Minireview

Drug delivery strategies to control macrophages for tissue repair and regeneration

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Abstract

Tissue repair and regeneration is a complex process. Our bodies have an excellent capacity to regenerate damaged tissues in many situations. However, tissue healing is impaired in injuries that exceed a critical size or are exacerbated by chronic inflammatory diseases like diabetes. In these instances, biomaterials and drug delivery strategies are often required to facilitate tissue regeneration by providing physical and biochemical cues. Inflammation is the body's response to injury. It is critical for wound healing and biomaterial integration and vascularization, as long as the timing is well controlled. For example, chronic inflammation is well known to impair healing in chronic wounds. In this review, we highlight the importance of a well-controlled inflammatory response, primarily mediated by macrophages in tissue repair and regeneration and discuss various strategies designed to promote regeneration by controlling macrophage behavior. These strategies include temporally controlled delivery of anti-inflammatory drugs, delivery of macrophages as cellular therapy, controlled release of cytokines that modulate macrophage phenotype, and the design of nanoparticles that exploit the inherent phagocytic behavior of macrophages. A clear outcome of this review is that a deeper understanding of the role and timing of complex macrophage phenotypes or activation states is required to fully harness their abilities with drug delivery strategies.

Keywords: Biomaterials, macrophage, drugs, wound, repair

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Introduction

At the heart of angiogenesis and tissue repair lies the inflammatory response, orchestrated primarily by macrophages. The normal response to injury constitutes a brief period of acute inflammation, followed by a cellular proliferation phase, and ending with a longer phase of tissue remodeling.[1] In the early stages of the response to injury, tissue resident macrophages initiate an inflammatory cascade in response to danger signals or invading pathogens.[2,3] These macrophages mediate recruitment of neutrophils and then monocytes into the wounded area. Monocytes differentiate into macrophages, which appear to control each subsequent phase of tissue repair to ensure successful progression through the healing process.[4] Although the mechanisms by which macrophages modulate healing are poorly understood, the critical role of macrophages in tissue repair has been repeatedly demonstrated by pharmacologic or genetic depletion of macrophages, which reduces or delays healing in many tissues, including skin,[5] muscle,[6] liver,[7] bone,[8,9] and cardiac tissue.[10] A landmark study by Godwin et al.[11] showed that macrophage depletion reduced limb regeneration in the salamander, which otherwise has a massive regenerative capacity. Similarly, macrophages are crucial for the integration of implanted tissues. For example, Li et al.[12] showed that liposome-mediated depletion of infiltrating macrophages in an autologous corneal transplantation mouse model impaired tissue integration and vascularization. Mice with depleted macrophages showed diminished inflammation, neovascularization, and infiltration of pericytes and myofibroblasts, resulting in the rejection of the transplanted cornea.[12] However, strict control over the timing of the inflammatory phase is crucial for successful healing, as evidenced by chronic wounds, in which sustained inflammation beyond the initial period impairs healing[13,14] (Figure 1).

As in wound healing, macrophages are crucial for the integration and vascularization of implanted biomaterials.[15,16] Indeed, some have suggested that the response of macrophages to a biomaterial *in vitro* may be indicative of its biocompatibility and success *in vivo*.[17,18] Just like the wound healing response, implanting a biomaterial stimulates an initial inflammatory phase that is characterized by the activation and accumulation of macrophages originating from both the tissue and from circulating monocytes. Ghanaati et al.[19] showed that osteoblasts seeded on a silk

biomaterial construct achieved robust vascularization and integration in part via actions of host macrophages. However, macrophages are also major contributors to the foreign body response, which often leads to fibrous encapsulation of biomaterials and their isolation from the rest of the body.[20–22] Thus, macrophage behavior determines the success or failure of an implanted biomaterial.

Biomaterials that control macrophage behavior would be advantageous to promote successful biomaterial integration and function *in vivo*. While anti-inflammatory drugs have been used for decades to inhibit the inflammatory activation of macrophages, in recent years, more sophisticated strategies have emerged that better incorporate consideration of the complexity of macrophage behavior during healing. In this review, we will introduce the importance of macrophages in biomaterial-mediated tissue repair and highlight emerging drug delivery strategies that target their complex behavior. The main strategies that will be discussed in this review include the temporally controlled delivery of anti-inflammatory drugs, the administration of macrophages as a cellular therapy, the recruitment of endogenous macrophages, the delivery of cytokines and drugs to control macrophage behavior, and the delivery of microparticles and nanoparticles that specifically target macrophages (Figure 2).

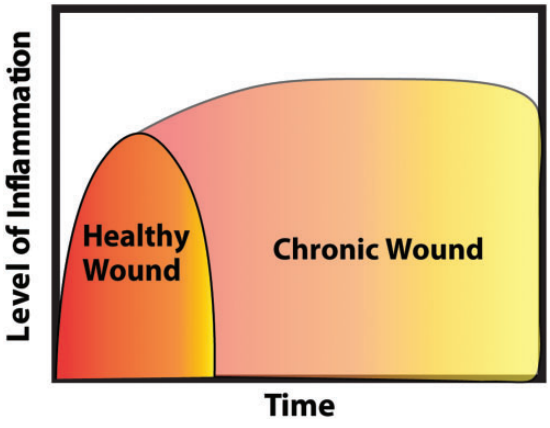

Figure 1 Differences in the level of inflammation in healthy vs. chronic wounds over time. While an initial period of inflammation is needed for successful healing, sustained inflammation is detrimental. (A color version of this figure is available in the online journal.)

Temporally controlled delivery of anti-inflammatory drugs

The initial inflammatory response to an injury is crucial to initiate a healthy wound healing cascade. Indeed, the use of non-steroidal anti-inflammatory drugs (NSAIDs) is contraindicated for patients with fracture bone injuries and spinal fusion surgeries.[8,23–25] NSAIDs have been also shown to reduce bone formation following cementless hip replacement surgery.[26] Jones et al.[27] found that NSAIDs impaired angiogenesis in an aortic ring assay. Clinicians are advised to treat NSAID drugs as a risk factor that may impair the healing of bone fracture patients.[28] Meanwhile, Glass et al.[29] showed that increasing inflammation via the administration of the inflammatory cytokine tumor necrosis factor-alpha (TNF-a) to a murine bone fracture model improved healing due to the enhanced recruitment of muscle-derived stromal cells.

On the other hand, sustained inflammation has been associated with impaired healing in diabetic ulcers,[14] chronic venous ulcers,[13] atherosclerotic lesions,[30] traumatic spinal cord injury,[31] and inflammatory renal disease.[32] Nassiri et al.[14] showed that healing diabetic ulcers were characterized by higher levels of inflammatory gene expression, which then subsided, compared to non-healing diabetic ulcers in humans. Taken together with the role of inflammation in stimulating angiogenesis[16] and wound healing,[10] these results suggest that acute inflammation is needed for normal wound healing, but sustained inflammation is detrimental for healing. Indeed, Wood et al.[33] showed that a one-time administration of the pro-inflammatory cytokine CCL2 immediately after injury improved healing of diabetic ulcers in mice. Similarly, temporal control over the administration of anti-inflammatory therapies may lead to positive outcomes. For example, Mirza et al.[34] showed that the delivery of an inhibitor of the inflammatory cytokine interleukin-1-beta (IL1b) improved healing of diabetic ulcers when it was applied at three days post wounding. However, more studies are required to systematically

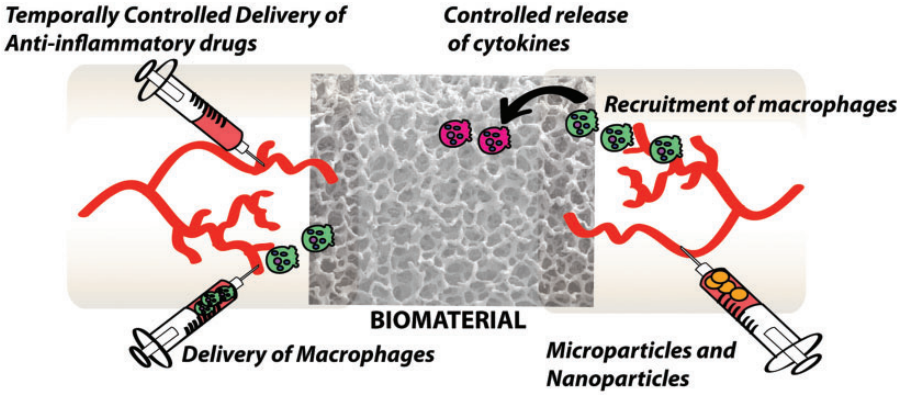

Figure 2 Drug delivery strategies to control macrophage behavior to promote tissue repair. (A color version of this figure is available in the online journal.)

evaluate the influence of timing of administration of pro- and anti-inflammatory therapeutics.

While it is not well understood why some biomaterials promote macrophage-mediated integration while others promote rejection via the foreign body response, delivery of anti-inflammatory drugs has been explored to inhibit the foreign body response. A study by Vacanti et al.[35] aimed to reduce fibrous capsule formation around electrospun fibers composed of synthetic polymers by conjugating the anti-inflammatory glucocorticosteroid dexamethasone to poly(l-lactic) acid and poly(ε-caprolactone) fibers.[35] This local delivery of dexamethasone reduced inflammation and the thickness of the fibrous capsule over four weeks following subcutaneous implantation in rats.[35] Similarly, the continuous delivery of dexamethasone from microdialysis tubing reduced fibrous capsule thickness in subcutaneous tissue of rats.[36] However, somewhat contradictory results were shown when Stevens et al.[37] delivered dexamethasone from a gelatin-based hydrogel implanted subcutaneously in mice. They found that the delivery of dexamethasone delayed but intensified the inflammatory response at day 7 postimplantation.[37] By day 21, they found comparable chronic inflammation between dexamethasone-loaded hydrogels and controls. The authors attributed these results to the short half-life of dexamethasone, so that an initial inhibition of inflammation was able to return once its bioactivity diminished around day 4 postimplantation.[37] It is possible that a strategy that allows a brief period of inflammation to occur prior to its inhibition would aid in biomaterial integration, like it aids chronic wound healing,[34,38] but this strategy has not been explored.

Other drug delivery strategies that have been shown to inhibit the foreign body response to biomaterials include the delivery of a blocking antibody to interleukin-4 (IL4), a cytokine that mediates macrophage fusion into foreign body giant cells *in vitro* and *in vivo*,[39,40] although it is important to note that the role of IL4 in the foreign body response remains controversial.[41] Other drug delivery strategies include the delivery of nitric oxide, which promotes angiogenesis and inhibits collagen deposition during wound healing.[42] For a review on other biomaterial strategies to control macrophages in the foreign body response, the reader is referred to Yu et al.[43] Further studies are required to thoroughly investigate the possibility of controlling inflammation to inhibit the foreign body response.

Delivery of macrophages as a cellular therapy for tissue repair

The significance of macrophage activity in promoting tissue repair and biomaterial vascularization has led many researchers to deliver macrophages to injured tissues to promote the healing response. For example, Zuloff-Shani et al.[44] evaluated the efficacy of allogeneic macrophage suspensions for treating chronic pressure ulcers. In their phase 3–4 clinical trial, 85.1% of the macrophage-treated group (120 out of 141 ulcers) achieved at least 50% closure in a time frame ranging from 14 to 171 days.[44] On the other hand, in the control group that was treated with the standard of care, only 41.3% (31 out of 75 ulcers) achieved 50% area closure in 26 to 368 days.[44]

Leor et al.[45] studied the impact of directly injecting macrophages to ischemic myocardial tissue in a rat model. Human macrophages were isolated from peripheral blood, activated *in vitro* by a novel method based on hypo-osmotic shock, and directly injected into the ischemic cardiac tissue 1 min after the induction of myocardial infarction.[45] Using magnetic resonance labeling, human macrophages were detected in the ischemic site until four to seven days postinjection.[45] Macrophage-treated rats exhibited improved myofibroblast accumulation, vascularization, and scar thickening, which overall prevented infarct expansion and led to better functional outcomes.[45] Local injection of macrophages also has been shown to improve vascularization and healing in bone,[46] ischemic hind limb tissue injury,[47] renal injury,[32,48] and spinal cord injury.[49] These positive outcomes likely result from the secretion of an array of beneficial cytokines and growth factors by macrophages, but future studies should focus on determining the precise mechanisms of action in order to facilitate clinical translation, especially considering there are disadvantages associated with delivering living cells. Because of the potential hurdles to clinical translation, it may be beneficial to recruit endogenous macrophages rather than delivering exogenous ones.

Drug delivery systems to recruit endogenous macrophages

Given the importance of macrophages for wound healing and biomaterial vascularization, as well as the benefit of administering them as a cell therapy, several groups have turned to recruitment of endogenous macrophages to sites of injury and implanted biomaterials to promote tissue regeneration. A landmark study by Roh et al.[50] showed that tissue-engineered vascular grafts transform into living blood vessels *in vivo* through processes mediated by the host inflammatory response. Biodegradable vascular grafts constructed from polyester tubes and seeded with human bone marrow cells (hBMCs) were implanted into the inferior vena cava of severe combined immunodeficient (SCID) mice.[50] Interestingly, tracking the hBMCs revealed that they were eliminated within one week *in vivo*, indicating that transformation of the seeded biodegradable scaffold into patent blood vessels was not driven by the transdifferentiation and proliferation of the seeded hBMCs.[50] Rather, the seeded hBMCs altered the kinetics of the early phase of vascular remodeling by expediting the recruitment of host monocytes/macrophages to the scaffold via secretion of monocyte chemoattractant protein-1 (MCP-1), a chemokine that plays a major role in monocyte/macrophage recruitment.[50] To test the effects of MCP-1 in inducing macrophage recruitment and vascular transformation, the team implanted non-cell-seeded grafts incorporating alginate microcapsules that released MCP-1.[50] The release of MCP-1 resulted in vascular transformation of the grafts to the same degree as those seeded with hBMCs.[50]

Hsu et al.[51] used intravital microscopy to highlight key cellular changes induced by the release of potent angiogenic factors from polyethylene glycol diacrylate hydrogels *in vivo* in a cornea angiogenesis assay in mice. They noted that the delivery of a combination of platelet-derived growth factor-BB (PDGFBB) and basic fibroblast growth factor induced more macrophage recruitment and potent angiogenesis relative to vascular endothelial growth factor (VEGF) delivery.[51] Macrophages were observed bridging adjacent blood vessel sprouts, suggesting a role for anastomosis; migrating down blood vessel sprouts that subsequently retracted, suggesting a role in pruning; and surrounding vessels like pericytes do, suggesting a role for maintaining vessel stability.[51] Finally, the authors showed that delivery of the macrophage-recruiting factor CSF1 enhanced the angiogenic response to VEGF.[51]

The recruitment of endogenous macrophages has also been shown to promote tissue healing in a bone defect model in rats.[52] Kim et al.[52] encapsulated gelatin/lactic acid-based micelles containing the macrophage-recruiting agent SEW2871 (an S1P1 receptor agonist) together with platelet-rich plasma (PRP) in gelatin hydrogels. The delivery of both factors together induced more macrophage recruitment compared to the delivery of either factor alone.[52] Increasing macrophage recruitment was coupled with increasing gene expression levels of the pro-inflammatory cytokine TNF-a three days postoperatively, along with a significant increase in IL10, osteoprotegerin, and transforming growth factor beta (TGFb)-1 10 days later. Delivery of PRP and SEW2871 lead to the highest bone regeneration outcomes.[52] Thus, recruitment of monocytes and macrophages via drug delivery is a viable strategy to promote vascularization of biomaterials and tissue regeneration.

Cell delivery of polarized macrophages

While delivery or recruitment of macrophages is beneficial for tissue regeneration, strategies that control-specific functions of macrophages may be even more successful. Macrophages exist on a diverse spectrum of phenotypes, with widely varying functions that depend on their specific activation state. These phenotypic differences are often described to range from classically activated macrophages (M1) to alternatively activated macrophages (M2), although many more distinct phenotypes have been identified, each with unique functions and gene expression profiles.[53] For an excellent review of macrophage phenotype, the reader is referred to Mosser and Edwards.[54]

The two most commonly studied phenotypes include M1, which are pro-inflammatory and pro-angiogenic, and M2, also known as M2a, which are anti-inflammatory. M1 macrophages are stimulated with pro-inflammatory stimuli, especially interferon-gamma (IFNg) and the bacterial cell wall component lipopolysaccharide (LPS), while M2a macrophages are stimulated with Th2 cytokines like IL4 and IL13. In the early stages of wound healing, M1 macrophages are among the first innate immune cells to infiltrate the region.[55] M1 macrophages secrete many pro-inflammatory cytokines such as IL1b and TNF-a that recruit other cells necessary for healing. In addition, they secrete high levels of VEGF, a potent initiator of angiogenesis and

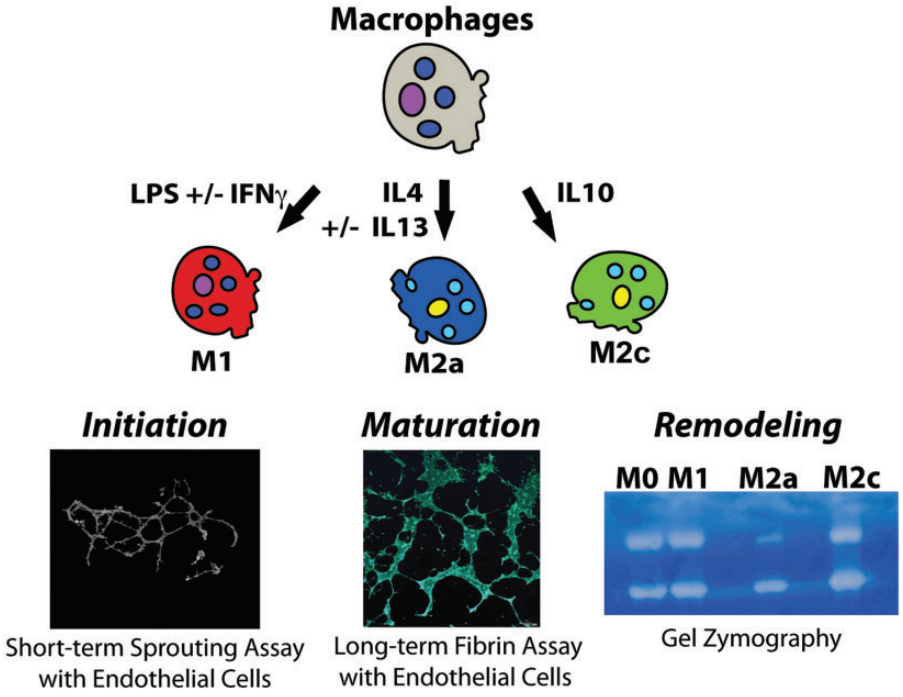

Figure 3 Role of macrophage phenotype in angiogenesis. M1 macrophages stimulate endothelial cell sprouting (Matrigel assay shown), M2a macrophages promote connection between adjacent sprouts (fibrin assay shown), and M2c macrophages may contribute to remodeling because they secrete high levels of MMPs (gel zymograph for MMP9 activity shown), but their roles in wound healing are still poorly understood. Images obtained with permission from Spiller et al.[16] (A color version of this figure is available in the online journal.)
LPS: lipopolysaccharide; IFNγ: interferon-gamma

promote endothelial cell sprouting in a Matrigel assay[16] (Figure 3). At later times after injury (four to seven days in mice), M2a macrophages secrete various anti-inflammatory cytokines like CCL18, CCL22, PDGFBB, and tissue inhibitor of metalloprotease 3.[16,55] M2a macrophages are anti-inflammatory and are associated with resolution of inflammation, cellular proliferation, stabilization of nascent blood vessels, and tissue maturation.[16,56] The sequential activities of M1 and M2a macrophages were demonstrated when endothelial cells were cultured on fibrin gel for one day in M1 macrophage-conditioned media followed by three days in M2a macrophage-conditioned media; they formed branching networks that were not observed in the presence of media from either phenotype alone[16] (Figure 3). However, perhaps because of their role in promoting cell proliferation and collagen deposition, M2a macrophages are also associated with fibrous encapsulation of biomaterials.[16,57] Indeed, delivery of the M2a-promoting factor IL4 exacerbates the foreign body response.[39] For more information on the contribution of M1 and M2a macrophages to tissue repair and regenerative medicine strategies, the reader is referred to Spiller et al.[58] and Nassiri et al.[59]

Another activation state of macrophages that is emerging as a major contributor to tissue repair and regeneration is the so-called M2c phenotype, which are stimulated with IL10 or glucocorticoids. M2c macrophages are characterized by the cell surface marker CD163, while M2a macrophages are characterized by CD206, at least for human macrophages.[16,60] M2a macrophages appear to promote blood vessel stabilization, while M2c macrophages appear to promote blood vessel sprouting and remodeling.[16] Recently, Spiller et al.[61] performed whole transcriptome analysis of human M0, M1, M2a, and M2c macrophages prepared *in vitro*. M2c macrophages expressed high levels of genes encoding potent extracellular matrix remodeling proteins such as matrix metalloprotease-7 (MMP7), MMP8, and MMP9, which was confirmed on the protein level.[61] Gel zymography showed that M2c macrophages secreted the highest levels of enzymatically active MMP9[16] (Figure 3). In addition, many genes associated with the M2c phenotype were found to increase at later times after injury (7–17 days) using data from human burn wounds.[61] Thus, the authors concluded that M2c macrophages play a major role in the later stages of wound healing that involve tissue remodeling,[61] although this role of M2c macrophages has yet to be definitively established. Future studies are needed to further understand the functions and the timing of M2c phenotype in tissue healing and regeneration.

The delivery of M1, M2a, or M2c macrophages into injured tissues has shed light on their distinct roles in tissue repair. Rybalko et al.[62] administered bone marrow-derived M1 macrophages (activated with inflammatory stimuli LPS and IFNg) to a tourniquet-induced ischemia/reperfusion muscle injury model. They showed 15% improvements in muscle tension and force 24 h after the activated macrophage infusion.[62] Moreover, they found that the delivery of activated macrophages accelerated myofiber repair and decreased fibrosis 14 days posttreatment.[62] Interestingly, the delivery of unactivated, or resting, macrophages impaired healing of the skeletal muscle.[62] Overall, their data indicate the importance of the initial inflammatory phase to improve healing outcomes, possibly by upregulating an earlier anti-inflammatory phase transition due to a more efficient clearance of apoptotic cells and higher vascularization mediated by activated macrophages.[62]

Wang et al.[32] used an immunocompromised mouse model of chronic inflammatory kidney disease to compare the effects of M1 (LPS stimulated) versus M2a (IL4 and IL13 stimulated) macrophages on renal injury. When injected at day 5 postinjury, macrophages of both phenotypes preferentially homed to the inflamed kidney.[32] Interestingly, the polarized macrophages maintained their phenotype *in vivo* until four weeks postinjection,[32] despite other studies that have shown that macrophages take on new phenotypes in response to their local microenvironment.[63] The administration of M1 macrophages led to an increase in secretion of pro-inflammatory cytokines and tissue damage.[32] In contrast, M2a macrophages exhibited a protective role, with a reduction in secretion of pro-inflammatory cytokines and reduced tissue damage, including tubular damage, interstitial volume, and glomerulosclerosis.[32] The precise mechanisms of how M2a macrophages protect tissue from damage are not known.

Lu et al.[48] showed that injecting *in vitro*-polarized murine M2c macrophages (stimulated with IL10 and TGFb) or M2a macrophages (stimulated with IL4 and IL13) to the same chronic inflammatory renal mouse model promoted different protective functions, though both M2a and M2c reduced CD4+ and CD8+ T cell infiltration and tissue inflammation.[48] M2c macrophages recruited regulatory T cells and caused a reduction in glomerulosclerosis, tubular atrophy, interstitial expansion, and proteinuria.[48] These findings highlight the important role of M2c macrophages in modulating the immune system and protecting against tissue damage. However, much is still unknown about the role of M2c macrophages *in vivo*.

In a study that highlighted the importance of timing of macrophage phenotype in wound repair, Jetten et al.[64] studied the effect of M2a or M2c macrophage administration in a cutaneous wound model in mice. They administered M2a (stimulated with IL4), M2c (stimulated with IL10), or unstimulated M0 macrophages immediately following cutaneous injury in both healthy and diabetic mice. While the macrophage treatment did not affect wound closure in healthy mice, administration of M2a or M2c macrophages resulted in significantly delayed wound closure relative to M0 macrophages or saline controls in diabetic mice.[64] The authors attributed this finding to a failure to allow an early inflammatory (M1) phase to occur prior to the administration of the macrophages, as had been done in the aforementioned studies of renal injury.[32,48] Clearly, more research is required to gain a better understanding of the optimal timing for their administration and their therapeutic functions in different tissues.

Delivery of cytokines to modulate phenotype of endogenous macrophages

Because of the importance of distinct macrophage phenotypes in tissue regeneration, biomaterials that recruit-specific

phenotypes of recruited macrophages, or that promote polarization of recruited macrophages, would be highly advantageous in regenerative medicine strategies. Awojoodu et al.[65] investigated drug delivery from thin films based on poly(lactic-co-glycolic acid) (PLGA) to control recruitment of macrophages to the site of an ischemic injury to promote vascularization. They showed that levels of MCP-1 peaked immediately after implantation and then decreased, while levels of stromal-derived factor-1-alpha (SDF-1a) peaked at three to seven days following implantation of the films.[65] Simultaneously, the level of M1 macrophages increased at early times after injury followed by peaks in the levels of M2 macrophages, as indicated by CD206 expression. Importantly, the initial inflammatory phase was coupled with high tissue angiogenesis.[65] The controlled release of FTY720, an agonist for sphingosine 1-phosphate receptor 3 ($S1P_3$), from the PLGA films further promoted microvascular stabilization and arteriogenesis via recruitment of M2 macrophages in response to endothelial cell-derived SDF-1a.[65] Similarly, Kreiger et al.[66] used PEG-DA hydrogels functionalized with desulfated heparin to create a localized gradient of SDF-1a, a heparin-binding cytokine. Release of SDF-1a was sustained over three days *in vitro* and preferentially recruited M2 macrophages via the CXCR4 receptor in a dorsal skinfold chamber in mice, which in turn promoted the stabilization of microvascular networks.[66]

Kim and Tabata[67] examined the effects of co-delivering SDF1 and an S1P1 agonist (SEW2871) from micelles within gelatin hydrogels on macrophage and mesenchymal stem cell (MSC) recruitment during cutaneous wound healing in healthy mice.[67] The release of SEW2871 increased recruitment of M1 and M2 macrophages at both one and three days after injury, while the combination of SDF1 and SEW2871 shifted the balance toward M2 macrophages.[67] The dual delivery of both chemokines accelerated wound closure, but because this strategy increased total macrophage recruitment as well as MSC recruitment, it is difficult to determine the mechanism of action.

Drug delivery methods can also be utilized to generate the desired macrophage phenotype from recruited monocytes/macrophages. Mokarram et al.[68] demonstrated that local delivery of cytokines can modulate macrophage phenotype and their effects on Schwann cell migration and axonal growth in a critically sized (15 mm) sciatic nerve gap model in rats. They implanted polysulfone nerve-bridging tubes filled with 0.7% agarose hydrogel mixed with either IFNg to promote the M1 phenotype or IL4 for M2a, or an unloaded control.[68] This drug delivery platform demonstrated release via diffusion over 24 h for both IFNg and IL4 *in vitro*.[68] Three weeks after implantation, an increase was observed in the total number of macrophages in both treatment groups relative to the control, but there was no difference between constructs releasing IFNg or IL4.[68] They found a significant increase in macrophages staining for CCR7 (an M1 marker) in the IFNg-treated group, and an increase in CD206 (M2 marker) in the IL4-treated group, confirming polarization of recruited macrophages via released cytokines.[68] The release of IL4 significantly enhanced Schwann cell infiltration and axonal growth three weeks postimplantation, while the release of IFNg had no effect.[68] In fact, the ratio of CD206+ to CCR7+ cells was directly proportional to the number of regenerated axons at the distal end of the nerve scaffold.[68] Thus, it appears that delivery of IL4 modulated the phenotype of the recruited macrophages to an M2 phenotype, with beneficial effects on neuronal regeneration.

Spiller et al.[71] examined the effects on scaffold vascularization of rapidly delivering IFNg followed by sustained IL4 to promote sequential M1-to-M2 macrophage activation. Using decellularized bone scaffolds as the drug delivery vehicle, IFNg was physically adsorbed to the scaffolds for rapid release (within 24 h), while IL4 was conjugated to the scaffolds using biotin–streptavidin interactions for a slower release profile (one to six days).[71] *In vitro* culture of primary human macrophages seeded on these scaffolds confirmed M1 and M2a polarization by scaffold release of IFNg and IL4 and showed that macrophages can simultaneously upregulate M1- and M2a-associated genes in the scaffolds that released both IFNg and IL4.[71] After two weeks of subcutaneous implantation in mice, scaffolds that released IFNg alone caused an approximately eightfold increase in the number of blood vessels compared to unmodified scaffolds. Considering that IFNg has been shown to inhibit endothelial cell sprouting *in vitro*,[69,70] the authors concluded that early activation of M1 macrophages enhanced scaffold vascularization. The release of IL4 alone or in combination with IFNg did not have a significant effect on scaffold vascularization, possibly because of overlapping M1 and M2a phases.[71]

Despite the importance of timing of macrophage activation, most biomaterials designs have not yet incorporated temporal control over macrophage behavior, in large part, because the optimal timing of macrophage activation has not been established. Several drug delivery strategies have been developed that will be useful in increasing our understanding of how the timing of macrophage activation affects biomaterial vascularization and tissue regeneration. For example, microdialysis tubing might be used to control the timing of cytokine infusion into the site of a wound. Keeler et al.[72] have used this strategy to deliver dexamethasone, which promotes M2c polarization and reduces fibrous encapsulation. Similarly, osmotic pumps have also been used to deliver IL4 to modulate M2a polarization and mitigate orthopedic implant wear particle-associated inflammation.[73] As another example, Reeves et al.[74] designed silk films with embedded IFNg or IL4 to promote M1 or M2a polarization, respectively, following macrophage-mediated release of the cytokines via phagocytosis. These films might be used in biomaterials coatings so that the release of macrophage-modulating cytokines is delayed until direct contact with macrophages.

Delivery of microparticles and nanoparticles to control macrophages

Macrophages are called ''professional'' phagocytic cells because they engulf cellular debris and foreign bodies via phagocytosis, macropinocytosis, or receptor-mediated endocytosis.[75,76] Similarly, microparticles and nanoparticles

designed for drug delivery purposes also are readily phagocytosed by macrophages,[77] a fact that may allow them to be exploited as vehicles to naturally target macrophages.[75] For example, the adsorption of the anti-inflammatory drug dexamethasone onto nanodiamond particles inhibited M1 activation of murine macrophages *in vitro*,[78] although phagocytosis was not evaluated. Controlling surface charge,[79] hydrophobicity,[80] and particle geometry and shape of nanoparticles also impacts their uptake by macrophages.[81,82] For example, macrophages upregulated gene expression of IL8 and IL10 following phagocytosis of gold nanorods displaying the charged molecule cetyltrimethylammoniumbromide.[83]

Using nanoparticles to target and control macrophages holds major therapeutic potential. For example, Harel-Adar et al.[84] designed microparticles to target macrophages in cardiac tissue following myocardial infarction. They designed a new strategy to promote the M2 phenotype transition by mimicking the natural process of efferocytosis, in which phagocytosis of apoptotic cells triggers the M1-to-M2 transition of macrophages. The team designed the liposomes to present phosphatidylserine (PS), an apoptotic cell signal, to macrophages upon phagocytosis.[84] Rat macrophages were treated with cytochalasin B, which inhibits non-specific particle uptake but not PS-mediated endocytosis, to show that uptake of the PS-presenting liposomes mimicked the process of apoptotic cell uptake. Uptake of these particles *in vitro* caused decreased expression of the M1-related marker CD86 and increased expression of the M2-related markers CD206 and IL10 by macrophages. Injection of these particles into the site of myocardial infarction caused a reduction in infarct size and increase in tissue vascularization compared to liposomes that did not mimic apoptotic cells by presenting PS.[84] Thus, modifying microparticles and nanoparticles to induce a specific macrophage behavior would constitute a major advantage in regenerative medicine strategies.

Conclusion and future directions

Many studies have demonstrated the importance of macrophages in wound healing and tissue regeneration. Drug delivery systems that target these cells and modulate their behavior and recruitment have been shown to promote a desirable healing outcome. The high complexity and heterogeneity of macrophage behavior is essential to achieving successful tissue regeneration. A deeper understanding of macrophage behavior in response to different environmental stimuli, including biomaterials and delivered drugs, is critical to advance the design of therapeutics that harness macrophages response as powerful healing agents *in vivo*. In particular, future drug delivery strategies must incorporate consideration of the appropriate timing of desired macrophage behaviors, such as sequential M1, M2a, and M2c behavior. In addition, detailed understanding of multiple macrophage phenotypes, especially M2c, is still lacking. Finally, there is still a need to develop more targeted drug delivery systems to separate out the effects of delivered cytokines on macrophages from off-target effects on other cell types in the vicinity. For example, the M2a-promoting cytokine IL4 can also potently inhibit angiogenesis via direct actions on endothelial cells,[85] which could potentially counteract any pro-angiogenic effects of M2a macrophage polarization. IL4 also has been shown to have a major impact on fibroblast gene expression[86] and their differentiation into myofibroblasts.[87] Thus, drug delivery strategies that release macrophage-modulating cytokines without specifically targeting macrophages will likely have unintended side effects on other cell types.

Despite these challenges, the critical role of macrophages in tissue healing and regeneration warrants increased research into controlling their behavior for therapeutic benefit. The development of advanced drug delivery strategies to target, recruit, and modulate macrophages *in vivo* holds potential to promote tissue regeneration via the body's natural healing mechanisms.

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DECLARATION OF CONFLICTING INTERESTS

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