophages also suggests direct signaling which has been shown to dominate at later time points of their interaction [11].

### 4.2. Macrophage phenotype in fusion

The phenotype of the macrophages involved has been shown to play an important role in biomaterial scaffold remodeling [10,11,52]. The fusion of adherent macrophages to FBGCs is typically associated with a phenotype switch of the macrophages over time, going from a more pro-inflammatory activation state (M1) to a more anti-inflammatory activation state (M2). M1 versus M2 macrophage activation has led to morphological variants of multinucleated giant cells *in vitro* [10]. The M2 activation cytokines IL-4 and IL-13 promote macrophage fusion and the formation of large FBGCs with randomly arranged nuclei and high degrees of cytoplasmic spreading, while the M1 activation cytokine IFN-$\gamma$ induces more limited degrees of macrophage fusion with resultant Langerhans-type giant cells. However, the activation state of fusing macrophages is neither M1-like, nor M2-like but rather an in-between state in the continuous spectrum of macrophage polarization. This suggests that biomaterial activation is unique in the process of inflammation [10,11].

The fusion of M2-activated macrophages into FBGCs is stimulated by IL-4 and IL-13, assumed to be secreted by activated T cells [11]. The precise origins of FBGC-inducing cytokines at the implant site remain unclear, with $T_H2$ cells, NK cells, eosinophils, basophils, and mast cells as possible candidates [31]. Both IL-4 and IL-13 were found to up-regulate mannose receptors on fusing macrophages, which mediate endo- and phagocytosis, with localization of the receptor at the fusion interface [10]. MCP-1 is also involved in FBGC formation though not by recruiting cells but rather by guiding macrophage chemotaxis toward each other [10,11].

Biomaterial-adherent macrophages and FBGCs seem to show combined action of biomaterial degradation and down-modulation of pro-inflammatory mediators. Perhaps the presence of macrophage fusion and FBGC formation on biomaterial surfaces represents host down-modulation of pro-inflammatory cytokine production, possibly via phagocytic removal of macrophages actively releasing these cytokines [31]. Next to promoting M2 phenotype and macrophage fusion, IL-4 prevents apoptosis of biomaterial-adherent macrophages by inducing shedding of TNF-$\alpha$ receptor I, preventing this TNF-$\alpha$-mediated process [11].

### 4.3. Biomaterial degradation and fibrosis

Macrophages and FBGCs mediate biomaterial degradation by concentrating phagocytic and oxidative activities at the interface between the cell and the biomaterial. During frustrated phagocytosis, macrophages and FBGCs release degradative mediators such as ROIs, degradative enzymes, and acid into the privileged zone between the cell membrane and the biomaterial surface such that immediate buffering or inhibition of these mediators is delayed or reduced [10]. In this process the phagocytic activity of macrophages decreases, while their degradative capacity increases [11].

FBGCs have the potential to be responsive to cellular signals via cell surface receptor expression as well as actively participate in the inflammatory response through the production of cytokines [10]. They produce anti-inflammatory cytokines, e.g. IL-10, which may be counter-regulated by the proteolytic and pro-oxidant microenvironment. Additionally, FBGCs are thought to release pro-fibrotic factors, e.g. TGF-β and PDGF, which trigger the action of fibroblasts and endothelial cells. Continuous action of FBGCs is assumed to result in prolonged fibroblast activation and excessive biomaterial-associated matrix deposition, leading to impaired wound healing and excessive fibrosis [11]. Therefore, FBGC formation has appeared to be an undesirable phenomenon with a negative impact on biocompatibility, producing cytokines that bias wound healing cells toward a fibrogenic phenotype [31]. Efforts in the design of the biomaterial for *in situ* tissue engineering of heart valves should enhance the biocompatibility, limiting macrophage fusion into FBGCs. Surface chemistry-dependent modulation of the protein layer may enable different receptor binding and signaling in the immune cells leading to altered cellular responses, promoting wound healing while sustaining implant function [11].

## 5. Modulating the immune response

The implantation of any biomaterial initiates an immune response. However, the extent and severity of this response can be modulated by adapting scaffold properties. As described in the previous sections, the immune response is a multi-phased cascade involving many different components. The combined effect of these components will determine the end-stage outcome of the immune response, ranging from pathological fibrotic repair to fully functional regeneration of the original tissue. By interfering with specific elements within this inflammatory cascade, the downstream outcome can be drastically affected, for better or for worse. In this plane of intersection, immuno-modulating scaffolds for *in situ* tissue engineered heart valves are being developed. The development of such a 'smart' scaffold bridges multiple length-scales and is dependent on a multitude of scaffold features. Biological scaffolds inherently come with a natural architecture and a cocktail of signaling components, which would be difficult to replicate with a synthetic counterpart. Synthetic scaffolds, on the other hand, offer a more dedicated control of individual elements in comparison to biological scaffolds. Either scaffold type can be modified within its own framework. There is a legion of possibilities to modify scaffolds over various interdependent scales, ranging from tuning

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biomaterial surface chemistry, scaffold architecture and mechanical properties, to the incorporation of bioactives and targeting of specific cell types. Apart from the scaffold itself, the implantation procedure contributes to the immune response. The method of implantation affects the degree of inflammation [27], and as such should be taken into account in scaffold design.

### 5.1. Biomaterial surface engineering

Biocompatibility and thrombogeneity are particularly important during the onset of the immune response. Immediately after implantation, blood proteins precipitate onto the biomaterial surface, creating an inflammatory milieu that determines the activation of the complement and coagulation cascades. Biomaterial surface chemistry influences the proteins that adsorb, which mediates subsequent interactions with immune cells and may lead to their activation [8]. Factors that affect the amount, composition, and conformation of proteins within the initial layer include the hydrophobicity/hydrophilicity of a surface, as well as its charge and the distribution of charged groups [32]. Incorporation of anti-fouling properties into the biomaterial surface has proven an efficient method to block non-specific protein binding and promote specific biomolecule-binding. This is typically achieved by modifications with hydrophilic polymers, such as poly(ethylene glycol) (PEG), that act as molecular spacers and create a hydrophilic microenvironment that can resist non-specific protein adsorption and cell binding [53].

Biomaterial surface topography and micron-scale architecture can modulate the cell-scaffold interactions that influence immune cell activation, alignment, infiltration, and fusion. Variations in surface roughness and topography affect cell adhesion, morphology, and cytokine secretion. The cell-surface interaction can change quantity and identity of secreted pro-inflammatory cytokines and chemokines, the gene expression pattern, and downstream remodeling events [11,47]. For example, one of the key cellular immune response mechanisms which can be targeted for control of biocompatibility is the mechanism for macrophage adhesion [31,54]. Macrophage fusion on biomaterial surfaces is material dependent, indicating that the surface must have an appropriate array of adsorbed proteins in order for adherent cells to adopt the necessary phenotype to fuse into FBGCs [10]. Furthermore, surface roughness of electrospun fibers has been shown to affect blood activation [55], illustrating the importance of appropriate surface engineering, in particular in the early phases of inflammation.

### 5.2. Scaffold architecture

Cell infiltration into the scaffold is one of the prerequisites for succesful tissue regeneration. It was shown that early infiltration of immune cells determines the degree of downstream ECM production and remodeling [56]. Cell infiltration is primarily determined by the scaffold architecture, or microstructure. Decellularized homograft/xenograft valves have shown limited cell infiltration, resulting in poor tissue remodeling and even degeneration. In contrast, decellularized *in vitro* tissue-engineered valves have shown fast repopulation with host cells and tissue remodeling following a distinct demarcation line. It has been suggested that this

critical difference in cell infiltration is due to a lack of the dense, native-like microstructural arrangement in tissue-engineered valves, as opposed to native valves [57].

For synthetic scaffolds, the importance of scaffold architecture is even more evident. In contrast to natural ECM, cells are typically unable to rapidly break down synthetic biomaterials in order to migrate. Therefore, cell infiltration into a synthetic scaffold is generally dependent on the available pore size, or void space [58,59]. Apart from overall cell infiltration, the pore size can also affect cell phenotype. For example, pore size has shown to be an important factor in the degree of macrophage fusion and material encapsulation. Porous implants with uniform spherical pores of 30-40 μm were shown to elicit healing with minimal fibrosis, high vascularity, and a higher M2/M1 macrophage ratio [52]. It has to be noted however, that the optimal pore size is not generic and has to be tailored to the application.

Synthetic scaffolds for heart valve tissue engineering typically consist of nano- or microfibers, with a high surface area-to-volume ratio. This fibrous architecture dictates the behavior of infiltrating cells. In addition to the void space, the fiber diameter and inter-fiber distance determine cell adhesion, spreading and proliferation [60,61]. Fiber diameter has also shown to affect platelet adhesion and coagulation activation [55]. Fiber alignment guides cell orientation and migration via contact guidance. Furthermore, it was shown that fiber alignment enhanced cell infiltration into a nanofibrous PLLA scaffold [62]. Novel processing techniques to produce fibrous 3D scaffolds with adjustable void space and/or aligned fibers (e.g. low-temperature electrospinning [63]), enhance the degrees of freedom in scaffold modification via 3D architecture (figure 4). For complex structures, such as the aortic valve, multi-layered scaffolds might be required to achieve suitable local cues [59,64].

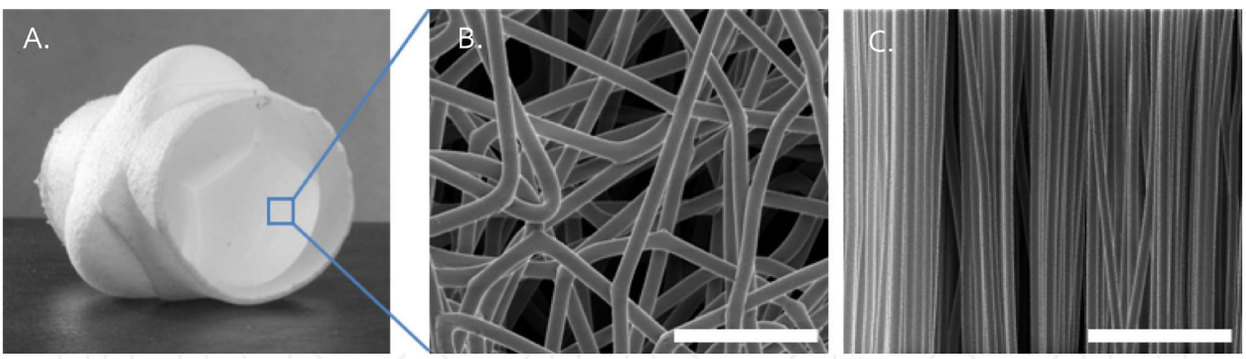


**Figure 4.** (A) Photograph of an electrospun poly(ε-caprolactone) heart valve demonstrating 3D valve architecture, and scanning electron micrographs of its microstructure showing either random (B) or aligned microfibers (C) (scalebar = 100 μm; images courtesy of M. Simonet and G. Argento).

### 5.3. Mechanical properties and degradation rate

Heart valve scaffolds require appropriate mechanical properties to endure the cyclic stresses and strains exerted by the hemodynamic environment. However, next to proper functioning in the hemodynamic environment, scaffold mechanical properties play an important role on the cellular level. The macromechanical properties are determined by the intrinsic material

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properties, the scaffold architecture and the degradation rate. The intrinsic material properties (e.g. stiffness) and the scaffold architecture (e.g. anisotropy) determine the local stresses and strains experienced by the cell. It is well recognized that mechanical conditioning is an important stimulus for ECM production and remodeling. It has been hypothesized that polymeric scaffolds can divert loads from the cells, so-called 'cell shielding', resulting in hampered ECM production. Furthermore, the scaffold or matrix stiffness can modulate the differentiation of cells into pathological phenotypes, e.g. osteoblastic or myofibroblastic, in response to mechanical and biochemical cues [5]. Therefore, efficient transduction of loads from the biomaterial to the cells is crucial. Elastomers typically exhibit adequate mechano-transduction properties, making them a favorable class of materials for application as synthetic scaffolds [25].

Mechanical integrity of the scaffold is dependent on the degradation rate of the material, or rather on the balance between material degradation and ECM production. Accelerated material degradation can result in mechanical instability and valve failure. On the other hand, long-term presence of the biomaterial results in prolonged macrophage activity. Macrophages typically persist at the implantation site until the biomaterial is completely resorbed. When uncontrolled, this may lead to excessive chronic inflammation resulting in fibrosis, calcification, and/or degeneration. Mineralization of synthetic or biologic scaffolds is end-stage pathology, generally irreversible and untreatable. This underlines the importance of timely degradation of the biomaterial. Apart from proper material selection, degradation rate of polymers is tunable by varying copolymer ratios [3]. Variations in degradation kinetics of materials are also employed for controlled delivery of bioactives, for example by introducing fast-degrading fibrin gel [65] or synthetic or biological microspheres [23,66] into the scaffold.

### 5.4. Incorporation of bioactives

Throughout the course of the immune response, signaling factors orchestrate the actions of the immune cells. By incorporating bioactive factors into the biomaterial scaffold, the cellular niche can be modulated locally. These biochemical factors can direct local cellular function, or promote recruitment of specific cell types via chemotaxis. Additionally, the crosstalk between immune cells and tissue cells can be enhanced, regulating the healing process [11]. Since these signaling factors play a role in a specific phase of the immune response, spatio-temporal control of growth factor or cytokine release has been the aim for many tissue engineering scaffolds. For example, long-term release of stromal cell-derived factor 1$\alpha$ (SDF-1$\alpha$) from porous PLGA scaffolds has demonstrated to result in reduced numbers and degranulation of mast cells at the scaffold in a subcutaneous mouse model. This led to downstream alterations in the inflammatory cascade, jumpstarting regeneration with enhanced participation of progenitor cells, increased angiogenesis and decreased fibrosis [67]. De Visscher *et al.* developed heart valves constructed from photo-oxidized bovine pericardium, which were impregnated with SDF-1$\alpha$ in combination with fibronectin to improve the SDF-1$\alpha$ presentation to the cells. Implanted in the pulmonary position in sheep, these valves demonstrated improved homing of primitive cells and normal functioning at 5 months follow-up [68]. Other pro-angiogenic factors, such as VEGF, have shown to play a similar role in vascularization and endotheliali-

zation via recruitment of bone marrow-derived circulating cells, with an essential paracrine role for myeloid cells [69]. Injectable hydrogels featuring a sustained release of VEGF, either or not combined with PDGF, have shown to enhance angiogenesis [70,71]. Dual delivery of MCP-1 and VEGF was applied to promote early monocyte invasion as well as angiogenesis. This was shown to increase mature vessel formation via enhanced endothelial and smooth muscle cell recruitment and displayed a trend of macrophage polarization to the M2 type in a time- and dose-dependent manner [66]. MCP-1 has demonstrated to be a potent immune-modulatory factor, leading to successful remodeling and regeneration of a PCL/PLLA blood vessel graft in mice [23]. Decellularized porcine aortic valves coated with a fusion protein of fibronectin and hepatocyte growth factor (HGF) demonstrated modest acceleration of infiltration of tissue cells, particularly in the valve leaflet, after implantation in a dog model [72].

Single extracellular molecules can impact both pro-inflammatory and anti-inflammatory pathways in different cell types participating in the repair response [35]. The shift from pro-inflammatory to anti-inflammatory response is generally mediated by lipoxins, protectins, and resolvins, actively promoting resolution of infection and tissue repair. Lipoxins are arachidonic acid (AA) derivatives generated by lipoxygenases, and stop the influx of neutrophils, promote the uptake of apoptotic neutrophils by macrophages and recruit additional monocytes to help clear away dead cells and tissue debris [28,39]. Incorporation of such bioactive components may enhance the resolution of the inflammatory response, avoiding uncontrolled chronic inflammation. Resolution of inflammation is also mediated by glucocorticoids, which inhibit inflammatory cell activation by withdrawing the synthesis of inflammatory mediators, and promote resolution of inflammation by enhancing anti-inflammatory cytokine release [29,54]. Glucocorticoids have been shown to modulate the phenotype of infiltrating macrophages and lymphocytes and could thus be used locally to regulate the cellular response [54].

An alternative to incorporating specific signaling moieties into a scaffold is to preseed the scaffold with cells that act as natural signaling factories. Cells, typically bone marrow-derived mononuclear cells, harvested from the host are directly seeded into a scaffold, which is subsequently implanted in a single operation. Although the preseeded cells are cleared from the scaffold within several days after implantation, they mediate the immune response via paracrine signaling by secreting a natural cocktail of growth factors and cytokines. This approach has shown prosperous results in clinical trials using synthetic blood vessel grafts [24]. Furthermore, a similar approach using decellularized tissue-engineered heart valves has shown promising short-term results after 4 week implantation in the pulmonary position in non-human primates [73].

Apart from boosting selected signaling molecules, biomaterials can be designed to tether endogenously released factors to promote a regenerative microenvironment. Natural occurring GAGs have been identified to bind and modify inflammatory factors like interleukins and chemokines, e.g. IL-10 [11]. Subtle differences in GAG structure and/or sequence might be sensed by signaling molecules, guiding their interaction with the ECM and mediating their presentation to leukocytes [11]. In this way, physiological cytokine concentrations are ensured, reducing the risk of adverse side-effects. Heparan sulfate is well-

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recognized as a natural binding site for many growth factors and cytokines, a feature which has been exploited by developing heparin-mimetic peptide nanofibers that are capable of binding growth factors such as VEGF and HGF [74].

Clearly, the use of bioactives or on-the-fly harvestable cells is a powerful tool to create immune-modulating scaffolds. Methods using covalent immobilization of factors [65], microspheres with controlled degradation profiles [66] or hollow-fiber electrospinning techniques [59] enable optimized spatio-temporal control of one or multiple factors. Furthermore, advanced hydrogels have been developed to offer on-demand, remote-controlled release using a magnetic field [75]. With state-of-the-art supramolecular polymers it is possible to engineer cell-responsive substrates [76], offering truly 'smart' scaffolds that can interact with their environment to mediate the host response to the biomaterial.

### 5.5. Cell recruitment and differentiation

The inflammatory response is mainly driven by colonization of the scaffold by blood-derived cells. The nature of the infiltrating cells and their differentiation were demonstrated of pivotal importance to control the delicate balance between fibrotic or functional regenerated ECM production. Of all the cells involved in the immune response, several cell populations can be identified as target cells for *in situ* heart valve tissue engineering.

Macrophages are the predominant mediators throughout the entire immune response, making them an attractive therapeutic target [77]. Although, the precise nature of macrophage plasticity and polarization has yet to be illuminated, it has been shown that early macrophage phenotype determines the end-stage outcome in various biological matrices. In particular, an increased ratio of M2/M1 macrophages correlates to enhanced remodeling, which is likely mediated by differential attraction of secondary cells [78]. By promoting the M2 phenotype, either via specific recruitment or local polarization, the inflammatory response may instantly be directed towards healing instead of inflammation [48,52]. With the identification of multiple subtypes, it is likely that the various macrophage phenotypes play a critical role throughout the various stages of acute inflammation, the healing phase and the resolution of inflammation (figure 5).

ECM production and remodeling is governed by the attraction of secondary cells to the scaffold, consisting of mature (myo-)fibroblasts and endothelial cells, as well as various stem/progenitor cells, released into the circulation by the bone marrow. Furthermore, it has been suggested that adult valve interstitial cells are continuously replenished via circulating endothelial or mesenchymal cell precursors derived from the bone marrow and subsequently undergo endothelial-to-mesenchymal-transition (EndoMT) [5]. Circulating progenitors, such as endothelial progenitor cells (EPC), can have a significant influence on the inflammatory response [4,12,79]. EPC are hypothesized to be an important target cell for endothelialization. Rapid formation of an endothelium over a scaffold is desirable as it acts as a dynamic and selective barrier by maintaining a nonthrombogenic surface, controlling the transfer of molecules across the layer, and regulating immune and inflammatory reactions. The endothelial layer also interacts with underlying cells to regulate their growth and proliferation [12].

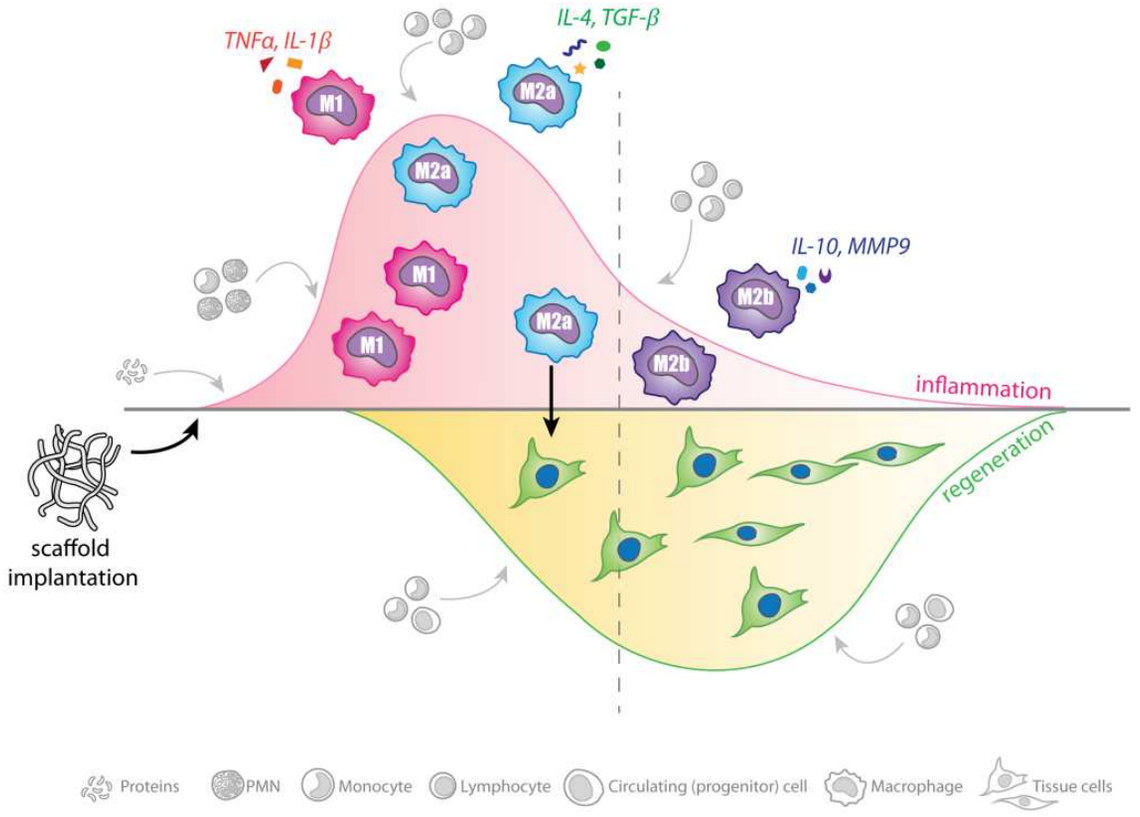


**Figure 5.** Hypothesized role of the various macrophage polarization states throughout the process of inflammation and tissue regeneration in response to scaffold implantation. Illustration by Anthal Smits.

Mesenchymal stem cells (MSCs) proliferate during the healing phase, directed by cytokines secreted by nearby cells, e.g. activated platelets and macrophages, and by ECM components such as collagen peptides and fibronectin [29]. MSCs produce an immunoprivileged environment by preventing the activation and proliferation of DCs, T cells, macrophages, and PMNs through direct cell-cell interactions and paracrine signaling [8]. Cells derived from immunoprivileged regions have been delivered to promote cell engraftment and protect grafts against autoimmune and allogeneic rejection. These cells secrete a range of factors, eg. TGF-β and IL-10, inducing regulatory T-cell differentiation/expansion, which enhances immunoprotection [8].

Recruitment and adhesion of target cell types can be achieved by offering binding domains on the scaffold, for example using supramolecular building blocks with cell-specific peptide sequences [76,79]. When combined with anti-fouling materials, such as PEG, this results in highly selective substrates.

### 5.6. Minimally invasive implantation methods

Independent of the biomaterial, the injury incurred during the implantation process will trigger an immune response, due to the disruption of host tissue and induction of cell damage. Besides substantial mortality and morbidity risks, invasive open heart surgery for heart valve

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replacement causes extensive tissue damage, giving rise to DAMPs, which prime the system for an enhanced immune response [29]. As an alternative, various transvascular, catheter-based techniques, as well as alternative minimally invasive surgical techniques, such as the transapical approach, have been developed [4,80,81]. This has implication for the scaffold design as the scaffold must be crimped and incorporated into a stent. Upon delivery at the valve annulus, the scaffold must also be able to expand properly, be held in place and instantly function within the hemodynamic environment. Transapical valve implantation of preseeded decellularized tissue engineered heart valves into both the aortic and pulmonary position has already proven feasible in pre-clinical models [82,83].

## 6. Challenges and pitfalls

*In situ* tissue engineering of heart valves represents a quick, cheap, and on-demand approach. Immunomodulatory scaffolds hold great promise for future application and commercialization. However, some priority challenges remain to be addressed in the translation from bench to bed.

### 6.1. ECM formation versus fibrosis

One of the main challenges for *in situ* tissue engineering is to stimulate functional ECM formation without inducing fibrosis. To maintain functionality of the valve, rapid ECM formation is required in order to overtake the load-bearing role of the degrading scaffold. However, cells and molecules that are stimulatory for ECM production have been designated as pro-fibrotic mediators. This poses a paradoxal challenge. Macrophage plasticity is a striking example. M2 macrophages have been identified as pro-wound healing cells, promoting ECM production by secretion of IL-4 and TGF-β. On the other hand, both IL-4 and TGF-β are strong inducers of fibrosis if not tightly regulated. Chemokines, such as MCP-1, have been identified as pro-fibrotic mediators by attracting fibrocytes and stimulation of M2 polarization [84,85]. On the other hand, MCP-1 inhibition leads to delayed or inhibited wound healing [86]. Fibrocytes are blood-borne mesenchymal stem cell progenitors with a fibroblast/myofibroblast-like phenotype ($CD34^+/CD45^+$/collagen type $I^+$) that similarly have been related to both ECM formation and fibrosis. The same holds for EndoMT-derived (myo-)fibroblasts. However, the local activation state of recruited myofibroblasts, rather than the source, determines their ECM remodeling activity. For example, TLR-signaling promotes fibroblasts to differentiate into collagen-producing myofibroblasts [84]. Valvular interstitial cells (VICs) in the adult valve have a quiescent myofibroblast-like phenotype. Regulating the activation state of colonizing myofibroblasts in the scaffold is pivotal in the prevention of fibrosis and obtaining a VIC-like population. The TGF-β pathway is one of the main players in this process. Furthermore, IL-10 has been shown to inhibit fibrosis in numerous animal models [84], underlining that timely resolution of inflammation is one of the main challenges for *in situ* tissue engineering.

### 6.2. Hemodynamic environment

In cardiovascular *in situ* tissue regeneration, the hemodynamic environment plays a key role by directing cell recruitment and cell differentiation. The mechanical load applied to the heart valves is a powerful regulator of cell phenotype, influencing many cell functions such as orientation, replication, growth factor production, and collagen synthesis [33]. In cardiovascular devices, apoptosis is often induced by shear stress arising from the blood flow [10]. Shear stress also has a significant effect on adhesion of circulating cells to the valve scaffold. Direct intimal binding of cells to the ventricular side of the leaflet is unlikely due to high shear forces during systole. In contrast, end-systolic and diastolic turbulations on the aortic side of the leaflet typically result in low shear stresses that allow for cell adhesion to the scaffold [4].

So far, the exact mechanism behind cell population of heart valve replacements with host cells remains elusive. For blood vessels, animal studies have identified trans-anastomotic ingrowth as the main source of host tissue cells in the scaffold [87]. However, this is most likely an animal model-dependent phenomenon, as it is known that trans-anastomotic ingrowth is very limited in humans [88]. Therefore, the use of humanized animal models or *in vitro* model systems [89] is indispensible in evaluating scaffold performance for future clinical applications.

### 6.3. Comorbidity and impaired wound healing

Little is known about the effect of the pathological status of a tissue, organ, or patient on the fate of a tissue engineered heart valve. It is reasonable to believe that the pre-existing pathology or existing risk factors would influence wound healing and long-term outcomes of valve implantation. One of the most complicated aspects of designing a replacement scaffold for diseased tissue would be the incorporation of measures which prevent the device from succumbing to the same fate as the diseased tissue it is replacing [12].

Impaired wound healing conditions include advanced age, diabetes mellitus (insulin resistance), vascular diseases (e.g. atherosclerosis), and obesity, in which adipose tissue functions as initiator of the chronic inflammatory response. Diabetic patients have significantly impaired wound healing as they are relatively immunocompromised and have higher blood glucose levels affecting leukocyte function [90]. Diabetes and advanced age are associated with delayed or impaired wound healing through a reduced ability to transition from an M1 to an M2 macrophage phenotype [52]. Malnutrition adversely affects wound healing by prolonging inflammation, inhibiting fibroblast function, and reducing angiogenesis and collagen deposition. For example, carbohydrates are needed for collagen synthesis, and ω-3-fatty acids are needed for modulation of the arachidonic acid pathway, resolving inflammation [90].

The patient's regenerative potential is dependent on age. The concentration of progenitor cells in human blood decreases with age [4]. Furthermore, aging typically leads to impaired angiogenesis and local immunity is altered due to lack of growth factors, increased neutrophil invasion and higher number of mature macrophages. Levels of TGF-β in wounds of elderly are, like fetal, markedly reduced, which is possibly related to reduced scarring with age [35]. Next to regeneration potential, the rate at which the scaffold degrades may also be age-specific due to variations in cell availability.

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Any chronic disease which affects the cardio-respiratory system may adversely affect the supply of oxygen and other nutrients required for wound healing. Although hypoxia is one of the chemoattractants for neutrophils and macrophages, oxygen is needed for their optimal function and to allow phagocytosis. Oxygen is also essential for collagen deposition as it acts as a substrate in the hydroxylation of proline and lysine residues. Smoking affects oxygen partial pressures and causes more wound healing complications and it is likely that smoking may also affect immune function and collagen deposition [90].

The use of diseased cells or tissues in humanized animal models or *in vitro* model systems [89] may aid in gaining insight in the effects of comorbidities on valve regeneration.

### 6.4. Patient heterogeneity

*In vivo* remodeling of tissue engineered heart valves displays considerable variability among patients, owing to biological heterogeneity among individuals in physiological tissue remodeling potential [5]. This heterogeneity could be a result of mutations or polymorphisms in key proteins central to ECM synthesis and remodeling [2]. The goal is to understand and potentially control human variation in different facets of biomaterial-tissue interaction and the healing process by developing robust or even patient-tailored scaffolds.

To cope with patient-to-patient heterogeneity, an important issue in tissue engineering of aortic heart valves will be the real-time noninvasive and non-destructive assessment of mechanical properties both *in vitro* and *in vivo* to ensure tissue quality and function [5]. The challenge here is to find appropriate methodologies to evaluate the evolving structural remodeling and functionality, especially in a noninvasive manner so that the valve can be followed over time [5]. One way of approaching this issue is developing imaging modalities and discovering new biomarkers of inflammation which would help further understanding of inflammatory diseases and discerning events related to inflammation in heart valve tissue-engineered implants [12]. When applied to engineered heart valves, developed biomarkers should correlate directly with success and failure in order to generate outcome measurements, such as laboratory assays or imaging results that substitute for and reflect the mechanism of a significant clinical event or characteristic, e.g. stenosis, calcification, or infection [5]. An important consideration is whether calcification, the major pathologic process in valve degeneration, will be problematic. Evidence suggests that calcification may not be a major problem as long as the scaffold is ultimately resorbed and/or not intrinsically mineralizable, the interstitial cells are viable, and the ECM is capable of remodeling [5].

For the translation from bench to bed, there must be understanding of the mechanisms involved and development of biomarkers, assays and tools for the assessment of valve regeneration. Surrogate and true endpoints must be defined to characterize and assure the quality of the tissue constructs, and predict outcomes as early as possible [5]. Key targets for characterizing tissue-engineered constructs include tissue composition, cellular gene expression and phenotype, ECM, and other key effectors of tissue remodeling and tissue quality [5].

## 7. Conclusion

The complexity of the immune response poses a challenging environment for *in situ* tissue engineering of heart valves [46]. Clearly, a better understanding of the underlying pathways appears crucial for controlling the fate of implanted biomaterial scaffolds and modulating inflammatory reactions in such a way as to induce tissue regeneration and remodeling and prevent fibrosis and/or degeneration [12]. Remaining largely unknown are the specifications of the optimal components (i.e. cells, scaffold and potentially biological modulators) and process conditions (mechanical and metabolic) that will facilitate the formation of optimal substitute heart valve tissues, whose function best emulates the structure, function, and extended durability of a natural valve in vivo [5]. However, the prosperous results of synthetic and biological scaffolds so far demonstrate the ground-breaking potential of *in situ* tissue engineering for heart valves.

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