Harnessing the Innate and Adaptive Immune System for Tissue Repair and Regeneration: Considering More than Macrophages

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Abstract

Tissue healing and regeneration is a complex, choreographed, spatiotemporal process involving a plethora of cell types, the activity of which is stringently regulated in order for effective tissue repair to ensue post injury. A number of globally prevalent conditions such as heart disease, organ failure, and severe musculoskeletal disorders require new therapeutic strategies to repair damaged or diseased tissue, particularly given an ageing population in which obesity, diabetes, and consequent tissue defects have reached epidemic proportions. This is further compounded by the lack of intrinsic healing and poor regenerative capacity of certain adult tissues. While vast progress has been made in the last decade regarding tissue regenerative strategies to direct self-healing, for example, through implantation of tissue engineered scaffolds, several challenges have hampered the clinical application of these technologies. Control of the immune response is growing as an attractive approach in regenerative medicine and it is becoming increasingly apparent that an in depth understanding of the interplay between cells of the immune system and tissue specific progenitor cells is of paramount importance. Furthermore, the integration of immunology and bioengineering promises to elevate the efficacy of biomaterial-based tissue repair and regeneration. In this review, we highlight the role played by individual immune cell subsets in tissue repair processes and describe new approaches that are being taken to direct appropriate healing outcomes via biomaterial mediated cytokine and drug delivery.

Keywords: Innate immunity, adaptive immunity, immune modulation, macrophage polarization, tissue engineering, tissue healing, tissue regeneration

Acronyms:

NK Natural Killer
ILC Innate Lymphoid Cell
TGF Transforming Growth Factor

VEGF Vascular Endothelial Growth Factor

PDGF Platelet-Derived Growth Factor

ECM Extracellular Matrix

BMSC Bone marrow-derived stem/stromal cells

MMP Matrix Metalloproteinase

LPS Lipopolysaccharide

IFN-γ Interferon-γ

IL- Interleukin-

ANXA1 Annexin A1

AMPK AMP-activated protein kinase

DAMP Danger Associated Molecular Pattern

CXCL- CX Chemokine Ligand

CCL- C Chemokine Ligand

DC Dendritic Cell

PGE2 Prostaglandin E2

NET Neutrophil Extracellular Trap

FBR Foreign Body Reaction

PBA 4-phenylbutarate

MI Myocardial Infarction

BM-MSC Bone marrow-derived mesenchymal stromal cells

ROS Reactive Oxygen Species

Th- T helper

TSLP Thymic Stromal Lymphopoietin

VAT Visceral Adipose Tissue

Treg Regulatory T Cells

LTi Lymphoid Tissue-Inducing Cells

PRR Pattern Recognition Receptor

pDC Plasmacytoid Dendritic Cell

IFN-α/β Type I Interferon

PBS Phosphate Buffered Saline

OPG Osteoprotegerin

RANKL Receptor Activator of Nuclear Factor Kappa B Ligand

SCID Severe Combined Immunodeficiency

WT Wild Type

IL-1R1 Interleukin-1 Receptor

IL-1Ra Interleukin-1 Receptor Agonist

PEG Polyethylene Glycol

NFkB Nuclear Factor Kappa B

EAE Experimental Autoimmune Encephalomyelitis

MS Multiple Sclerosis

GVD Graft Vs. Host Disease

TLR Toll-Like Receptor

MSN Mesoporous Silica Nanoparticles

SPM Specialized Pro-Resolving Mediator

AT-RvD1 Aspirin-Triggered Resolving D1

MCP Monocyte Chemoattractant Protein

iDC Immature Dendritic Cell

siRNA Small Interfering RNA

miRNA Micro RNA

EV Extracellular vesicle

1. Introduction

The limited capacity of many tissues and organs to regenerate is at the heart of numerous healthcare challenges. When controlled, the tissue healing response allows for resolution of inflammation and restoration of tissue architecture and homeostasis. A dysfunctional response, on the other hand, often leads to fibrosis and scarring at the injury site which can impair healing and result in organ failure [1]. A number of globally prevalent conditions such as heart disease, severe musculoskeletal defects, and organ failure comprise those most urgently demanding improved therapeutic strategies that enhance the tissue repair process. A thorough understanding of the cellular processes underlying tissue healing and repair is therefore crucial in the development of appropriate and effective therapies that engineer regenerative mechanisms to combat debilitating disease processes.

As the number of tissue repair models used in research has grown and evolved over the last few decades, our understanding of tissue repair and the ability to integrate this knowledge with the pathogenesis of relevant diseases has also expanded. A prevailing concept derived from study findings across a number of species indicates that the tissue regenerative capacity inherent to organisms lesser in complexity, such as fish and amphibians, diminishes as the immune

competency of more complex organisms, namely mammals, increases [2, 3]. It is now well accepted that the immune system plays a pivotal role in tissue healing, with immune cells directly affecting the host response at the injury site and influencing the activity of related tissue-specific cell populations and both recruited and tissue-resident stem cells. Indeed, the immune system has been shown to both positively and negatively regulate the tissue repair process, resulting in either effective tissue regeneration or fibrosis and scarring, respectively [2, 4].

In this review, we highlight the contribution of a number of immune cell subsets across the innate and adaptive arms of the immune system, and the underlying cellular mechanisms that impact on the tissue healing process. We focus not just on macrophage populations which have been at the center of immunology-based tissue engineering strategies and hence the majority of such reviews to date, but also consider the contribution of neutrophils, natural killer (NK) cells and the more recently characterized innate lymphoid cell (ILC) subsets as members of the innate immune system at play in tissue regeneration. Furthermore, we highlight the role played by cells of the adaptive immune system and move to summarize current efforts to integrate immunology and biomaterial-mediated therapeutic development in order to elicit a favorable immune response to facilitate tissue repair and regeneration.

2. Immune Cell Mediators of Tissue Healing and Repair

In mammals, the normal tissue healing process progresses in four overlapping phases: hemostasis, inflammation, repair (also termed proliferation or granulation), and remodeling (also termed maturation [5] (*Figure 1*). To ensure optimal healing, these phases must progress in a tightly controlled manner, with aberration resulting in impairment that can lead to fibrosis and heightened potential for organ failure. The progression of these phases is heavily reliant on the contribution of immune cells, particularly during the inflammatory phase which dictates the efficacy of subsequent tissue repair and remodeling [6, 7].

Inflammation begins with neutrophil influx, followed by monocyte infiltration and their subsequent differentiation into macrophages, in a process that is dependent on location and stimuli received [8]. Signals from these innate cells recruit lymphocytes to the site of injury, which engage in crosstalk and influence further innate immune responses. This phase resolves naturally in the

case of acute wounds and is marked primarily by the transition of inflammatory macrophages at the injury site to a pro-reparative population. The subsequent repair phase is characterized by the onset of angiogenesis along with collagen synthesis and the deposition of ECM, with the final phase of regeneration involving the maturation of the newly formed vessels and remodeling of tissue. Immune cells exert critical influence over the processes of remodeling and regeneration in their clearance of cellular debris, along with their effects on the proliferation and differentiation of tissue specific stem/progenitor cell populations. This is exemplified in the case of bone regeneration, in which the crosstalk between immune cells and osteoprogenitor cells is integral to the initiation and propagation of osteogenesis and associated activities. The concerted activity of macrophages and bone marrow derived stem/stromal cells (BMSCs) is particularly well-studied in this regard, exemplified by demonstration of the initial M1-like macrophage population's ability to enhance bone mineralization by BMSCs *in vitro*, pointing to the importance of the M1-like macrophage in the initiation of osteogenesis [9].

An ever-growing wealth of evidence supports our understanding that aberrant cellular activity in the inflammatory phase often results in impaired tissue healing and defective host responses, with over-fibrosis and scarring a major outcome of such. Given the complex role immune cells of both the innate and adaptive immune system play in the tissue repair process, a thorough understanding of their individual contributions to healing and their interaction with other cells that are involved in tissue repair and regeneration, particularly the aforementioned tissue specific stem/progenitor cells, is required.

2.1 Macrophages in Tissue Healing and Repair

Whilst tissue healing and tissue repair requires the presence and concerted actions of a plethora of cell types, the involvement of macrophages across all stages of tissue repair and their extensive interaction with stem and progenitor cells has indicated that targeting macrophage-mediated responses holds wide-ranging therapeutic potential [10]. Their crucial role in the repair process across multiple tissue types has been demonstrated by depleting macrophage populations in several models of tissue injury. Seminal works in salamander and murine models exemplified the detrimental effect of macrophage depletion on limb regeneration and muscle repair [6, 11].

recently bolstered by a comparative study in which single-cell RNA-seq was used to compare immune cell populations between salamander and murine models of tissue regeneration, showing an earlier recruitment of macrophages exhibiting heightened expression of matrix metalloproteinases (MMPs) in the salamander as compared to the more immuno-competent murine model [12].

Macrophages originate from myeloid precursor cells in the bone marrow compartment and persist in the host system either within the circulation where they differentiate from monocytes through extravasation through the endothelium or residing in tissues in which they rapidly proliferate in response to injury [13]. There is evidence to support the belief that these subsets can play opposing but important roles in healing in a tissue-dependent manner, with studies involving inhibition or depletion of monocytes and macrophages showing a more pro-inflammatory role for macrophages derived from monocytes, in comparison to a more regenerative role for those macrophages that are tissue resident [14-16].

An early paradigm introduced in 2000 that has greatly evolved in recent years is that depicting macrophage plasticity and polarization states. These states exist across a spectrum of diverse, plastic phenotypes, with one end of the spectrum correlating to an "M1-like" or pro-inflammatory macrophage phenotype and the other referring to an anti-inflammatory "M2-like" macrophage phenotype, which in turn determines their functionality [17].

Broadly speaking, M1-like macrophages are induced by pro-inflammatory stimuli (*in vitro*, the classic "M1-like" activators are lipopolysaccharide (LPS) and interferon-γ (IFN-γ)) and they act to orchestrate the initial acute inflammatory response to tissue assault. M1-like macrophages engage in phagocytosis of foreign particles and clearance of debris at the wound site, a process which propagates a pro-inflammatory secretome. The release of pro-inflammatory cytokines and growth factors acts to counter the hypoxic conditions at the site of tissue damage or injury, with molecules including platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF-A) prompting formation of new vessels and cellular proliferation.

M2-like macrophages are induced *in vitro* and in certain *in vivo* settings by anti-inflammatory cytokines such as interleukin (IL)-4 and IL-13, along with glucocorticoids, prostaglandins, and

modulators of metabolism, and these macrophages play a key role in tissue healing and remodeling [18]. They secrete factors including TGF-β1 and anti-inflammatory cytokine IL-10 that instruct cells at the injury site such as fibroblasts to move towards a reparative phenotype and deposit extracellular matrix (ECM) [19, 20]. The latter phases of repair and remodeling require precedence of the M2-like macrophage population, facilitating dampening of inflammation and destructive processes and upregulation of healing-associated activities. Under normal healing conditions, the initial predominant population of M1-like macrophages shifts towards M2-like as the reparative phase begins [21]. This shift from M1-like to M2-like can be attributed to the effect of a number of stimulatory molecules, and was recently shown in a murine model of musculoskeletal injury to be particularly dependent on the protein annexin A1 (ANXA1), a major driver of resolution of inflammation signalling through the AMP-activated protein kinase (AMPK)-governed pathway [22].

Indeed, a large number of studies have shown that higher M2/M1 ratios correlate with heightened tissue repair and a reduction in fibrosis and scarring in the wake of tissue injury, largely caused by a local reduction in inflammatory mediators [23-26]. A caveat to macrophage modulation lies in the critical temporal roles of various macrophage phenotypes and functions, with their delicate balance and inextricable interplay with cells of the adaptive immune system rendering them both an attractive therapeutic target and a potential hinderance to immunomodulation. Supporting this, a recent study uncovering the contribution of macrophages to deleterious collagen deposition during scar formation in both a zebrafish and a murine model of cardiac tissue repair implicates macrophages as an apparent ‘double-edged sword’ in tissue restorative processes [27], further pointing to the importance of macrophages in immunoengineering research and indicating that they should continue to be considered as either central or peripheral components in the majority of immunomodulation strategies, depending on the intended target cell population or populations and tissue types. An in-depth account of macrophages in tissue healing can be found in the following recent reviews [28, 29].

2.2 Neutrophils in Tissue Healing and Repair

Like macrophages, neutrophils function on the frontline of the innate immune system and are the first immune cell to arrive at the site of injury. They are derived from common myeloid progenitors in the immune reservoirs of the body (the bone marrow and spleen) and function by secreting antimicrobial agents and proteases that control infection at the wound site [30]. They predominate during the beginning of the acute inflammatory response and large numbers are recruited from the peripheral blood by chemoattractants released by cells in response to damage associated molecular patterns (DAMPs) following injury [30, 31]. The activated neutrophils are known to secrete immuno-regulatory factors, namely CX chemokine ligand (CXCL) 8 (a target of neutrophils), C chemokine ligand (CCL) 2, and CCL4 (both chemoattractants and activators of monocytes, macrophages, immature dendritic cells (DCs), and lymphocytes) [32].

Generally regarded as being short-lived, it is now accepted that neutrophils can persist at the injury site in a cyclical relationship with inflammation for days beyond assault [33]. Here, they release granules or undergo the respiratory burst to facilitate degradation of necrotic tissue in addition to their phagocytic action. Regulation of neutrophils, primarily mediated by macrophages and dependent on prostaglandin E2 (PGE2), is a vital component of the healing process, with the shift towards the reparative macrophage subset leading to clearance of neutrophils that would otherwise result in matrix degradation and delayed deposition of collagen [34, 35]. Upon increased monocyte infiltration to the injury site in response to neutrophil derived CCL2 and CCL4, neutrophil recruitment is suppressed and, without further signalling, neutrophils apoptose and are cleared from the site. Importantly, macrophages that clear apoptosed neutrophils by phagocytosis have been shown to polarize to an M2-like, pro-reparative phenotype, facilitating progression of the normal tissue repair process [36, 37].

In addition to resolution of neutrophil activation by macrophages, neutrophils also employ the process of neutrophil extracellular trap (NET)-osis, which typically involves release of neutrophil extracellular traps (NETs) and functions at the site of injury to trap and sequester pathogens and can dampen inflammation. While this process has been shown to aid the healing response, it has also been shown to play a role in a sustained foreign body reaction (FBR), a severe result of aberrant healing in the case of encountering a non-native material, and resultant production of a dense fibrotic matrix that can preclude further repair [38-40].

Further supporting a role for neutrophils in tissue healing is evidence that neutrophils function as players in angiogenesis, a crucial requirement for the onset of repair and precursor to tissue remodeling. This occurs via the secretion of MMP-9 by neutrophils which has been demonstrated to be a VEGF-independent mechanism of initiating the switch to angiogenesis [41]. Moreover, they have been implicated in wound repair in a murine model of spinal cord injury, in which Ly6G leukocyte depletion correlated with impaired healing ability [42].

Similar to the M1/M2-like macrophage paradigm, recent interest has grown in the concept of an N1/N2-like neutrophil spectrum of polarization states. In the field of cardiovascular disease and atherosclerosis, investigation is being conducted into the differential effects of neutrophil polarization on disease states and resolution. Neutrophils polarized to a pro-inflammatory state upon LPS stimulation, as characterized by the expression of inflammatory mediators such as CD11b, MMP-9, and Dectin-1, were shown to contribute to an exacerbated atherosclerotic state in a murine model. The converse was seen following transfer of neutrophils polarized to an anti-inflammatory state by the stimulation of LPS-stimulated neutrophils with 4-phenylbutarate (PBA) [43], concomitant with a reduction in the expression of the inflammatory mediators expressed by the N1 phenotype. As with macrophages, polarized neutrophils as defined by Ly6G and CD206 expression have been shown to exert similar temporal effects post myocardial infarction (MI), with the authors defining $Ly6G^{+}CD206^{+}$ neutrophils as 'N1', shifting to 'N2' upon loss of CD206 expression [44]. This implicates the targeting of neutrophil polarization as a potential immunomodulatory strategy in a similar manner to the targeting of macrophage polarization that has dominated the field thus far, with additional considerations for therapeutic design and experimental investigation lying in the relatively short lifespan of primary neutrophils both in culture and *in vivo*. Given the 'first responder' role of neutrophils in the healing response and their ability to initiate angiogenesis, targeting of neutrophil phenotypes has the potential to influence subsequent immunological events to gear the immune response towards that of a pro-regenerative state by acting at an earlier stage to that of macrophage infiltration, introducing the possibility of an attractive new key player in the field of immunoengineering.

2.3 Natural Killer Cells in Tissue Healing and Repair

Natural killer (NK) cells function as the sentinels of the innate immune system. Depending on their received stimuli, NK cells are instructed to either tolerate or attack an invading pathogen [45], and parallels are drawn between NK cells and cytotoxic CD8+ T cells of the adaptive immune system regarding their functionality [46]. They are well-defined in terms of their regulation of the adaptive immune system, with a growing body of evidence detailing their efficacy in mediating host immunity to viral pathogens [47]. In the case of tissue healing, NK cells have been shown to function as regulators of the inflammatory phase, with evidence to date indicating a net negative effect of NK cells on the tissue repair process. Using a murine polytrauma model, NK depletion resulted in 50% mortality reduction and an overall decrease in inflammatory response as measured by IL-6 expression [48]. One study investigating femur fracture in Rag1-null mice, a murine model of adaptive immunodeficiency, implicated NK cells as a major contributor of IFN-γ during fracture healing, which indicates that that they have the ability to polarize macrophages towards a proinflammatory phenotype, particularly when highly activated as they are known to secrete large amounts of IFN-γ in this state [49, 50]. IFN-γ production by NK cells has been shown to be enhanced by IL-33 (in combination with IL-12), a cytokine which is constitutively expressed in the bone and has been hypothesized to play opposing roles in bone homeostasis depending on the differentiation stage of the osteoclasts it acts to stimulate [51, 52]. From a pro-healing perspective, one investigation used a model of corneal epithelial abrasion to demonstrate that NK depletion led to neutrophil influx to the wound site and was reported to hamper nerve regeneration and healing [53].

Studies so far largely indicate the ability of NK cells to influence inflammation as being their major contribution to tissue healing. This is supported by investigation into NK cell interaction with bone marrow-derived mesenchymal stromal cells (BM-MSCs) in the context of bone fracture repair, with pro-inflammatory cytokine-activated NK cells inducing reactive oxygen species (ROS) generation within BM-MSCs and leading to decreased MSC viability [54]. It should be noted that MSCs have been shown to inhibit the cytotoxic capabilities of NK cells in turn, supporting a need for more thorough characterization of NK cell-MSC interactions [55-57], additionally advocating for further consideration of MSCs as potential complementary components of immunomodulatory

strategies and therapeutic design, particularly in NK-cell focused immunotherapeutics such as those employed in cancer immunotherapies.

2.4 Innate Lymphoid Cells in Tissue Healing and Repair

Innate lymphoid cells (ILCs), a recently classified family of innate immune cells, have emerged as cellular populations that share properties with cells of the adaptive immune system but do not display antigen-specific receptors. In this way, they are often described as the "innate counterparts of T cells" [58]. ILCs can be found in both lymphoid organs and non-lymphoid tissues. They are primarily tissue-resident and found in abundance in mucosal tissues, interacting with immune cells to promote immunity and mediate inflammation and tissue repair [59]. Of particular relevance to tissue healing, ILCs are enriched in barrier tissues such as the skin, intestine, and the lung and have been documented as playing a role in the maintenance of tissue integrity and promotion of chronic inflammatory disease [60]. They are grouped into three distinctive subsets: ILC1s, ILC2s, and ILC3s which are functionally considered to correspond to the adaptive immune cell subsets $CD4^+$ T helper (Th)1, Th2, and Th17 cells in their ability to respond to intracellular pathogens, response to extracellular pathogens and mediation of allergic responses, and response to microbial pathogens respectively [61].

ILC1s share features with NK cells in their expression of overlapping markers such as NKp46 (humans and mice) and Tbet, similar effector functions, and production of IFN-γ. ILC1s are activated by cytokines such as IL-12, IL-15, and IL-18, and respond quickly to infections and certain bacteria [61]. They are distinguished from NK cells by their production of CD127 and IL-7R (a receptor for the pro-survival cytokine IL-7), and their lack of the eomesodermin (Eomes) transcription factor. Whilst their active role in tissue regeneration and repair is less established than that of ILC2s and ILC3s, a recent study also indicated ILC1s as being drivers of intestinal epithelial matrix remodeling by their secretion of TGF-β1 and its consequent promotion of WNT/β-catenin pathway signalling via upregulation of CD44v6, a variant of the CD44 cell surface adhesion receptor [62]. ILC2s respond to cytokines IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) and, like their functional Th2 counterparts, have been shown to be producers of IL-5 and the M2-like macrophage polarizing cytokine IL-13 in murine visceral

adipose tissue (VAT) and a model of allergic asthma [63, 64]. They are also producers of the M2-like macrophage polarizing IL-4 and have been shown to contribute to the expansion of the anti-inflammatory regulatory T cell (Treg) subset, helping to establish disease resistance in a murine model of experimental cerebral malaria [65]. This role as a type 2 cytokine producing cellular subset and demonstrated effects on M2-like macrophage polarization and Treg expansion advocate for ILC2s as a notable player in pro-healing responses. In addition, ILC2-secreted IL-13 has recently been shown to promote the renewal of stem cells via the β-catenin pathway in both human and murine intestinal stem cells [66]. A subset of ILC2s that are dependent on epithelial cell-derived IL-33 to drive their responses were also shown to improve wound healing in a cutaneous wound model [67]. ILC2s have been shown to drive Th2 cell polarization by inhibition of Th1 cells, supporting their role in tissue repair and regeneration given the pro-regenerative functions of the Th2 cell subset [68].

ILC3s exert a protective role by the secretion of IL-22, a cytokine that has been linked to reduced liver fibrosis and hepatocyte cell death, in addition to having been shown to drive thymic tissue regeneration in mice [69-71]. During tissue healing, ILC3s exert an immunomodulatory influence as shown by their recruitment to the wound site, with depletion of this subset leading to hampered skin repair in a murine model [72]. Furthermore, a subset of ILC3s referred to as lymphoid tissue-inducing cells (LTis) has been shown to play a role in promoting the formation of secondary lymphoid organs during embryonic development and mediating tissue repair [73].

Taken together, delineation of the direct contribution of each ILC subset to tissue specific repair processes may prove a promising avenue of exploration for identification of novel targets for enhanced tissue healing, with the potential for incorporation of each subset into both innate and adaptive immunomodulatory strategies as either direct influencers or peripheral contributors.

2.5 Dendritic Cells in Tissue Healing and Repair

Dendritic cells (DCs) are considered as “bridging” cells between the innate and adaptive arms of the immune system. DCs play a similar phagocytic role to macrophages in their recognition of invading pathogens and are heavily involved in the instruction of T and B cell responses post-

trauma, primarily in their antigen-presenting capabilities. They can also activate the pro-reparative T cell subset, regulatory T cells (Tregs), an adaptive immune cell subset vital for regeneration [74].

DCs express pattern recognition receptors (PRRs) which recognize pathogenic and foreign materials and propagate signalling. Depending on the PRR stimulated, DCs exhibit an immunogenic or tolerogenic phenotype, the latter of which is responsible for the activation of various immunosuppressive pathways allowing for tolerance of self-antigen [75]. Tolerogenic DCs, in turn, polarize T cells towards a similarly tolerant state and can potentially reduce the extent of inflammation and thus attenuate aberrant host responses that can preclude tissue healing.

DCs play a putative immunoprotective role in the heart following MI in their regulation of anti-inflammatory monocytes and M2-like macrophage recruitment to the infarcted myocardium, with DC ablation resulting in sustained inflammation [76]. There is also evidence that enrichment of DCs facilitates wound closure in a murine model of burn wound healing, with resultant increased TGFβ1 expression and enhancement of fibroblast proliferation confirmed not to adversely affect quality of wound healing by contributing to scar formation, supporting a beneficial role of DCs in the host healing response [77]. Furthermore, depletion of plasmacytoid DC (pDCs), a specialized type of DC that produces large amounts of type I interferon (IFN-α/β), was found to delay reepithelization of skin wounds in a murine model of skin injury, indicating a requirement for pro-inflammatory IFN-α/β and an acute inflammatory response in the initiation of epithelialization [78]. This can be likened to the requirement for an initial pro-inflammatory macrophage response in the tissue healing process and further implicates the inflammatory response as a major modulator of tissue repair. While these studies highlight the important role of DCs in the tissue healing process across varied tissue types, it appears likely that they primarily play a regulatory role, particularly in their signaling to macrophages and T cells, rather than acting as a pronounced component of tissue repair and remodeling.

2.6 B Cells in Tissue Healing and Repair

The major role of B cells is in their presentation of antigen to T cells and resultant influence of the adaptive immune response. While studies regarding the role of B cells in tissue repair and wound healing are limited in number as compared to T cells, it has been shown that CD19-null mice (a murine model of B cell deficiency) in which cutaneous wound healing was examined, a lack of B cells resulted in inhibited healing accompanied by an increase in pro-inflammatory cellular infiltration and cytokine expression [79]. Mature B cells have been shown to accelerate wound healing when administered topically as a purified cell suspension in phosphate-buffered saline (PBS) to wounds sustained in a murine model of skin wound healing, with efficiency of wound healing scored by collagen deposition, angiogenesis, and nerve regeneration [80]. In this study, splenic mature naïve B cells were shown to significantly accelerate wound closure in both wild-type and diabetic murine wounds, showing their efficacy in the case of both acute and chronic wounds. Of note, a marked neutrophil influx was also observed at early time points in acute wounds treated with the mature B cell suspension, followed by neutrophil depletion at an accelerated rate when compared to the saline-only control cohort and further indicating accelerated tissue repair as a result of B cell administration. B cell depletion has been shown to result in delayed wound healing, with B cell reintroduction leading to the rescue of the healing phenotype in a splenectomized murine model [81].

B cells have been shown to predominate at the later stages of bone healing in a murine model of femoral fracture [82]. Under normal tissue homeostasis B cells secrete osteoprotegerin (OPG), an anti-osteoclastogenic factor. Under inflammatory conditions, however, B cells are known to secrete pro-osteoclastogenic factors such as receptor activator of nuclear factor kappa-B ligand (RANKL) [83, 84]. While this is supportive of a temporal role for B cells in fracture healing, there is still much more extensive characterization required regarding the contribution for B cells and their specific secreted factors in tissue repair in various tissue types. Nonetheless, targeting of B cells during healing may prove an important strategy for enhanced tissue repair, particularly in the case of bone and skin repair, when considered in the context of inflammation-modulating strategy design.

2.7 T Cells in Tissue Healing and Repair

There is now ample evidence supporting the crucial role of T cells in tissue repair and regeneration through modulation of inflammation during the healing process. Naïve T cells differentiate into either CD8+ cytotoxic T cells or CD4+ T helper (Th) cells which can exert positive or negative effects on tissue healing. Seminal work in the field has indicated that cytotoxic CD8+ T cell presence at the injury site is associated with poor tissue regenerative outcomes, with a high number of CD8+ cytotoxic T cells at the bone fracture site having a negative impact on bone regeneration in both mice and humans [85, 86]. In contrast, a prevalence of CD4+ Th cells has been shown to contribute to effective tissue regeneration [87]. The main CD4+ T cell subset involved in tissue repair is the CD4+ Th subset, largely functioning in its ability to switch from a Th1 to Th2 phenotype analogous to the M1-like to M2-like macrophage transition and is marked by a pro to anti-inflammatory cytokine expression profile promoting the resolution of inflammation and resultant regeneration of tissue. Both Th1 and Th2 subtypes of CD4+ Th cells play a role in the chronic inflammatory phase of healing by the production of cytokines that modulate the inflammatory state of the injury site. An early study demonstrated CD4+ T cells to be the predominant T cell population persisting throughout the tissue repair process in a murine model of dermal wound healing. This study also demonstrated the importance of both CD4+ and CD8+ T cell presence for tempering of inflammation during the healing process, with severe-compromised immunodeficient (SCID) mice exhibiting exacerbated inflammation, heightened scarring, and decreased formation of new vessels post-dermal wound introduction, yet displayed accelerated wound closure time compared to their WT counterparts [88]. This additionally indicates that the speed at which wound closure processes occur is not to be taken as definitive indicator of superior tissue repair, with a number of factors to be considered when addressing the question of tissue regeneration. It becomes necessary in experimental investigation to further define the criteria that must be met to identify ‘superior’ healing and tissue regeneration, and recognize the elements, such as faster wound closure, that appear to be beneficial but ultimately prove preclusive to adequate regeneration of functional host tissue.

Notably as regards tissue repair, one study which involved repopulating Rag1-null mice with wild-type (WT) CD4+ T cells post-injury resulted in improved running capacity accompanied by upregulated Th2-associated cytokine IL-4 at 3 weeks post repopulation, further supporting a role

for Th2 cells in injury-related repair [87]. A recent investigation into bladder tissue regeneration using chymase-deficient mice, which do not undergo natural exfoliation of bladder tissue during infection to the same extent as their WT counterparts, found that bladder infection and requirement for tissue regeneration stimulated a Th2-biased response to promote epithelial cell proliferation in the WT cohort at the expense of host ability to clear bacteria and control infection, indicating a dampening of the Th1-mediated anti-bacterial response in favor of a Th2-biased pro-reparative immune response. This study exemplifies the tissue-specific action of certain immune cell subsets, and the potential compromises resulting from such, to be an additional consideration in therapeutic design [89]. It is worth considering investigation into immune cell activity not only in the general context of tissue repair, but with added scrutiny of the tissue-specific microenvironment with which immune cells interact, given certain tissues may require particular immune responses to facilitate repair that would otherwise prove disadvantageous.

Regulatory T cells (Tregs) exert an influential role in tissue regeneration by engaging in crosstalk with neutrophils, directing macrophage polarization, and regulating helper T cells, and have been shown in a number of tissue repair models to be regulators of the healing response [90, 91]. Tregs polarize macrophages towards an M2-like phenotype and are seen to persist as a T cell subpopulation at the injury site even as overall T cell numbers decrease [92, 93]. Treg depletion has been shown to result in increased muscle damage and inflammation in a murine model of muscular dystrophy, while mice treated with compounds promoting Treg differentiation and development displayed reduced muscle damage when compared to untreated control mice [94, 95], highlighting the necessity of Tregs for tissue regeneration. Mechanistically, Tregs homing to an injury site are drawn by mast cell-secreted growth factor amphiregulin [96] (also secreted by the ILC2 subset and γδT cells), and can then in turn secrete their own amphiregulin, which plays a role in tissue regeneration and is required for the differentiation and proliferation of surrounding immune cells [96-98]. The dependency on paracrine signalling by Tregs for cardiomyocyte proliferation, a critical factor in regeneration of the myocardium, has been demonstrated in a murine model of Treg depletion and re-supplementation with six Treg-secreted factors (Cst7, Tnfsf11, Il33, Fgl2, Matn2, and Igf2) as chosen by performance of an *in silico* analysis followed

by a test of ability to induce cardiomyocyte proliferation, resulting in rescue of the Treg-dependent therapeutic effect [99].

CD4+ Tregs are also known to mediate immunosuppression and the presence of these cells has been reported to have a pro-reparative role in the repair of skin, kidney, lung, and skeletal muscle [90, 100, 101]. Tregs have been shown to modulate monocyte and macrophage phenotypes to promote tissue healing post-MI and promote pro-resolving macrophage activity in the regression of atherosclerotic inflammation and tissue remodeling [97, 102]. A recent review highlights the necessity for Tregs in repair of skin injury in governing interaction between lymphocytes and nonhematopoietic cells during the healing process [103]. On this basis, it is reasonable to suggest that Treg-targeting strategies may be employed in immunomodulatory therapeutic design in which it is sought to influence a number of immune cell subsets. In particular, strategies that facilitate the tissue repair process by inhibition of deleterious immune cell contributions, namely those of T cell subsets such as CD8+ cytotoxic T cells and Th1 cells, would stand to benefit from harnessing the immunosuppressive functions of Tregs.

Other T cell subsets have been shown to play a role in orchestrating the healing response. Th17 cells and the Th17 secreted pro-inflammatory cytokine IL-17 are emerging as players in the development of fibrosis. A recent body of work has shed light upon the previously unexplored dynamic between Th17 cells and senescent cells in the context of tissue regeneration. It was shown that Th17 cells and senescent fibroblasts engage in a positive feedback loop that fosters chronic inflammation and ultimately inhibits tissue repair processes, with *in vitro* application of Th17 cells inducing senescence in healthy fibroblasts, and senescent fibroblasts in turn skewing differentiation of naive T cells towards Th17. Using a murine model of osteoarthritis, it was then shown that clearance of senescent cells in turn reduced the prevalence of Th17 cells and thus resulted in decreased tissue damage, also highlighting the requirement for IL-4 post clearance to allow healing activity to ensue [104].

Furthermore, a recent study highlighted an IL-36γ-producing macrophage subset as the driver of IL-17 mediated fibrosis [105]. It was shown that application of urine-derived stem cells downregulated Th17 (as well as Th1) immune responses, leading to decreased inflammation and improved healing in a murine colitis model [106]. Further subsets of T cells include the αβT cell

and γδT cell subpopulations; to date, αβT have been shown to exert both positive and negative effects on tissue regeneration, whilst γδT cells have primarily been demonstrated to display pro-reparative functions. This T cell subpopulation is particularly enriched in the dermis, and has been shown in murine models to be required for wound repair [107]. As seen with Tregs, γδT cells are also contributors of pro-healing factor amphiregulin, with γδT cell-secreted amphiregulin demonstrated to be crucial in the maintenance of barrier tissue homeostasis [108]. In the regeneration of bone tissue, an important pro-regenerative role for IL-17A-production by γδT cells has been demonstrated in vivo, in which it was shown that this pro-inflammatory cytokine plays a role in promoting the osteoblastic differentiation of mesenchymal progenitor cells [109].

Thus, it is well established that various T cell subsets and T cell specific cytokines are critical modulators of inflammation driven tissue repair, however details of their interactions with stem cells and progenitor, the major contributors of ECM deposition, is relatively underexplored and therefore warrants further investigation.

3. Immunomodulation for Tissue Repair and Regeneration

With an array of immune cell subtypes implicated as crucial players in tissue repair and regeneration it is unsurprising that a vast range of immunomodulatory strategies are employed in the design of regenerative therapeutics. Although these strategies have advanced the field of regenerative medicine to a considerable degree, there still exist multiple limitations and challenges when attempting to target various components of such a complex and interdependent system during tissue regeneration. The field of immunomodulatory bioengineering, or immunoengineering, a field which marries the benefits of immunomodulatory therapeutics with those of biomaterial intervention, either alone or in combination with stem cells and growth factors, has the capacity to address many of these challenges. Here, we provide an overview of means by which immune cell processes have be targeted to enhance tissue repair and regeneration, touching upon the limitations of each, and providing examples of how the incorporation of biomaterials has led to the advancement of regenerative therapeutics.

3.1 Immunomodulation Through Targeting Pro-Inflammatory Factors

Given the critical role of inflammation in tissue repair and the extent to which inflammation dictates the subsequent reparative or destructive processes, the targeting of inflammatory pathways has proven to be a potent immunomodulatory strategy. In this regard many investigations into modulation of the immune response have focused on targeting pro and anti-inflammatory mediators, and there exists in-depth review of biomaterial-mediated induction of the inflammatory response *via* formation of the inflammasome [110]. Therapeutic strategies targeting inflammation are of particular relevance to biomaterial-mediated tissue regeneration in that they also pose an avenue for modulation or tempering of the foreign body reaction (FBR) and potential fibrosis and scarring that can result from inflammation in the wake of biomaterial implantation.

The pro-inflammatory cytokine TNFα has been implicated as a major contributor to detrimental outcomes post-injury. This has been demonstrated particularly in the context of macrophage-mediated responses, and reduction or inhibition of TNFα as an associated effect of common painkillers such as aspirin and ibuprofen has shown promise in the context of tissue repair and regeneration [3, 111]. Serving as an example of the beneficial effect of TNFα inhibition, bone tissue regeneration was shown to be positively influenced by the biomaterial mediated delivery of anti-TNFα antibodies in a diabetic rat model [112]. Cardiac tissue, too, has been shown to benefit from inhibiting TNFα, with the use of gold nanoparticles to knockdown TNFα in RAW264.7 macrophages *in vitro* and subsequently in an *in vivo* rodent model of MI, resulting in improved cardiac function [113].

Hyaluronic acid, shown to exert an anti-inflammatory effect in its high molecular weight form [114], has also proven effective in delivering anti-TNFα to modulate inflammation in a burn wound model, resulting in a decrease in nonviable tissue and lessened pro-inflammatory cytokine secretion at the wound site when compared to HA alone and saline controls [115]. The efficacy of TNFα targeting, therefore, provides not only an effective strategy to modulate inflammation, but also supports the targeting of major pro-inflammatory cytokines to achieve a more pro-regenerative immune cell landscape.

Similar to our earlier caveat regarding the modulation of macrophage phenotype in the development of immunomodulatory strategies, TNFα too has been implicated to play a beneficial

role at particular points during bone tissue repair [3]. A recent study particularly highlighted the role of TNFα and its interaction with TNFR2, expressed on many cells of the immune system, in mediating anti-inflammatory and immunosuppressive responses in the context of MSC-mediated immunomodulation, indicating TNFα as not only useful in inhibitory strategies, but also a potential deliverable therapeutic in a finely-tuned system [116].

Targeting of the pro-inflammatory cytokine interleukin-1 (IL-1) and its receptor (IL-1R1) is particularly notable in the context of biomaterial-mediated immunomodulation, with inhibition of IL-1R1 signalling shown to enhance the tissue regenerative activity of MSCs [117]. A recent investigation building on this demonstrated the negative impact of IL-1R1 activation on the osteo-regenerative capabilities of MSCs [118]. In this study, the inhibition of IL-1 pro-inflammatory signalling through the co-delivery of IL-1 receptor agonist (IL-1Ra) and pro-osteogenic growth factors BMP-2 and PDGF-BB via a fibrin-based biomaterial scaffold resulted in enhanced bone regeneration *in vivo* when compared to growth factors alone.

Further exemplifying the immunomodulatory potential of targeting pro-inflammatory IL-1R1 signalling with biomaterial intervention, it was demonstrated that electrodes coated with poly-ethylene glycol (PEG) functionalized with IL-1Ra improved overall neuronal survival *in vitro* due to the imbued, controlled anti-inflammatory capability of the biomaterial [119], suggesting that in circumstances in which cell death and resultant inflammation triggered by necrotic cells and their secretome prove preclusive to tissue repair, inhibition of IL-1R1 may offer a combative strategy to enhance cell survival.

Given the potency of targeting pro-inflammatory signalling towards a pro-regenerative outcome, therapeutic strategies that seek to target the nuclear factor kappa b (NFkB) signalling pathway, a central pathway in pro-inflammatory mediation across a range of diseases and regenerative processes [3, 120], have also proven useful in aiding tissue repair and regeneration. For example, it has been shown that curcumin-loaded nanoparticles effectively inhibited the activation of NF-kB and downregulated inflammatory markers alongside decreased infiltration of inflammatory monocytes in an *in vivo* model of experimental autoimmune encephalomyelitis (EAE), holding promise for the future combatting of degenerative or destructive diseases such as multiple sclerosis

(MS) and graft vs. host (GVD) [121]. In the context of bone regeneration, pro-inflammatory cytokine-stimulated NFkB signalling has previously been shown to inhibit osteogenic differentiation of MSCs [122], making inhibition of NFkB signalling an attractive target in bone regeneration. Small interfering RNA (siRNA) based NFkB targeting has also risen as an effective means of blocking NFkB signalling, with use of hybrid micelles to co-deliver siRNA targeting p65 (RelA, a member of the NFkB signalling family) and dexamethasone, a glucocorticoid that inhibits transcription of NFkB and is commonly employed in treatment of rheumatoid arthritis, effectively reducing inflammation and inducing polarization of macrophages from a pro- to and anti-inflammatory state in a murine model of collagen-induced arthritis [123].

NFkB targeting has been shown to be an effective modulator of inflammation across a range of tissue types, with biomaterial mediated delivery aiding localization and elevating efficacy. This means of NFkB targeting can also be employed to enhance signalling through the NFkB pathway, with recent evidence implicating the NFkB/IL-1 signalling pathway as critical in mediating murine lung tissue repair post influenza infection as part of a specific inflammatory niche required for removal of damaged tissue and activation of the required stem and progenitor cell populations for regeneration [124].

Toll-like receptors (TLRs), a set of pattern-recognition receptors expressed on many immune cell subsets, have been shown to play a role in tissue regeneration which can be attributed in part to their activation of the pro-inflammatory NFkB signalling pathway, as well as the induction of TNFα among other pro-inflammatory cytokines. They therefore pose a potential upstream target of pro-inflammatory signalling and have thus been employed in biomaterial-mediated immunomodulation, with TLR4 targeting proving particularly effective in tissue regeneration. TLR4 activation in murine renal and cardiac ischemia-reperfusion models has been shown to contribute to pro-inflammatory responses that mediate organ failure, with TLR4-/- mice shown to exhibit decreased inflammation, smaller infarct sizes, and protection against tubular damage and immune cell infiltration [125, 126]. However, it is clear that inhibiting certain influencers of inflammatory signalling must be considered in the context of the complex cellular interplay at the defect site, a caveat supported by a recent *in vitro* study using LPS-primed MSCs in which TLR4 had been silenced that displayed diminished wound healing ability, in contrast with their LPS-

primed only MSC controls which promoted neutrophil activation, NET formation, and increased healing [127]. TLR4 signalling, too, has been demonstrated to be a regulator of wound healing in vivo, with TLR4-/- mice exhibiting slower wound healing, which was postulated to be as a result of TLR4-induced TGF-β and CCL5 activation, an immune cell chemokine [128].

3.2 Immunomodulation Through Targeting Anti-Inflammatory Factors

In contrast to the targeting of pro-inflammatory pathways, which generally aims to inhibit or dampen the activity of pro-inflammatory mediators, anti-inflammatory strategies usually seek to promote host responses towards a desired state typically *via* the delivery of cytokines known to have a direct anti-inflammatory function. The appropriate temporal dampening or modulation of inflammation is often desirable in the context of tissue regeneration, with biomaterial-mediated delivery of anti-inflammatory factors representing a promising route towards immunomodulation for tissue repair.

Delivery of IL-4, an M2-like macrophage polarizing cytokine, via mesoporous silica nanoparticles (MSNs) has been shown to induce M2-like macrophage polarization *in vivo* and lower ROS production, demonstrating the potential of exogenous IL-4 delivery for improving tissue repair [129]. Similarly, functionalization of TiO_2 nanotubes with IL-4 and attachment of RGD peptide resulted in M2-like macrophage polarization and enhanced production of the pro-reparative cytokine IL-10, and also appears to improve MSC osteogenic differentiation [130]. IL-4-conjugated gold nanoparticles implanted into murine skeletal muscle were shown to lead to a twofold increase in the M2a-like macrophage population (one of the four distinct M2-like macrophage subsets and most strongly associated with anti-inflammatory and wound healing actions [131]) and a twofold decrease in M1-like macrophages [132]. Another recent study has found that alginate hydrogels loaded with anti-inflammatory molecules, either CSF-1 alone or in conjunction with IL-4, or IL-4, IL-6, and IL-13, have resulted in faster tissue repair *in vivo* using a rat model of ischemia-reperfusion. Subsequent injection of biomaterials loaded with CSF-1 and IL-4 were then shown to result in reduced fibrosis and increased cardiac function in a rat MI model, supporting use of biomaterial mediated cytokine delivery for the healing of damaged cardiac tissue [133].

It is worth noting that, given the complex and spatiotemporal role of macrophage phenotype in tissue repair, appreciation for the need of an M1-like phenotype early on in repair process has come to light. Demonstrating this, a bone scaffold that sequentially delivered M1-like and M2-like polarizing cytokines via physically adsorbed IFN-γ and biotinylated IL-4 respectively has been investigated in a murine subcutaneous implantation model. Overall, this sequential cytokine delivery method resulted in improved vascularization *in vivo* in comparison to either an IFN-γ or IL-4 scaffold alone, exemplifying the necessity of controlled transition of M1-like to M2-like macrophages required for vascularization and angiogenesis [134], and highlighting the tissue-specific requirements for a spectrum of inflammation states at different stages of repair as a consideration in therapeutic delivery of immunomodulatory cytokines.

In addition to loading of IL-4 as an M2-polarizing cytokine, delivery of IL-10 has also been investigated as a means of inducing M2-like macrophage polarization and promoting tissue repair. Indeed, loading of alginate nanoparticles with IL-10 has displayed a potent polarization towards a reparative phenotype in a rat model of arthritis [135], while more recently it has been demonstrated that direct supplementation of recombinant IL-10 in culture media resulted in induction of robust BMP2, ALP and osteopontin gene expression in human MSCs, demonstrating a role for IL-10 not only in macrophage polarization but also in promoting osteogenesis [136]. Further supporting the therapeutic use of targeted IL-10 delivery, an investigation by Kim et al. has demonstrated that delivery of IL-10 via a biomaterial nanocarrier resulted in significant regression of atherosclerotic plaques in a murine model of atherosclerosis. This employment of nanocarriers as delivery molecules also resulted in greater IL-10 accumulation in the atherosclerotic lesions, surmounting the issue of rapid clearance encountered with delivery of free cytokine [137].

Of particular note, a recent study by Sok et al. employed PEG-hydrogels to locally deliver IL-10 and the specialized pro-resolving mediator (SPM) aspirin-triggered resolving D1 (AT-RvD1) resulting in increased infiltration of pro-regenerative macrophages, dendritic cells, and T cells in a murine model of tissue injury, highlighting the efficacy of local bioactive molecule delivery in inducing the desired response of multiple immune cell subtypes [138].

The TGF-β isoforms 1-3 have been shown to play a key role in multiple stages of tissue healing and regeneration [139], with the TGF-β1 isoform particularly implicated in skeletal muscle repair [140] and scar formation [20]. TGF-β1, although known to hold pro-inflammatory capabilities, has also been shown to function as a major anti-inflammatory modulator in tissue regeneration and therefore indicated as a therapeutic target. Early work demonstrated *tgfb*-null SCID mice exhibited inferior tissue healing in comparison to their SCID only counterparts, implicating TGB-β1 as a crucial factor in healing and tissue regeneration [141]. The implantation of TGF-β1 releasing PLG scaffolds into a murine model of diabetes was then shown to result in a 40% decrease the expression of pro-inflammatory mediators TNFα, IL-12, and MCP-1, and a decrease in subsequent leukocyte infiltration postulated to be as a result of the earlier tempering of inflammation [142]. TGFβ1 and IL-10 are frequently used to influence dendritic cell activity in vitro, and it was demonstrated that immobilization of TGFβ1 and IL-10 on PEG hydrogel surfaces inhibited immature dendritic cell (iDC) maturation, advocating for the efficacy of designing an immunosuppressive biomaterial for therapeutic benefit [143].

TGFβ has been also characterized as a key regulator of the immunosuppressive and anti-inflammatory activities of Tregs [144], with knockout of FoxP3+ Treg-specific TGFβ receptor 1 (TGF-βR1) leading to a decreased ability to modulate inflammation in the colon in a murine model of colitis [145]. Taken together, studies thus far indicate that TGFβ1 poses a promising target to employ in biomaterial-mediated drug delivery for an immunomodulatory goal, with the potential to exert pro-regenerative effects on multiple immune cell types involved in tissue repair.

Given the potency of targeting inflammatory signalling in the context of the immune response and the wealth of evidence strongly suggesting that such responses can be modulated *via* biomaterial intervention, it is reasonable to suggest that biomaterial-mediated targeting of inflammatory activities represents a particularly promising means of generating pro-regenerative environments *in vivo*.

4. Concluding Remarks and Future Directions

Despite a wealth of advances that have furthered our understanding of different immune cell subsets and their extensive interplay with tissue repair processes, macrophages remain the most characterized cell type when determining the role of (and targeting) the immune system in tissue regeneration. However, there is now growing evidence to suggest that various other immune cell subsets can both positively and negatively influence tissue regeneration in a tissue specific manner. For example, Th2 cells, Tregs, and γδ T cells have been shown to be supportive of a more pro-regenerative phenotype, and indeed direct polarization of these subsets through cytokine delivery or blockade may prove a plausible and worthy strategy to enhance tissue repair. While less well characterized, a new appreciation for the temporal role of B cells, NK cells and ILCs in tissue healing positions these cells as potential candidates to target, although greater efforts must be made to extensively characterize and establish their role in the regeneration of multiple tissue types. Understanding the role of these immune cells in the context of specific tissue microenvironments will be central to the design of elegant, tissue-specific immunomodulatory strategies.

The incorporation of biomaterials into immunomodulatory therapeutics has the potential to transform the field of tissue engineering and regenerative medicine. Existing approaches to manipulate immune cell responses at sites of injury generally aim to target pro or anti-inflammatory activities at play in the tissue microenvironment, with a particular focus on dampening the inflammatory activities of immune cells with the goal of promoting superior healing and tissue regeneration. Incorporating biomaterials into the design of such therapies enables the localized delivery and temporally controlled release of cytokines and molecules targeting inflammatory processes, in addition to providing a physical structure to guide the tissue repair process. Perhaps equally important, the physiochemical properties of biomaterials themselves will have a significant impact on the host immune system [3, 111, 146], which can be leveraged to design scaffolds and biomaterials that will invoke programmable immune responses. Future immunomodulatory therapeutics will likely leverage such biomaterial properties as well as their capacity to control the delivery of cytokines, chemokines and other regulatory factors.

As immunotherapies in tissue engineering and regenerative medicine develop in this way, it will become necessary to consider the host reaction to diverse immunomodulatory inputs. For example, how will the host-biomaterial response, which is commonly pro-inflammatory and skewed towards

the adverse outcomes of fibrosis and scar formation, be influenced by the local delivery of anti-inflammatory cytokines from the same biomaterial? Furthermore, much of our understanding of how the physiochemical properties of biomaterials, for example how surface topography or substrate stiffness can be tuned to promote anti-inflammatory macrophage polarization *in vitro* [147-151] needs to be reevaluated in the context of in vivo microenvironment where potentially pro- or anti-inflammatory factors are being released from the same device. Careful fine-tuning of biomolecule release will be central to the success of such therapeutics. Indeed, such advances hold the potential to inform further immunological studies and elucidate the precise effects or modulate the action of immunomodulatory factors to a greater extent than has been achieved without the backdrop of biomaterial design.

There also remains a substantial gap in our knowledge of cell-cell interactions between immune cells and tissue specific stem and progenitor cells, and further studies may reveal mechanisms by which tissue healing and regeneration takes place in addition to identifying novel therapeutic targets that could be used to enhance the tissue repair process. As well as the interaction of immune cells with stem and progenitor cells, other cells types continue to be identified as players in tissue regeneration and the immune response to biomaterials. A recent review summarizes the role of pericytes, mural cells of blood microvessels that facilitate immune cell extravasation, and their role in angiogenesis and potential to contribute to scar formation [3], highlighting not only pericytes as additional cellular players in tissue repair, but also the potential for targeting a range of cellular subsets closely linked to immune cell activity as another level to be considered in biomaterial mediated immunotherapeutic design.

While current studies are scarce, therapeutic strategies involving siRNA-mediated gene silencing or regulating the immune system through miRNAs and extracellular vesicles (EVs) are underway and offer alternative strategies to loading of exogenous cytokines and antibodies, proving beneficial to tissue healing. Another key remaining challenge lies in the development of biomaterials that can control specific immune system mediated responses for optimal healing and complete regaining of tissue function *in situ*. As our understanding and characterization of the role of the immune system in tissue healing and regeneration continues to grow, compounded with ongoing investigation into the varied and potent effects of biomaterial composition and properties

on immune cell function, it is reasonable to assume that we will continue to see a vast expansion of our knowledge and resources in this area over the coming years.

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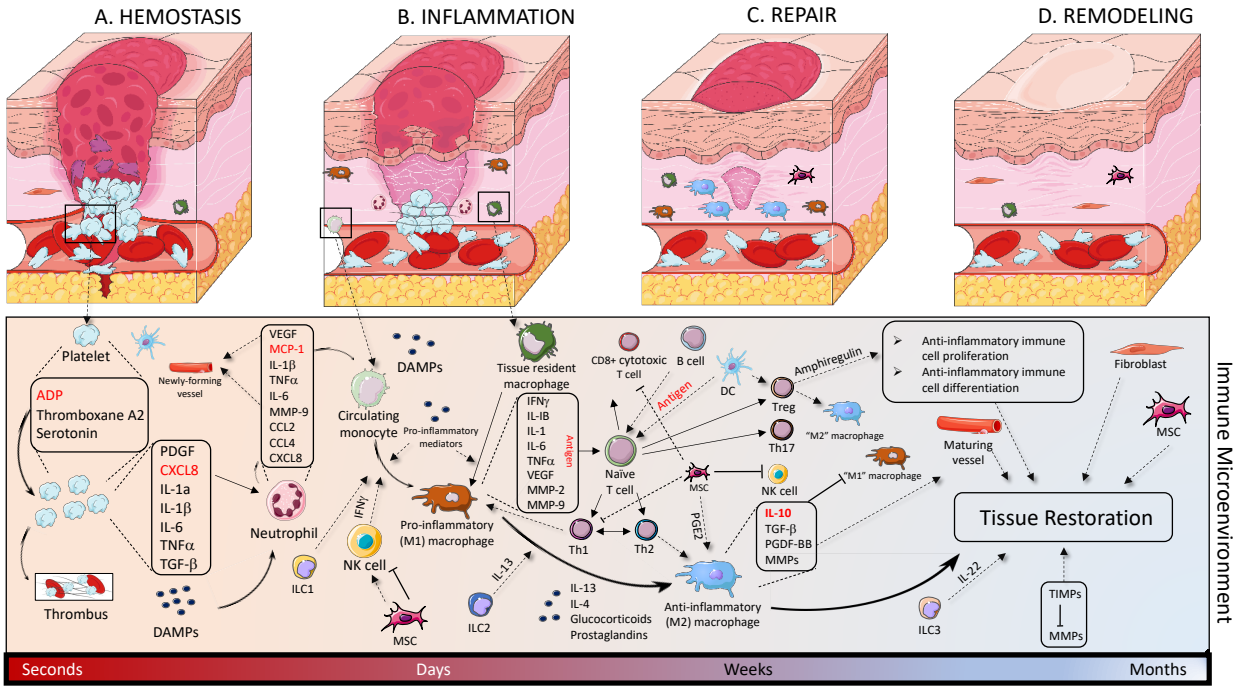
1 Fig. 1



2

3

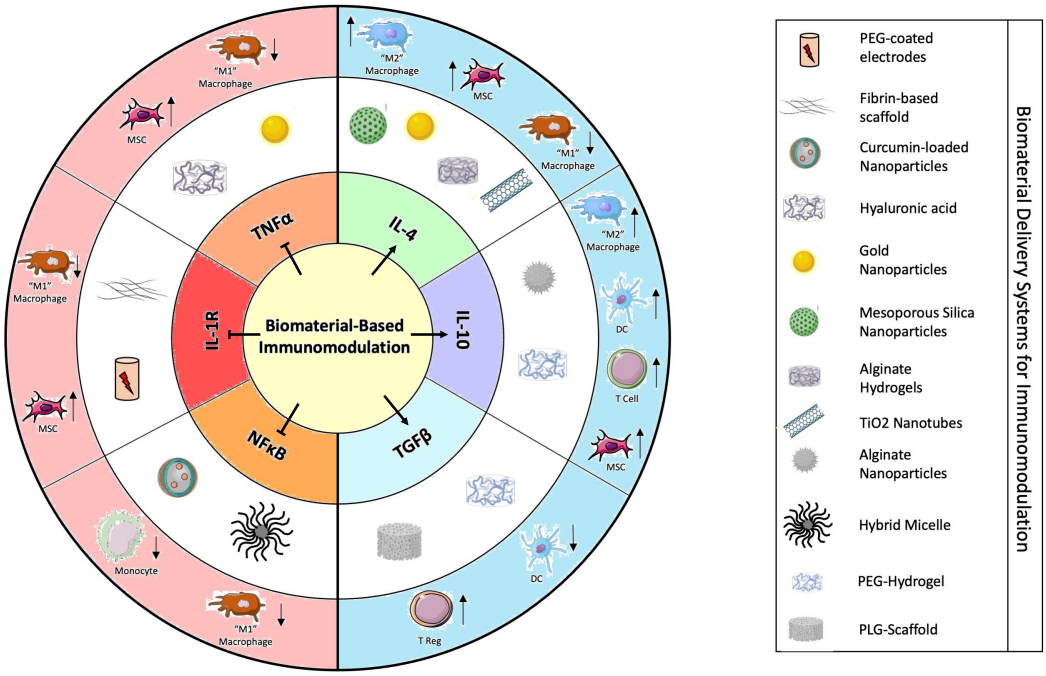
Fig. 1: The Immune Microenvironment in Tissue Healing

a) Immediately post-injury, platelets are recruited and secrete chemoattractant factors such as ADP, thromboxane A2, and serotonin, to further recruit platelets to the injury site. Thrombocytes and leukocytes migrate to a fibrin-rich provisional matrix where platelets then degranulate and activate the complement cascade, with resultant DAMPs facilitating inflammatory immune cell activation. **b)** Neutrophils first migrate to the site of injury, facilitated by the chemoattractant CXCL8. Here they secrete antimicrobial factors including ROS and sequester and phagocytose pathogens assisted by the process of NETosis. They secrete a variety of growth factors and cytokines, stimulate recruitment of inflammatory cells, and facilitate angiogenesis and proliferation of cells such as fibroblasts and epithelial cells. MCP-1, also secreted by these neutrophils, stimulates circulating monocytes to migrate from the surrounding blood vessels and differentiate into macrophages upon encountering further pro-inflammatory stimuli in the injury microenvironment. At this point, ILC1s and NK cells contribute to the inflammatory microenvironment in their secretion of pro-inflammatory cytokine IFNγ. A population of tissue resident macrophages are also stimulated to proliferate and differentiate by pro-inflammatory mediators at the injury site. The predominant macrophage subpopulation during this inflammatory phase of tissue healing are pro-inflammatory/M1-like. The pro-inflammatory secretome of M1-like macrophages perpetuates inflammatory immune cell recruitment. **c)** As the phases progress from inflammation to repair, the macrophage subpopulation shifts from being predominately M1-like to M2-like (this can be driven by ILC2 derived IL-13) and secrete anti-inflammatory and pro-resolutory cytokines such as TGF-β1, while also depositing ECM. M2-like macrophages exert potent anti-inflammatory effects, including suppression of the M1-like phenotype by their secretion of IL-10 and maturation of newly-formed vessels via secretion of PGDF-BB. T cells are recruited by macrophage derived cytokines and the two most crucial T cell subsets in tissue repair and remodeling are the Th2 and Treg subsets, both of which secrete factors contributing to matrix formation such as TGF-β1, IL-4, -5, -13, and -21. Treg-derived amphiregulin stimulates immune cells to differentiate and proliferate and exert anti-inflammatory functions in the latter stages of healing. **d)** During the final phase of repair and remodeling macrophages release MMPs such as MMP-2, -12, and -19, as well as contribute to deposition of type VIII collagen, necessary to maintain the integrity of newly formed tissue. Cellular expression of TIMPs also rise in the latter stages to inhibit MMPs and facilitate repair and regain of function.

4

5

1 Fig. 2



2

Fig. 2: Summary of Reviewed Biomaterial-Based Targeting of Pro and Anti-Inflammatory Factors for Immunomodulation

A summary schematic of each of the biomaterials discussed in this review as examples of biomaterial-based immunomodulation by targeting pro and anti-inflammatory factors, plus a legend to identify key biomaterials discussed in this paper. From the center outwards this depicts the factor targeted whether by inhibition or delivery, the biomaterial used to implement this, and the cell type influenced.

From the upper right segment and going clockwise: Mesoporous silica nanoparticles, gold nanoparticles, alginate hydrogels, titanium dioxide (TiO_2) nanotubes delivering IL-4 enhance M2-like macrophage polarization and increases the M2:M1 macrophage ratio. Loading of alginate hydrogels with anti-inflammatory IL-10 increases the M2:M1 macrophage ratio and enhances osteogenesis. PEG-hydrogel delivery of IL-10 facilitates immunomodulation. Delivery of TGFβ *via* PEG-hydrogels and PLG scaffolds tempers inflammation and reduces iDC maturation, and can enhance immunosuppressive functions of Tregs. Inhibition of NFkB signaling through hybrid micelles delivering siRNA, or curcumin-loaded nanoparticles leads to increased anti-inflammatory macrophages and monocytes respectively. Coating of electrodes with IL-1R1-functionalized PEG resulted in improved neuronal survival *in vitro*. Inhibition of IL-1R by delivering IL-1R agonist alongside pro-osteogenic growth factors on a fibrin-based matrix led to enhanced bone regeneration in a murine model. Knockdown of TNFα with gold nanoparticles and delivery of anti-TNFα through hyaluronic acid (HA) based hydrogels resulted in lessened pro-inflammatory cytokine secretion and enhanced the immunomodulatory capabilities of MSCs.

3