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METHODS TO MODULATE MACROPHAGE POLARIZATION IN TISSUE REGENERATION VIA BIOMATERIALS

Faculty of Medicine and Health Technology
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TIIVISTELMÄ

Lotta Hiihtola: Makrofagien polarisaation säätely kudosten uusiutumisessa biomateriaaleilla

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Immuunivastetta muokkaavat biomateriaalit ovat nousseet lupaavaksi uudeksi strategiaksi regeneratiivisen lääketieteen alalla auttaen muun muassa parantamaan kroonisia haavoja tai tuki- ja liikuntaelin vaurioita. Biomateriaaleilla kyetään muokkaamaan makrofagien fenotyyppiä ja sen muutoksista aiheutuvaa immuunireaktiota, joka on läsnä vahingoittuneiden kudosten uusiutumisessa ja siitä aiheutuvassa tulehdusreaktiossa.

Makrofagit ovat tärkeä osa ihmisen luonnollista immuniteettia toimien syöjäsoluina ja tasapainon ylläpitäjinä. Makrofageja esiintyy useissa eri kehon kudoksissa, joissa niillä on myös kudoksille ominaisia tehtäviä. Kyky säädellä immuunireaktiota on seurausta makrofagien välittämistä sytokiineista, jotka toimivat viestiaineina solujen välillä. Yleisesti makrofagit pystyvät muuttamaan fenotyyppiään mikroympäristönsä mukaan tulehdusta edistävän- ja tulehdusta rauhoittavan fenotyypin välillä. Tulehdusta rauhoittavan fenotyypin tiedetään myös edistävän kudosten uusiutumista.

Fenotyypin oikea-aikainen muutos on hyvin tärkeä kudosten uusiutumisen kannalta, ja ilman sitä vauriosta johtuva tulehdus voi kroonistua. Tulehdus on kuitenkin tärkeä osa kudoksen parantumista, joten sitä ei kannata kokonaan estää. Tämän lisäksi makrofagit ovat mukana muun muassa säätelemässä angiogeneesiä ja esisolujen aktivaatiota, ja siten ollen myös isossa roolissa kudosten uusiutumisessa.

Makrofagien polarisaatioon vaikuttavia tekijöitä on useita, esimerkiksi niiden tarttumapinnan fyysiset ja kemialliset ominaisuudet, joten kehon sisäisten siirrännäisten biomateriaalin ominaisuuksilla on merkitystä. Makrofagit kohtaavat kehoon joutuneen vierasesineen ensimmäisten solujen joukossa, pyrkien kiinnittymään ja fagosytosoimaan kyseisen vierasesineen. Tämän epäonnistuessa vierasesine pyritään eristämään kehosta vierasesinereaktion avulla. Biomateriaalien fyysiset ja kemialliset ominaisuudet vaikuttavat suuresti makrofagien kiinnittymiseen ja niiden ohjaamaan immuunivasteeseen. Bioaktiivisten tekijöiden hallitulla vapauttamisella on vielä mahdollista hienosäätää polarisaatiota. Näitä materiaalien ominaisuuksia hyödyntämällä voidaan ohjata oikeansuuntaista ja -aikaista makrofagien polarisaation muutosta.

Tässä kirjallisuuskatsauksessa tarkastellaan makrofagien fenotyypin polarisaation roolia kudosten uusiutumisessa ja miten tätä voidaan säädellä biomateriaalien kemiallisten ja fysikaalisten ominaisuuksien avulla. Kirjallisuuskatsauksessa tutustutaan myös erilaisiin biomateriaaleihin sisällytettäviin bioaktiivisiin tekijöihin ja niiden vaikutukseen kehon immuunivasteessa.

Avainsanat: Makrofagi, polarisaatio, biomateriaali, regeneratiivinen lääketiede

ABSTRACT

Lotta Hiihtola: Methods to modulate macrophage polarization in tissue regeneration via biomaterials

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Immunomodulation via biomaterials has become a promising strategy for regenerative medicine, helping to heal chronic wounds and assisting to guide the natural healing process. Particularly, modulation of macrophage phenotype via biomaterial properties has raised interest in recent years due to macrophages heterogenous phenotype and their involvement in tissue regeneration and inflammation.

Macrophages are an important part of the innate immune system as they act as first responders to inflammation. They can be found from numerous tissues, where they maintain the natural homeostasis by phagocytosing intrusive pathogens, apoptotic cells, and cell debris. In addition, macrophages secrete cytokines and chemokines thus regulating function and migration of surrounding cells. Generally, macrophages can switch their phenotype from pro-inflammatory M1 to anti-inflammatory M2 type, depending on the microenvironment. This change in phenotype is crucial for the proper tissue healing process and without it, chronic inflammation can take over. Besides inflammation, macrophages have shown for instance, to be involved in regulating angiogenesis and progenitor cell stimulation.

The polarization of macrophages is influenced by various factors, such as physical and chemical properties of the foreign body or cytokines in the microenvironment. Therefore, harnessing biomaterial physical and chemical properties for modulating macrophage polarization is a viable strategy. In addition, controlled release of bioactive substances can be utilized to fine-tune the reaction timing and magnitude.

This thesis introduces the role of macrophages in tissue regeneration and discusses the interaction of biomaterials with them. Moreover, the strategies for modulation of macrophage polarization are showcased with recent advances in biomaterial design. Also, the usage of bioactive substances in biomaterials for fine-tuning the macrophage respond is discussed.

Keywords: Macrophage, polarization, biomaterial, regenerative medicine

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1. INTRODUCTION

Macrophages are an important component of the innate immune system, and they can be found in numerous tissues in human bodies (Mao et al., 2022). Macrophages act as a first responders to inflammation alongside other immune cells such as neutrophils and they contribute immensely to tissue homeostasis and regeneration (Martin & García, 2021). They are an appealing target for biomaterial-directed regenerative medicine approaches considering their role in tissue regeneration and their ability to steer the healing process into right direction (Kharaziha et al., 2021; Martin & García, 2021).

Biomaterial-directed regenerative medicine approaches could solve and help multitude of different medical problems. One of these problems in the clinic are musculoskeletal injuries, some which still lack effective treatment, or the patient is having to face many disadvantages such as complications, rejection and infection with existing treatments. (Mao et al., 2022) In addition, humans endogenous repairing ability decreases with age and some pathological conditions such as diabetes. In diabetic injuries, the injury process has difficulties to shift away from the initial inflammation state, which is why modulating macrophage polarizations has shown promising results to treat these kinds of injuries. (Mao et al., 2022; Z. Wu et al., 2022)

This thesis begins by introducing the concept of macrophage polarization and function in tissue regeneration processes in various tissues. It will then go on to explain biomaterial-macrophage interaction and its complications. The remaining part of the thesis will discuss the biomaterial related strategies to modulate macrophage polarization in order to regulate tissue regeneration and what will the future prospects be for these applications.

2. MACROPHAGE POLARIZATION

Macrophages can be derived from different origins. Tissue-resident macrophages originate from embryonic yolk sac and fetal liver progenitors whereas monocyte-derived macrophages are derived from bone marrow progenitors. Monocytes migrate to tissues through the circulatory system from blood, bone marrow or spleen. Depending on their origin and environment, macrophages phenotypes can vary greatly and their plastic nature allows them to possess diverse roles as key regulators in complex processes. (Martin & García, 2021) Since their phenotype is difficult to define, some approaches of classification have been developed to group similar of phenotypes to-

gether. In a simplified manner, macrophages could be grouped in to classically activated pro-inflammatory M1 type and alternatively activated anti-inflammatory pro-regenerative M2 type, referring to activated T helper cell phenotypes type 1 (Th1) and type 2 (TH2). (Liu & Segura, 2020; Mao et al., 2022) Characterization of these phenotypes is done in vitro and in the intricate in vivo environment the M1 and M2 types are just two extremes of the entire spectrum of phenotypes in between them. This can be seen as co-expression of M1 and M2 markers in vivo. (Ye et al., 2021) The M2 type can then be further divided into at least 4 subtypes (M2a, M2b, M2c and M2d) (Martin & García, 2021). From these 4 subtypes, M2a and M2c are most prominent in tissue regeneration and fibrotic processes although the characterization model might still be an oversimplification of the actual in vivo phenotypes (Kharaziha et al., 2021; Martin & García, 2021). The spectrum of phenotypes is presented in figure 1. In this thesis, for the sake of clarity, the bipolar nomenclature M1/M2 will be mostly used from here on as it is commonly utilized practice.

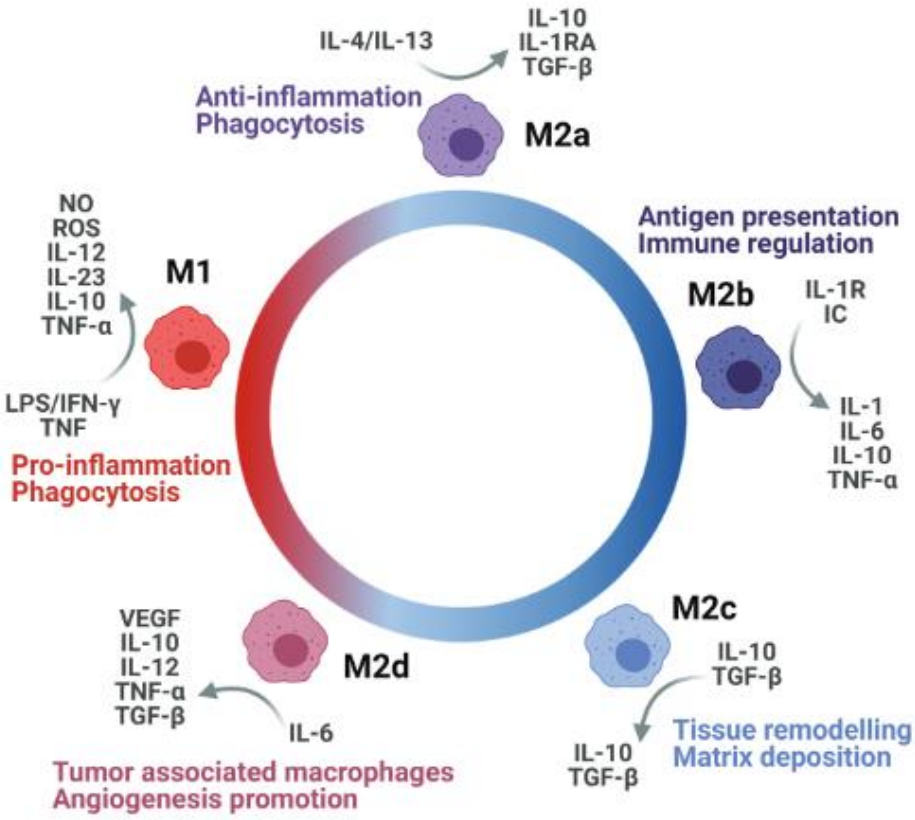

Figure 1. *Presentation of the macrophage phenotype and its fluidity between the different subtypes (Antmen et al., 2021).*

In vitro experiments have been used to determine the activating factors of these different phenotypes and as a result it is recognized that the M1 macrophages are induced by danger signals such as danger-associated patterns (DAMPs), pathogen-associated molecular patterns (PAMPs) and cytokines including TNF-α and IFN-γ. Likewise M2 macrophages are induced by cytokines such as Interleukin -10 (IL-10), IL-4, IL-13 and IL-33, in addition M2 phenotype can be stimulated by hormones and immune complexes. (Shen et al., 2021; Ye et al., 2021) As described earlier, M1 macrophages express pro-inflammatory phenotype that induces the inflammatory state by releasing oxidative metabolites, antimicrobial peptides, cytokines such as IL-6, IL-12 and tumor necrosis factor α (TNFα). In addition to those, M1 macrophages work as phagocytic cells and as antigen presenting cells further inducing the type 1 immune reaction through activation of T helper 1 cells. On the contrary, M2 macrophages express an anti-inflammatory phenotype that promotes tissue regeneration by secreting anti-inflammatory cytokines and growth factors, such as IL-10, Platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and Transforming growth factor β (TGF- β). These anti-inflammatory mediators stabilize angiogenesis, activate progenitor cell ingrowth and proliferation, and promotes extra cellular matrix (ECM) construction therefore assisting tissue regeneration processes. (Kharaziha et al., 2021; Martin & García, 2021)

3. THE ROLE OF MACROPHAGES IN TISSUE REGENERATION

One of macrophages many roles is participating in tissue regeneration and repair amongst other immune cells in the tissue. Macrophages are known to be scavenger cells that contribute as a first defence of innate immunity by phagocytizing and destroying intrusive pathogens, apoptotic cells, and cellular debris (Martin & García, 2021). Additionally, they are crucial to tissue repair by releasing numerous cytokines and chemokines thus attracting and regulating other cells (Lee et al., 2018). An overview of the regression of tissue regeneration process and macrophage polarization is described in figure 2. However, functions of macrophages are diverse and depending on the tissue macrophages could act differently.

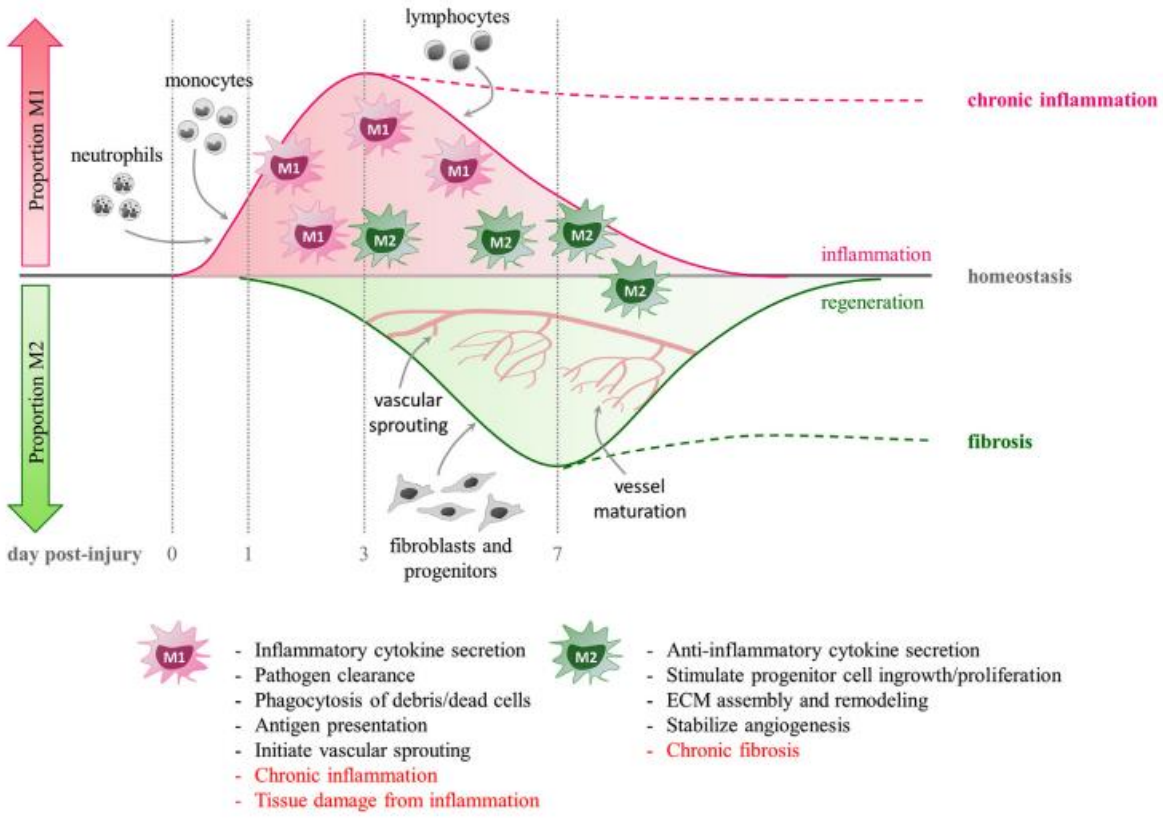

Figure 2. *Timeline of tissue regeneration processes and macrophage polarization following tissue injury (Martin & García, 2021).*

Both, tissue resident macrophages and monocyte-derived macrophages contribute to tissue regeneration, however monocyte-derived macrophages takeover after the initial inflammatory response. Nonetheless, tissue-resident macrophages aid to initiate the local inflammatory response by recognition of the danger signals such as DAMPs within minutes of injury thus polarizing into M1 macrophages. Local inflammatory cells, including macrophages, secrete inflammatory chemokines and cytokines such as monocyte chemoattractant protein-1 (MCP1), TNF-α, and IFN-γ that attract circulating monocytes to the damage site where they acquire M1 phenotype hence further intensifying inflammation response. (Liu & Segura, 2020; Shen et al., 2021) As stated earlier, macrophages are responsible of phagocyting pathogens and dead cells. This function is carried through by M1 macrophages that also function as potent antigen-presenting cells that help to activate Th1 cells and therefore induce the inflammatory response (Mao et al., 2022; Martin & García, 2021).

M1 phenotype is predominant in the acute inflammation state however the prevalent phenotype starts to shift towards M2 around 3-4 days after initial injury (Lee et al., 2018; Martin & García, 2021). M2 macrophages steer adaptive immune system by interacting with Th2 and regulatory T

cells helping to inhibit the inflammation (Martin & García, 2021). The shift towards M2 phenotype aids to resolve the inflammatory environment and to initiate reparative state by secreting anti-inflammatory cytokines and growth factors that guide other cells to differentiate at the damage site for instance due to these factors fibroblasts acquire more reparative phenotype and deposit ECM. (Martin & García, 2021; Shanley et al., 2021) At the reparative state, M2 macrophages are crucial to tissue angiogenesis, ECM assembly and remodelling and stimulating progenitor cells to proliferate.

Angiogenesis is a necessary process in correct tissue regeneration of various tissues and macrophages have vital role in the regulation of this process. Already in early stages of tissue repair, M1 macrophages release high levels of vascular endothelial growth factor (VEGF) which is pro-angiogenic and promotes vasculogenesis. M1 macrophages regulate endothelial cell differentiation into the tip cell phenotype, which is associated with early vascular sprouting as well as regulating endothelial cell migration and proliferation. This means that angiogenesis initiates already in the inflammation phase and requires M1 macrophages. In a later phase of tissue repair, M2 macrophages partake in the maturation and maintenance of the newly formed blood vessels. They secrete high amounts of PDGF-BB, which aids to guide pericytes and mesenchymal stem cells (MSC) to the damage site thus stabilizing the vaculature. In addition, M2 macrophages interact with endothelial cells by promoting gene expression related to pericyte cell and smooth muscle cell differentiation. This is associated with late stages of vascular maturation. (Mao et al., 2022; Martin & García, 2021)

In a more specific manner, macrophages are crucial part of regulation of wound healing. The principal of this is the same as described above, M1 macrophages dominate in the initial inflammation stage and subsequently M2 macrophages dominate in the later stages of regeneration. In the regeneration stage of the wound healing, fibroblasts, keratinocytes, and endothelial cells are recruited to the wound site by various factors. By secreting TGF-β, macrophages are able to promote collagen release from myofibroblasts thus strengthening the healing tissue to the level of healthy tissue. (Mao et al., 2022)

Apart from wounds, macrophages possess complex roles also repairing musculoskeletal system injuries such as cartilage, tendon/ligament, muscle, and bone injuries. As with wounds, macrophages main processes stay the same in musculoskeletal tissue regeneration although with some nuances. In cartilage, macrophages are located in the synovial lining of the joints. Ye et al. states in their review that M2 macrophages are able to upregulate the release of TGF-β and insulin-like growth factor (IGF) thus stimulating cartilage regeneration and synthesis of glycosamino-

glycans and collagen type II. In the case of tendons and ligaments, the precise roles of macrophage phenotypes are yet to be specified, however studies show that there are different functions between phenotypes. Muscle tissue is dependent on myogenic precursor cells (MPCs), which differentiation and proliferation is regulated by M1 macrophages by releasing IFN-γ and tumor necrose factor (TNF) in the initial inflammation phase. M2 macrophages play an important role in muscle differentiation and growth. (Ye et al., 2021) Likewise, in bone tissue regeneration, macrophages steer the healing process by releasing cytokines, which regulate inflammation, differentiation of MSCs and osteoblast function (Lee et al., 2018). Acute inflammation occurs in the early stages of bone repair, initiating tissue regeneration and therefore duration and timing of the M1 inflammation state are crucial to bone repair. Too early inhibition of the inflammatory state, can lead to incorrect bone regeneration outcome. (Martin & García, 2021) In summary, macrophages are involved in musculoskeletal tissue regeneration in various ways.

Following traumatic peripheral nerve injury (PNI) and central nerve system (CNS) injury, macrophages take part in neural regeneration. The main processes behind the regeneration are the same as in any tissue regeneration process. After initial injury, macrophages and monocytes are being recruited to the damage site where tissue-resident macrophages and monocyte-derived macrophages acquire M1 phenotype and start inducing the inflammation response. Furthermore, M1 macrophages possesses the vital role of phagocytosing debris and promoting angiogenesis at the neural injury site. The polarization of M1 macrophages is activated by DAMPs in PNI and by microglia secreted pro-inflammatory cytokines in CNS injury however microglia are therefore activated by DAMPs. While in PNI, macrophages work closely with Schwann cells, in CNS injury macrophages collaborate with astrocytes as well as other cells near injury. After acute inflammation state, macrophages start acquiring the M2 phenotype, which is crucial to the tissue repair state. M2 macrophages in CNS injury are also in charge of degradation of chondroitin sulphate proteoglycan (CSPG) by releasing metalloproteases. (Dervan et al., 2021) The fate of neural stem cells (NSCs) can be regulated by different macrophage phenotypes. While cultured in vitro with M1 macrophage conditional serum, NSCs differentiated into astrocytes. In contrast, when cultured with M2 type of macrophage conditional serum, NSCs differentiated into neurons or oligodendrocytes. (Xiao et al., 2021)

Because M1 and M2 macrophages have such distinctive roles in tissue regeneration, the switch between phenotypes needs to happen in a highly regulated manner. Likewise, the return to homeostatic state needs to follow the regeneration phase in a correct time. Despite the fact that inflammation is often seen as harmful, too early resolution of the initial inflammation can lead to in-

complete repair of the tissue, for instance M1 macrophage mediated inflammation is necessary for the fracture repair and too early swich to M2 phenotype can cause improper bone repair. Moreover, too quickly resolved M1 phase, can cause too weak inflammation response hence causing pathogens to stay at the injury site without proper phagocytotic cleaning. (Martin & García, 2021; Ye et al., 2021) It is also good to take in account, that M1 macrophages partake in the initiation of angiogenesis, which is essential for tissue regeneration (Mao et al., 2022). However, unresolved M1 phase can cause chronic inflammation and therefore lead to harmful conditions such as fibrosis or cancer (Mao et al., 2022). Depending on the host, injured tissue, and the severity of the injury, strength of the inflammation and the duration of immune responses can deviate from each other. For instance, in liver tissues M2 macrophages can take part in resolving fibrosis, while also can influence or lead to increased fibrosis. In this instance, timing of the M2 response seems to be a determining factor, if the soft tissue injury is able to go through tissue regeneration and follow up with stable homeostatic state. If this timing is somehow disturbed, the injury might result in chronic fibrosis. (Martin & García, 2021)

4. MACROPHAGE POLARIZATION VIA BIOMATERIALS

Biomaterials are broadly used in tissue engineering, since they can be utilized in variety of treatments and applications. For instance, they can be designed to possess the ability to provide mechanical support or have the ability to deliver therapeutic molecules to the injury site. (Lee et al., 2018) Particularly immunomodulatory biomaterials have gained interest in recent years in the field of regenerative medicine and instead of just trying to suppress the immune response, now biomaterials are designed to interact and steer the immune response to be as desired (Martin & García, 2021). Macrophages make an intriguing target for immunomodulation because of their involvement in tissue regeneration and foreign body response (FBR), which is one of the common complications of implanted biomaterials (Lee et al., 2018; Liu & Segura, 2020). The inflammatory response following biomaterial implantation can be described as a “double-edged sword” due to the fact that it can be beneficial and create an optimal microenvironment for tissue regeneration, but the immune response can also go in to the opposite direction and cause harmful conditions such as chronic inflammation and formation of fibrous capsules (Ye et al., 2021). It is possible to modulate macrophage polarization by controlling the microenvironmental cues through bio-

material properties which can be roughly divided to biological, physical, and chemical properties (Lee et al., 2018). In addition, material composition and additional cues can affect macrophage polarization (Shen et al., 2021).

Physical properties such as stiffness, topography, pore size, and particle size can regulate macrophage polarization. It is still unclear as to, what are the molecular mechanisms behind some physical cues, such as stiffness of the material, although recent evidence suggest that softer materials induce macrophage polarization to M2 phenotype while stiffer materials led to FBR. Additionally, it is observed that surface topography affects the adhesion, extension, spreading, and movement of the macrophages therefore regulating the polarization process, for instance macrophages tend to acquire M2 phenotype when elongated utilizing surface topography. (Mao et al., 2022) Pore size is widely studied material property, although the optimal pore sizes for macrophage polarization is yet to be determined. However, studies have shown that by decreasing the pore size, macrophages acquire more likely anti-inflammatory M2 phenotype due to limited actin polymerization. This results in decreased nuclear translocation of myocardin-related transcription factor-A, which is actin dependent transcription factor that affects the inflammatory response. (Mao et al., 2022; Shen et al., 2021)

Alongside of physical cues such as surface topography, surface chemistry is an important factor in design of macrophage focused immunomodulating biomaterials. (Liu & Segura, 2020) For example, it can affect protein absorption to the surface. Immediately after the biomaterial implantation, proteins from blood and intestinal fluids grip to the surface and possibly undergo conformational changes to expose domains that can be recognised by immune cells. This induces the inflammatory response and activates phagocytosis. (Ye et al., 2021)

Biomaterial-based delivery systems are used to fine-tune the immune response of the material and further regulate macrophage polarization. Biomaterials can be used to deliver cytokines, growth factors, anti-inflammatory drugs and genes to the injury site to control for instance the time or duration of M1/M2 switch. These delivery systems are delicate processes that need to be taken in consideration the complex microenvironment and macrophage functions in vivo. Some nanoparticles have been used as carriers for drug deliveries to the target area due to their colloidal stability. (Ye et al., 2021)

4.1 Role of macrophages in foreign body response

FBR is known to be one of the most common complications following biomaterial implantation. In FBR, the implanted biomaterial gets encapsulated by fibrotic tissues which leads to improper integration of biomaterial. It is a host response to the foreign material that is implanted into the body and it is highly regulated by macrophages that supervise the inflammation response. (Liu & Segura, 2020) Progression of the innate immune response following the implantation of biologically inert biomaterial is demonstrated in figure 3. As demonstrated in the figure 3, the initial protein absorption response happens within minutes of implantation (Sridharan et al., 2015). In the early stages of the host response, neutrophils dominate the implantation site, but after 1 hour circulating monocytes start to migrate to the implantation site hence becoming the primary cell type after 2 days (Martin & García, 2021; Sridharan et al., 2015). Their primary function in the implantation site is to eliminate debris and external material through phagocytosis, rearrange the local matrix by secreting enzymes, and releasing substances to regulate surrounding cells such as fibroblasts (Liu & Segura, 2020). Once adhered to the surface of the biomaterial, macrophages seek to degrade the foreign material by fusing into foreign body giant cells, which are large cells that retain multinucleated phenotype. Foreign body giant cells aim to engulf the biomaterial and therefore destroy it, but since they are not able to reach around it, they try to degrade the biomaterial by releasing reactive oxygen species and enzymes. This response is called frustrated phagocytosis. Since most of the biomaterials implanted cannot be fully degraded by macrophages, they recruit fibroblasts to form a wall of collagen layers around the biomaterial that assemble a fibrotic capsule. (Liu & Segura, 2020; Martin & García, 2021) This happens in a range of 5 to 28 days post implantation, when fibroblasts start to migrate near the biomaterial. (Martin & García, 2021; Sridharan et al., 2015)

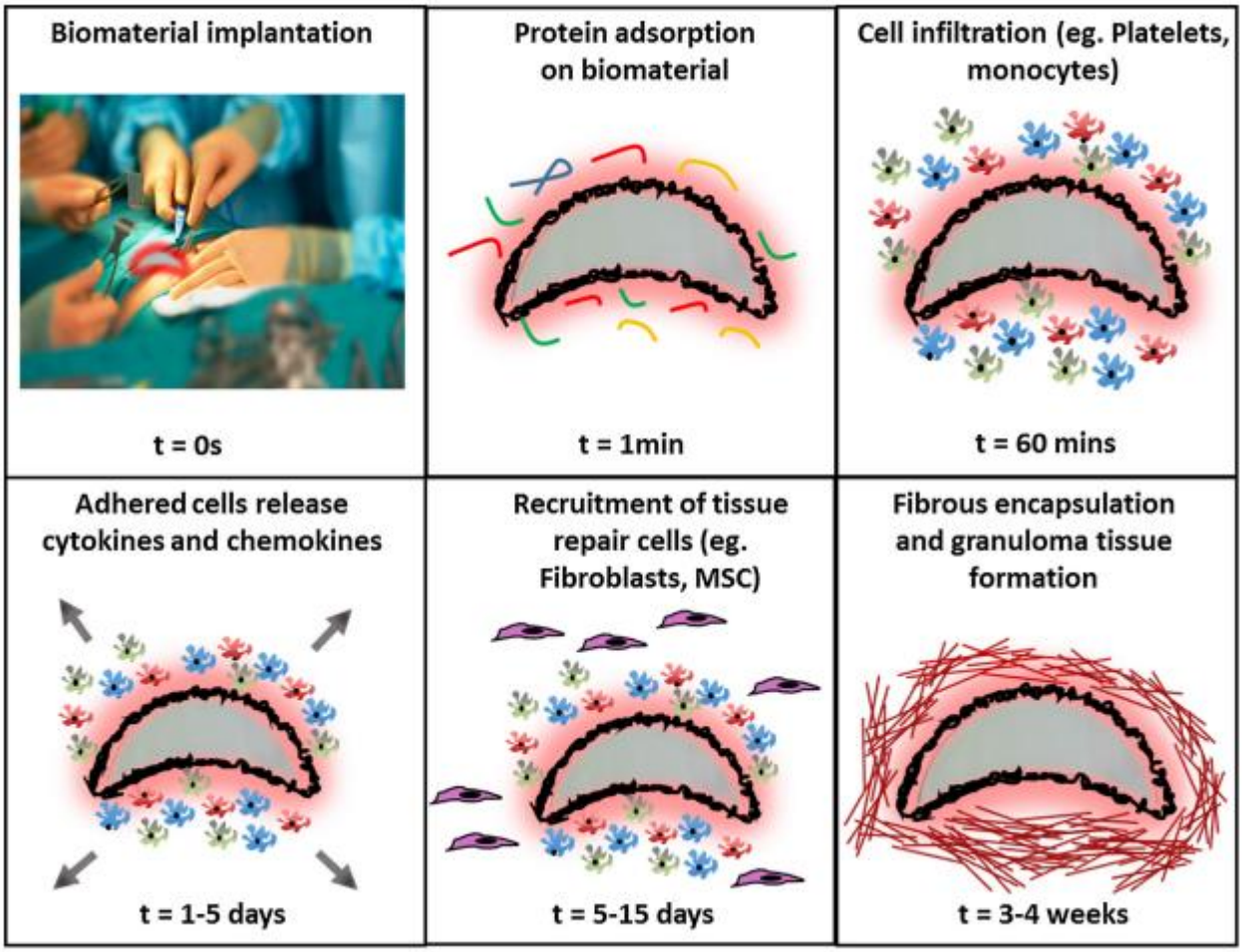

Figure 3. *The progression of innate immune reaction to implantation of biologically inert biomaterial (Sridharan et al., 2015).*

Macrophage polarization has been studied in various immune responses and FBR is not an exception, although the research results are conflicted on this issue. Studies have connected cytokines such as PDGF, TGF-β that are commonly secreted by M2 macrophages to the formation of FBR. In contrast cytokines and chemokines such as TNF-α and MCP-1 have been connected to be part of the same response, linking both phenotypes to FBR. This can be result of two different scenarios, one being that both phenotypes are present near the biomaterial, and both contribute to the process of FBR. On the other hand, since macrophage phenotype is fluid, it could be possible to have macrophages expressing both M1 and M2 phenotype markers and signalling molecules. (Liu & Segura, 2020) Alternatively, some studies have demonstrated M1 being the dominate phenotype by reducing the activation of M1 macrophages and rising the M2/M1 ratio which resulted in declined formation of fibrosis and foreign body giant cells. (Martin & García, 2021)

5. BIOMATERIAL BASED STRATEGIES

Macrophage polarization is crucial for healthy tissue regeneration, and therefore controlled macrophage function can be used to promote regeneration of various tissues. This can be executed via immunomodulatory biomaterials since they can provide structural support to the healing tissue o they can be utilized to deliver pro-regenerative substances such as drugs and cytokines to the damage site. (Lee et al., 2018) This chapter will give an overview to the recent strategies to modulate macrophage polarization via biomaterials to promote tissue regeneration and reduce negative complications such as FBR, fibrosis and chronic inflammation. The extent of different strategies that can be utilized is pictured in figure 3. As introduced in figure 3, biomaterials can also be combined with external forces, however it exceeds the focus of this thesis.

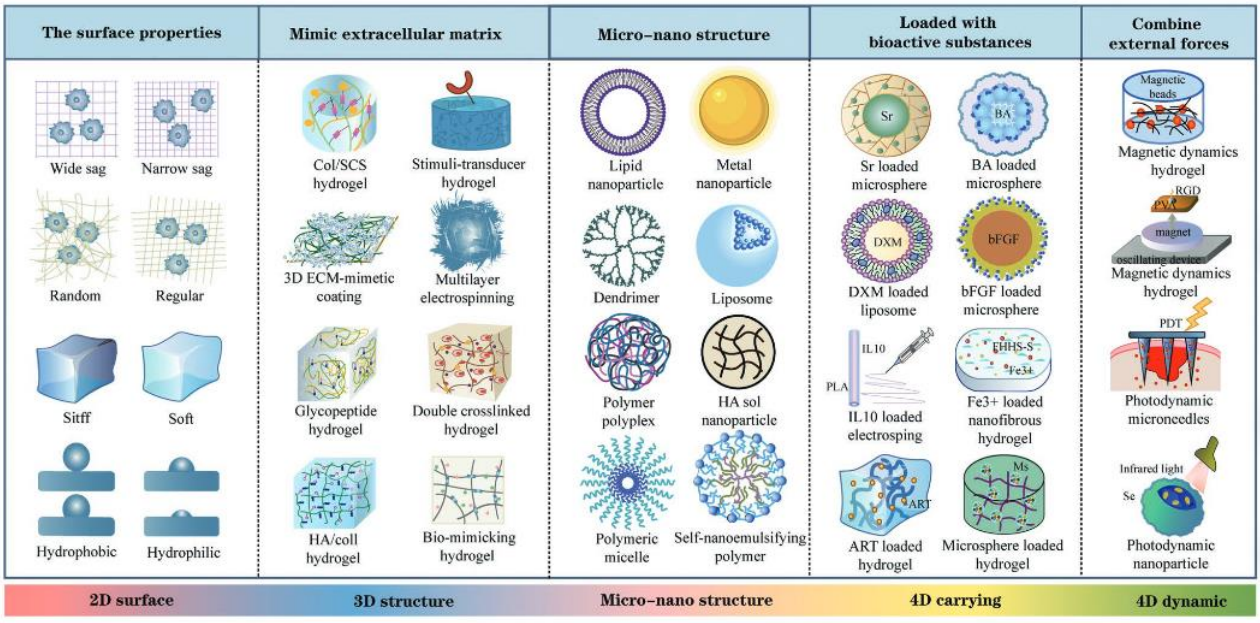

Figure 4. *Schematic picture of the range of strategies utilized in biomaterial induced regulation of macrophage polarization (Mao et al., 2022).*

5.1 Strategies utilizing physical and chemical properties

Extensive research has shown that chemical and physical properties of biomaterials are valuable tools in modulating macrophage polarization. The initial immune response is dependent on the

surface properties of the biomaterial, because once the biomaterial has been implanted, wide range of proteins such as fibrinogen, vitronectin, fibronectin and complement proteins flow through fractured blood vessels and adsorb to the surface of the biomaterial which are accompanied by macrophages that adhere to the surface. Protein adsorption is highly dependent on the surface properties such as wettability, stiffness and surface chemistry while also affecting macrophage polarization. (Ye et al., 2021)

It is an important distinction whether biomaterial is hydrophobic or hydrophilic since wettability of material can affect integration to the host. It has been demonstrated that hydrophobic implants induce M1 macrophage phenotype and the release of pro-inflammatory proteins in vitro whereas hydrophilic biomaterials have shown to favour anti-inflammatory M2 phenotype (Mao et al., 2022). There are variety of strategies to make surfaces more hydrophilic in order to promote pro-regenerative phenotype in macrophages. For instance, plasma modification and hydrolysis are available methods for making surface hydrophilic. (Lee et al., 2018). Also, studies have shown that hydrogels are suitable for keeping the wound site moist by improving hydrophilicity, therefore promoting macrophage polarization to M2 in naturally diagnosed diabetic mice (Mao et al., 2022). Chitosan-heparan sulfate multilayer coating can also be used in order to polarize macrophages into M2 phenotype because of its high wettability properties (Z. Li & Bratlie, 2021). The effect of wettability on macrophage polarization can be related to the integrin interactions with the surface, phagocytosis, and regulation of activation (Mao et al., 2022).

Recent studies have gathered that surface topography and more specifically surface roughness has a huge impact on macrophage polarization by influencing the adhesion, motility, diffusion, and elongation of macrophages (Mao et al., 2022). One approach on utilizing topography is to create micropatterns such as microgrooves on the surface of the biomaterial. These microgrooves affect the morphology and polarization of macrophages by elongating them and thus guiding the macrophages to polarize into M2 phenotype. (Dervan et al., 2021; Mao et al., 2022) It has been demonstrated that the influence of these grooves on M2 polarization peaked on titanium surface when the grooves were 400-500 nm wide, however wider grooves than this induced M1 macrophage polarization while narrower grooves induced M2 polarization (Mao et al., 2022).

In addition to surface microgrooves, the arrangement and orientation of nanofibers have been shown to guide macrophage polarization through morphology changes of macrophages. Study of electrospun poly(l-lactide-ε-caprolactone) (P(LLA-CL)) nanofibers established the effect of nanofiber arrangement on macrophage polarization by comparing randomly and neatly oriented nanofibers on nerve guidance conduit. Neatly arranged nanofiber scaffolds increased significantly M2

polarization while randomly oriented nanofibers induced more M1 phenotype. P(LLA-CL) could be used in applications focused on peripheral nerve regeneration. (Mao et al., 2022) Recent studies have shown a common interest in polymeric electrospun nanofibers as implanted biomaterials resulting in variety of materials studied. In fact, correspondingly Xiao et al. utilized electrospun poly(L-lactide)(PLLA) nanofibers in their research hence PLLA has been proven to have good biocompatibility and is easy to process. It was shown that neatly aligned nanofibers induced M2 polarization as expected. (Xiao et al., 2021). With similar results Pisani et al. studied the interaction of electrospun biomaterials and macrophages by using the block copolymer poly-L-lactide-co-poly-ε-caprolactone (PLA-PCL) of 70:30 ratio in vitro. (Pisani et al., 2021) Although electrospun nanofibers have shown significant results, there are other methods to produce oriented scaffolds, for instance, lithographic methods, self-assembly, phase-induced separation and mold compression (Xiao et al., 2021).

Morphological modulation of macrophage phenotype can also be exploited by designing materials with different porosity and pore size. Although this property has been widely studied, there are a few contradicting results about the issue and the optimal pore size is yet to be determined. In a recent study, it was demonstrated that pores of 40μm in size promoted M2 polarization the most, being the smallest scaffold pore size in the study. This indicated that the smaller pore sizes induce more anti-inflammatory state by elongating macrophage cell shape. (Mao et al., 2022) On the contrary, other research have shown that in poly(2-hydroxyethyl methacrylate) (pHEMA) and poly(methyl methacrylate) (PMMA) scaffolds pores in size 34μm had higher expression of M1 related markers such as iNOS and IL-1R1 in vivo (Lee et al., 2018). Still, it has been also reported that biomaterials with pore size of 160μm stimulated M2 polarization in macrophages (Dervan et al., 2021). More research needs to be conducted on this matter to specify the optimal parameters for tissue regeneration in varying tissues.

In addition to previous properties, material stiffness has become an attractive target for research of medical biomaterial applications. Softer materials induce more anti-inflammatory M2 phenotype in macrophages whereas harder substrates lack of this function. This was done by investigating collagen-coated poly-acrylamide gels with varying stiffness noticing that for the softer gels, TNF-α expression decreased and the expression of IL-10 increased, indicating M2 polarization. (Mao et al., 2022) Gels composed of zwitterionic polymethacrylic acid sulfobetaine (poly-SBMA) hydrogels were studied with varying cross-linking agent contents and the results demonstrated that having softer and more viscous properties, effect macrophage polarization pro-regeneratively

(Mao et al., 2022). Varying strategies can be utilized to modify stiffness of the material for instance additional hydrogel cross-linking increases stiffness of the hydrogel. (Liu & Segura, 2020)

Crosslinking can be also utilized to connect substances together in order to modulate macrophage phenotype (Kharaziha et al., 2021). Crosslinking can be divided into physical and chemical crosslinking with the distinction that chemical crosslinking is connected to covalent interactions and physical crosslinking is related to hydrogen bonding, self-assembly and electrostatic interactions of macromolecules (Kharaziha et al., 2021). Crosslinking can be used for instance to encapsulate cells and biomolecules sufficiently into 3D hydrogels by in situ chemical crosslinking by incorporating short ultra violet light or visible light radiation (Kharaziha et al., 2021). There are wide range of cross-linking agents that can be used, for instance formaldehyde, hexamethylene diisocyanate, glutaraldehyde, polyepoxy componds, carbodiimides (EDAC, EDC) and genipin (Dervan et al., 2021). All of these will have different effects on macrophage polarization. As an example, collagen can be crosslinked with 1-ethyl-3-(3 dimethylaminopropyl)-carbodiimide and N-hydroxysuccin-imide (EDC/NHS) in order to decrease inflammatory cytokine expression such as TNF-α and C-C motif chemokine ligand 22 (CCL22). Moreover, collagen can be crosslinked with glutaraldehyde in the aim to decrease M2/M1 ratio and increase pro-inflammatory response. (Kharaziha et al., 2021) When the influence of cross-linking done by genipin and formaldehyde on nerve guidance conduits made of collagen were compared, it was demonstrated that genipin cross-linking increased the level of IL-10 and decreased the level of TNF-α, indicating M2 polarization. Formaldehyde cross-linking did not state similar results, suggesting that genipin could be applied in aim to modulate macrophage polarization towards anti-inflammatory state. (Dervan et al., 2021)

Biomaterials can also possess different effects on macrophage polarization depending on their structure and origin. For instance, naturally-derived and synthetic materials can function differently as implantations (Ye et al., 2021). Biomaterials, derived from decellularized ECM have been seen to improve wound healing and promote macrophage polarization into M2 phenotype this can be related to the fact that ECM scaffolds and decellularized tissues contain cell-binding motifs such as integrins which can induce specific immune responses in macrophages (Liu & Segura, 2020; Ye et al., 2021). Furthermore, natural polymers, such as fibrin, elastin, collagen and hyaluronic acid, used in hydrogels have shown immense biocompatibility and cell affinity (Kharaziha et al., 2021; Shen et al., 2021). In addition, natural polysaccharides have some advantages compared to synthetic polysaccharides, for instance they can be easily harvested from various organisms and they better resemble the polysaccharides in inherent ECM-

microenvironment. (Z. Li & Bratlie, 2021) In contrast, synthetically obtained materials and macromolecules can provide controllable parameters that can be optimized to suit the wanted application, for example synthetic polymers can be used to generate electrospun nanofibers in order to create specific surface topography for modulating macrophage polarization (Lee et al., 2018).

Synthetic and natural materials can be created to mimic ECM physical and chemical properties. These biomimetic materials have shown beneficial characteristics in modulating macrophage polarization into more pro-regenerative phenotype. For instance, hydrogel composed of non-sulphated glycosaminoglycan (GAG) and hyaluronic acid (HA)/collagen was made in order to mimic natural ECM composition and induce polarization in to M2 phenotype. (Mao et al., 2022) GAG is a natural component in ECM and controls macrophage behaviour by binding on to surface receptors or by regulating the availability and activity of growth factors and cytokines (Z. Li & Bratlie, 2021; Mao et al., 2022). In addition, HA has naturally anti-inflammatory characteristics that can be affected by its molecular weight, hence heavier HA molecules seem to resolve inflammation. The release of sulphated hyaluronan further helps to reduce inflammation and polarize macrophages into M2 phenotype. (Mao et al., 2022) Like GAG, chitosan is also a polysaccharide that has shown promising results for modulating macrophage polarization, although by itself it has tendency to modulate the phenotype towards more inflammatory state (Kharaziha et al., 2021; Mao et al., 2022). However sulphated chitosan mimics heparin that occur naturally in ECM and it can be used in combination with collagen I in order to create hydrogel that has the ability to modulate macrophage phenotype into M2 (Kharaziha et al., 2021). In addition, … fat can be coupled into nanofiber hydrogel composite in order to mimic ECM microarchitecture and biological function of adipose tissue. This composite has shown to promote macrophage modulation to M2 phenotype and enhance the vascularization process in soft-tissue wounds. (Henn et al., 2022)

To combine these physical and biochemical cues, it is important to take into account how they affect each other. For instance, in hydrogels that are chemically cross-linked, the substrate stiffness affects also material degradation, mesh size and ligand density. This means that while enhancing stiffness of these hydrogels, the degradation rate slows down, mesh size gets smaller and local ligand density increases thus making it harder to combine different cues together with stiffness for modulation of macrophage phenotype. In addition, while designing biomaterials it is crucial to consider factors beyond macrophage phenotype in order to achieve synergistic responses. (Liu & Segura, 2020) To conclude, macrophage polarization can be modulated through physical and chemical cues in various strategies utilizing natural and synthetic components. There is still a lot

to uncover about the interactions between macrophages and biomaterials and how to implement that knowledge into regenerative medicine.

5.2 Nanoparticle polarization strategies

Nanomaterials such as nanoparticles are emerging strategy for directing macrophage polarization. They are commonly used as drug carriers due to their unique physiochemical properties and nanoscale size, ranging from 1 nm to 1000nm diameter. However, also pristine nanoparticles, such as dendrimers, metal nanoparticles, liposomes, and polymeric micelles, can be used to direct macrophage polarization. (Mao et al., 2022; Medrano-Bosch et al., 2021) The logical way of using nanoparticles in regenerative medicine is to use them to target macrophages, since macrophages are in the first line of detecting and phagocytising foreign bodies in blood and tissues. Macrophages intake nanoparticles via endocytosis and therefore nanoparticles go through endolysomal compartment, where they are degraded. Nanoparticles can bypass the degradation process by destabilizing the lysosomal membrane with cationic ligands or surfactants. (Medrano-Bosch et al., 2021) As previously stated, polarization of macrophages towards M2 phenotype is commonly used strategy for tissue regenerative applications.

As professional phagocytes, macrophages intake nanoparticles through endocytosis. This process can be regulated by designing nanoparticle surface to bind various receptors, such as scavenger receptors, mannose receptor, Fc receptors, folate receptors, and CD44 receptor on macrophage membrane. (Medrano-Bosch et al., 2021) CD44 receptor has been shown to be overexpressed on activated macrophages, particularly on M1 phenotypic macrophages, and thus making it a promising target for nanoparticle attachment. Hyaluronic acid coating has been demonstrated to promote CD44-mediated endocytosis and combined with IL-4 or IL-10 plasmid DNA, they are able to polarize macrophages from M1 to M2 phenotype. Also, polyethyleneimine can be utilized in these nanoparticles in order to improve plasmid DNA escape from degradation. (Z. Li & Bratlie, 2021; Medrano-Bosch et al., 2021) In addition, silica nanoparticles modified with konjac glucomannan has shown to stimulate and upregulate mannose receptor and therefore these nanoparticles are able to polarize naïve macrophages to M2 phenotype (Mao et al., 2022). In contrary, β-glucan nanoparticles can bind to Dectin-1 receptor, which is overexpressed on M2 macrophages and therefore modulate the shift from M2 to M1 phenotype (Mao et al., 2022).

Pristine nanoparticles, such as metal nanoparticles, could be harnessed in regenerative medicine to modulate macrophage polarization. For instance, gold nanoparticles have shown to enhance anti-inflammatory markers and downregulate pro-inflammatory markers in murine macrophages

upon LPS induced inflammation. With similar results, gold nanoparticles coated with arginine-glycine-aspartic acid peptide or Cys-Leu-Pro-Phe-Phe-Asp hexapeptide, pushed macrophage marker expression towards M2 type. Gold nanoparticles have proved to be promising strategy for cardiac regeneration. (Corsi et al., 2020) Also, Cerium oxide nanoparticles have shown promising results in regenerative studies, and they have been proven to polarize macrophages into M2 phenotype (Corsi et al., 2020). In contrast to the inorganic particles, nanoparticles constructed from hydrolysed galactomannans, which are natural polysaccharides of galactose and mannose, have the ability to sway macrophage polarization towards M2 phenotype (Mao et al., 2022). Another natural polymer, PLGA, has also been demonstrated to decrease M1-marker levels and enhance M2-marker levels in vivo. Pristine PLGA nanoparticles were injected daily for 7 days following spinal cord hemisection and results were gathered after 7 and 84 days from injury. (Dervan et al., 2021)

5.3 Polarization by delivery of Bioactive Substances

By implementing delivery systems into biomaterials, it is possible to fine-tune the timing and magnitude of the macrophage response and polarization even further than only just with materials physical and chemical cues. Strategies for implementing bioactive substances into materials have been developed and researched in recent years. Some of these strategies include the release of cytokines, peptides, ions, drug molecules, hormones and genetic material.

5.3.1 Delivery of peptides and macromolecules

Biomaterials can also be used to deliver proteins and macromolecules such as cytokines, peptides, lipids, and polysaccharides as seen in table 1. Cytokines commonly secreted by immune cells can be implemented into biomaterials in order to regulate the immune response caused by the implanted material (Mao et al., 2022). Controlled release of cytokines such as IL-4, IL-10, TGF-β1 and IFN-γ have been utilized in biomaterials to modulate macrophage polarization, thus regulating tissue regeneration process (Lee et al., 2018; Mao et al., 2022; Ye et al., 2021). IL-4, IL-10 and TGF-β1 have been shown to sway the macrophages towards M2 phenotype and in contrast IFN-γ has shown to induce M1 phenotype (Ye et al., 2021). Cytokines can be implemented into the material in various ways, for instance Lee et al. introduces mesoporous nanoparticles with pore size of 30nm loaded with IL-4, which have been shown to promote M2 phenotype after injection to mouse model (Lee et al., 2018). With similar effect, IL-4 can be loaded into silk fibroin functionalized electrospun polycapolactone nanofibers through layer-by-layer assembly to

induce M2 phenotype (Lee et al., 2018). In addition, polycaprolactone (PCL) nanofiborous scaffold loaded with IL-10 has shown to polarize macrophages towards M2 in vivo thus it could be useful in peripheral nervous system repair (Dervan et al., 2021). In cartilage regeneration, thermosensitive photocrosslinkable hydrogel loaded with TGF-β1, has shown to promote shift from M1 to M2 phenotype (Ye et al., 2021). Accordingly, bone morphogenic protein-4 (BMP-4) from TGF-β1 superfamily has shown to induce macrophage polarization to M2 phenotype. Sun et al. demonstrated the usage of mesoporous nanoparticles loaded with BMP-4 to regulate macrophage polarization in controlled manner. (Sun et al., 2021)

Since the dominant macrophage phenotype varies during tissue regeneration process, it is important to take that into consideration as well. Therefore, controlled release of cytokines during different phases of healing process can be beneficial (Ye et al., 2021). Even though M1 macrophages are seen as proinflammatory phenotype, the initial wave of M1 macrophages is crucial to the initiation of angiogenesis (Mao et al., 2022). In order to imitate this shift, controlled release of one cytokine can be utilized. For instance, IL-4 implemented into bilayer sol-gel coated TIO_2 nanotubes is released slowly in the beginning, letting the macrophages to polarize into M1 phenotype. Later in the healing process, increased amount of IL-4 is released, promoting the shift from M1 to M2, which helps to resolve the inflammation (Mao et al., 2022). Correspondingly, IL-10 can be implemented into electrospun fiber by “inner-outer” design, which has cascade release behaviour. The first burst or IL-10 is in the inflammation phase to polarize macrophages into M2 phenotype thus helping to resolve excessive inflammation and inhibit over proliferation of dermal fibroblasts. The second burst of IL-10 happens in the proliferative phase. (Mao et al., 2022) To further modulate the macrophage polarization, two cytokines can be released at different timepoints. As mentioned above, TIO_2 nanotubes can be loaded with IL-4, but with double hydrogel coating IFN-γ can also be implemented into the structure. IL-4 is continuously released from the nanotubes, but IFN-γ is only released in the beginning. Due to initial release of IFN-γ in the first 3 days, macrophages shift into M1 phenotype. After 4 days, M2 takes place as the dominant phenotype. (Ye et al., 2021) In contrary, biphasic release of cytokines can also be used to first recruit naïve macrophages to the injury site with chemokine MCP-1, where then IL-4 promotes macrophage polarization into M2 phenotype in order to resolve inflammation (Kharaziha et al., 2021). As consequence of varying nature of the macrophage phenotype, controlled cytokine release combining two cytokines, seems promising strategy for modulating macrophage polarization through biomaterials. On the other hand, due to complexity of in vivo environment, cytokine dosage can vary depending on different factors, such as age (Ye et al., 2021).

In addition to cytokines, some research has been done about the controlled delivery of peptides and how they affect macrophage polarization in tissue regeneration. For instance, collagen VI has been shown to be a novel regulator of macrophage polarization and recruitment in peripheral nerve regeneration and therefore can be used in biomaterials to promote pro-regenerative M2 phenotype (Chen et al., 2015). Sustained release of collagen VI from electrospun PCL scaffold has shown to shift macrophage phenotype into M2 thus enhancing sciatic nerve regeneration (Dervan et al., 2021). Peptides can also be used as carriers for free metal ions such as copper ions, since peptides seem to be easier to release with control than free metal ions. According to Zhou et al. the tripeptide GHK and Cu^{2+} complex (GHK-Cu) promotes macrophage polarization towards M2 phenotype and controlled release of this peptide showed to be beneficial to bone tissue regeneration and vascularization. Controlled release was done with 3D silk-based scaffold which has been coated with polydopamine. (Zhou et al., 2021)

Table 1.*Summary of strategies utilizing controlled delivery of macromolecules (cytokines, peptides) to modulate macrophage polarization in tissue regeneration.*

Bioactive substance	biomaterial composition	effect on macrophages	source
cytokine	IL-4 loaded into silk fibroin functionalized electrospun PCL nanofibers	Promotion of M2 phenotype	(Lee et al., 2018)
	IL-4 loaded into mesoporous nanoparticles with a porosity of 30 nm.	Promotion of M2 phenotype	(Lee et al., 2018)
	Controlled release of IL-4 from bilayer sol-gel coated TiO_2 nanotubes	Promotion of M2 phenotype.	(Mao et al., 2022)
	IL4 loaded TiO_2 nanotubes with double hydrogel coating containing IFN-γ between the layers.	IFN-γ promoted polarization to M1 phenotype in the first 3 days and after 4 days, dominant phenotype turned to M2 due to IL-4.	(Ye et al., 2021)
	TGF-β1 loaded thermosensitive and photocrosslinkable hydrogel.	Induced the shift from M1 phenotype to M2 in cartilage tissue.	(Ye et al., 2021)
	"Inner-outer" IL-10 loaded electrospun fiber that has cascade release behaviour.	Initial IL-10 release in the inflammation phase polarizes macrophages to M2 phenotype.	(Mao et al., 2022)
	PCL nanofibrous scaffold loaded with IL-10	When conjugated to the surface of nerve fibers, material modulates macrophage polarization towards M2 in vivo.	(Dervan et al., 2021)

	chemokine fractalkine containing agarose implemented into polysulfone-based scaffold	Fractaline showed to promote polarization to M2 phenotype	(Dervan et al., 2021)
	Mesoporous silica nanoparticles loaded with bone morphogenic protein-4 (BMP-4)	BMP-4 promotes macrophage polarization to M2.	(Sun et al., 2021)
	IL-4 and monocyte chemotactic protein-1 (MCP-1) loaded self-assembling peptide/heparin hydrogel	Recruitment of naïve macrophages to the wound site and polarizing them to M2 phenotype.	(Kharaziha et al., 2021)
peptide	Collagen VI containing electrospun PCL conduit	Sustained release of collagen VI promoted M2 polarization	(Dervan et al., 2021)
	3D-printed silk based scaffold containing tripeptide glycyl-l-histidyl-l-lysine/copper complex (GHK-Cu)	Sustained release of GHK-Cu promoted polarization to M2.	(Zhou et al., 2021)

5.3.2 Delivery of ions

Delivery of inorganic ions have shown promising results in macrophage polarization and thus could be used in combination with biomaterials to enhance tissue regeneration. Summarized in table 2, inorganic ions of substances such as copper (Cu), calcium (Ca), strontium (Sr), iron (Fe), lithium (Li) and silicon (Si), has already been implemented into biomaterials and researched as regulators of macrophage phenotype. Usually, ion release from biomaterials is due to the breakdown of the material. (Ye et al., 2021) For instance, sustained release of calcium ions from biphasic calcium phosphate ceramic consisting of hyaluronic acid and beta-tricalcium phosphate have shown to polarize macrophages into M2 phenotype (Zhang et al., 2021). In addition, ionic degrading products of bioactive glass promote polarization of macrophages into M2 phenotype. Components of bioactive glass are SiO_2, CaO, Na_2O and P_2O_5 which can also rise the pH value of the injury site, if bioactive glass is used as a pure powder. Therefore bioactive glass can be mixed with sodium alginate in order to keep the pH in balance and form easily injectable hydrogel. (Zhu et al., 2020) In similar manner, gelatin methacryloyl (GelMA) and bioactive glass can be combined and decorated with lithium ions thus ensuring controlled release of Li^+, Si^{4+}, PO_4^{3-}, and Ca^{2+} ions which showed to polarize macrophages towards M2 phenotype. Added LiCl concentration is crucial for cell adhesion and proliferation and 5% molar ratio in hydrogel has shown best results. (Z. Wu et al., 2022) Concentration of the metallic ions seem to play a pivotal role in

macrophage polarization. As an example, bioactive glass/ceramic scaffold loaded with copper ions seem to direct macrophage polarization toward M1 phenotype in higher copper ion concentrations such as 28.3ppm. However, in concentrations between 0.5-16ppm the scaffold showed to promote macrophage polarization to M2. (Ye et al., 2021) In addition, bioactive glass microspheres containing strontium has shown to shift macrophages phenotype towards M2 due to released strontium ions (Mao et al., 2022). Similarly, silk-fibroin, borosilicate and methacryloyloxy containing composite system releases borate ions, which have been demonstrated to modulate macrophage polarization towards M2 phenotype (Mao et al., 2022). In the other hand, not all of the ion delivery systems rely on biomaterial decomposition outside of the cell. Superparamagnetic iron dioxide containing nanoparticles loaded with fibroblast growth factor have shown to deliver iron ions intracellularly through macrophage nanoparticle uptake (Mao et al., 2022).

Because macrophage phenotype is not stationary in healthy tissue injury healing process, dual release of bioactive factors has shown promising results. It has been proven that silicone ions promote macrophage polarization into M2 phenotype and therefore can be used in combination with IFN-γ in order to direct macrophage polarization in two phases. This can be seen utilized in a 5% (w/v) calcium silicate/β-tricalcium phosphate scaffold loaded with IFN-γ, which has an initial release of IFN-γ in the first three days while silicon ions get gradually released from the degrading scaffold and induce a shift from M1 to M2 after three days. (Ye et al., 2021)

Table 2. *Summary of strategies utilizing controlled delivery of ions to modulate macrophage polarization in tissue regeneration.*

Bioactive substance	biomaterial composition	effect on macrophages	source
ions	Composite system consisting of silk fibroin and borosilicate hydrogels that are modified with methacryloyloxy	Polarization towards M2 phenotype due to BO ions.	(Mao et al., 2022)
	Strontium (Sr) containing bioactive glass microspheres	Polarization towards M2 phenotype due to Sr ions.	(Mao et al., 2022)
	superparamagnetic iron oxide nanoparticles (bFGF-HDC@Fe_3O_4) nanoparticles loaded with fibroblast growth factor (FGF)	Polarization towards M2 phenotype.	(Mao et al., 2022)
	Cu^{2+} ions loaded into bioactive glass-ceramic scaffold	Copper ions seem to induce M2 phenotype at concentration of 0.5-16ppm and M1 type at 28.3ppm.	(Ye et al., 2021)

	biphasic calcium phosphate ceramic	Calcium ions promote M2 polarization in macrophages	(Zhang et al., 2021)
	Bioactive glass and sodium alginate hydrogel	Ionic products of bioactive glass promote macrophage polarization to M2 phenotype	(Zhu et al., 2020)
	Hydrogel combining gelatin methacryloyl (GelMA) and bioglass loaded with lithium ions	decreased amount of M1 macrophages and increased amount of M2 macrophages	(Z. Wu et al., 2022)
ion + cytokine	5% (w/v) calcium silicate/β-tricalcium phosphate scaffold with IFN- γ loaded onto it.	Due to initial release of IFN- γ, M1 phenotype dominated in the first 3 days, after which silicone ions start to release from the degrading scaffold and polarizing macrophages into M2 phenotype.	(Ye et al., 2021)

5.3.3 Delivery of genetic material

Genetic material such as plasmid DNA, microRNAs (miRNA), and small interfering RNAs (siRNA) can be used in variety of therapeutic applications including in regenerative medicine as regulators of macrophage polarization. Summary of these methods can be found in table 3. Vectors utilized to carry the genetic material need to be able to overcome in vivo barriers such as cell membrane. Particularly in macrophages, the carrier needs to keep the genetic material from degrading after internalization due to the intracellular degradative enzymes. Gene delivery approach usually works by regulating the expression of the target gene thus shifting macrophage polarization. (Kharaziha et al., 2021)

Plasmid DNA can be used as an alternative for cytokines delivered in protein form. For instance, plasmid DNA of IL-10 can be implemented into collagen scaffold or tuftsin coated alginate nanoparticles which both modulate macrophage polarization towards M2 phenotype thus showing the effect of IL-10 plasmid DNA on macrophages. (Shen et al., 2021; Ye et al., 2021) In a similar manner miRNAs can be utilized, although there is limited amount of research yet to be done about biomaterial delivered miRNA therapeutics. However, miRNAs such as miRNA-124, miRNA-125a-3p, miRNA-223, let-7c, miRNA-132, miRNA-146a and miRNA-21 have shown to promote macrophage polarization from M1 to M2.(X. Li et al., 2022; Shen et al., 2021) For instance, miR-

NA-21 have been reported to shift the polarization towards M2 phenotype and miRNA-155 promotes polarization to M1 phenotype. These two miRNAs can be delivered sequentially in positive nanocarriers to initially induce M1 phenotype and only then M2 phenotype, which is demonstrated to be beneficial for angiogenesis and healthy tissue regeneration. (X. Li et al., 2022) In addition miRNA-223-5p mimic loaded hyaluronic acid nanoparticles have been integrated into GelMA hydrogels and its efficiency to polarize macrophages into M2 phenotype has been demonstrated. (Shen et al., 2021) According to Sharifiaghdam et al. siRNA can be used in wound healing applications to induce macrophage polarization towards M2 phenotype through knockdown of interferon regulatory factor 5. Interferon regulatory factor 5-siRNA is delivered into macrophages with selenium based layer-by-layer nanocomplexes coated with polyethyleneimine. (Sharifiaghdam et al., 2021)

Table 3. *Summary of strategies utilizing controlled delivery of genetic material to modulate macrophage polarization in tissue regeneration.*

Bioactive substance	**biomaterial composition**	**effect on macrophages**	**source**
genetic material	Collagen scaffold containing IL-10 plasmids.	Downregulation of M1 macrophages and upregulation of M2 macrophages.	(Shen et al., 2021)
	Tuftsin peptide coated non-condensing alginate nanoparticles loaded with IL-10 plasmid DNA	Promotion of M2 type synovial macrophages.	(Ye et al., 2021)
	adhesive GelMA hydrogel loaded with miR-223 5p mimic hyaluronic acid nanoparticles	induce macrophage polerization to M2	(Shen et al., 2021)
	selenium based layer-by-layer nanocomplexes coated with polyethyleneimine and loaded with irf5-siRNA	induce macrophage polarization to M2	(Sharifiaghdam et al., 2021)
	miRNA-155 and miRNA-21 delivered in positively charged nanocarriers	First polarization towards M1 phenotype due to miRNA-155 and after M2 polarization due to miRNA-21	(X. Li et al., 2022)

5.3.4 Delivery of drugs and small molecules

Effective drug delivery systems can be created trough biomaterials in order to regulate macrophage polarization in controlled manner. As presented in table 4, electrospun scaffolds, hydrogels and nanoparticles has shown to be suitable for drug delivery applications for tissue regeneration applications and wide variety of different drugs and small molecules can be utilized. For instance, 7,8- dihydroxyflavone, a small-molecule agonist for tropomyosin receptor kinase B, has been demonstrated to promote macrophage polarization towards M2 phenotype and can be implemented into PLLA electrospun fibers which also has the ability to induce macrophage polarization to M2 phenotype by itself. This kind of scaffold could be suitable for neural stem cell-based regenerative applications. (Xiao et al., 2021) In like manner, an angiotensin 2 receptor blocker, telmisartan can be loaded into polycaprolactone/polyvinylpyrrolidone nanofibrous scaffold made by electrospinning. Telmisartan loaded scaffold showed to promote M2 macrophage polarization and bone regeneration in vivo. (S. Wu et al., 2022) In addition, plant-derived drug artemisinin can be loaded into membranes composed of poly(lactic-co-glycolic acid) (PLGA) and filamentous cellulose through electrospinning technique. The membrane displayed ability to polarize macrophages into anti-inflammatory phenotype thus enhancing the skin regeneration process. (Mao et al., 2022)

Hydrogel based materials can act as a molecule carrier for drugs and small-molecules. For instance, a natural compound silibinin can be implemented into GelMA hydrogel, from which it is released due to degradation of the material. Inhibition of M1 polarization and upregulation of M2 polarization is the result of the released silibinin. (Xu et al., 2022) In their research, Mi et al. constructed hyaluronic acid-based hydrogel combined with IRE-1α inhibitor APY29 and endothelial-derived exomes in order to enhance bone regeneration with sustained release of these substances. APY29 is a promising modulator of macrophage phenotype in bone regeneration applications since it induces M2 polarization as well as inhibits osteoclastogenesis. (Mi et al., 2022) Similarly, injectable hydrogel composite, consisting of Dimethyloxalyl glycine loaded hydroxypropyl chitin hydro-gel and kartogenin conjucated chitosan porous microspheres, can be utilized in cartilage repair through modulating macrophage polarization. This is a result of HIF prolylhydroxylase inhibitor dimethyloxalyl glycine, which is reported to promote M2 polarization in macrophages. (Ji et al., 2022) In addition, for tissue regeneration applications it is beneficial to combine two bioactive substances such as IL-4 and immunosuppressive drug dexamethasone, which can be loaded into injectable silk hydrogel in order to further polarize macrophages to M2 phenotype since both IL-4 and dexamethasone have shown to enhance M2 polarization. (Shen et al., 2021)

Similarly, dexamethasone phosphate can be delivered in liposomes which are surface modified with hormone phosphatidylserine. Phosphatidylserine has also been proven to modulate macrophage polarization to M2 phenotype and can be utilized without dexamethasone as phosphatidylserine-presenting liposomes. (Mao et al., 2022; Medrano-Bosch et al., 2021) However, dexamethasone can be used alone loaded into polydimethylsiloxane -based microporous 3D scaffold. (Shen et al., 2021)

Nanoparticles are suitable drug carriers as they can be designed to protect the cargo and release it in controlled manner. For instance, the anti-inflammatory antibiotic minocycline can be loaded into polycaprolactone nanoparticles, which has been reported to shift macrophage polarization towards M2 phenotype after 7 days in compressed mouse spinal cord. Polycaprolactone nanoparticles deliver their cargo inside of macrophages and microglial cells, making the delivery specific for those cells. (Dervan et al., 2021) In a similar manner, a natural polyphenol resveratrol can be delivered into macrophages by thermosensitive PLGA vesicles with quadrilateral ruthenium nanoparticle core with dextran sulphate surface modifications, which bind to scavenger receptors on macrophage cell membrane (Mao et al., 2022; Ye et al., 2021).

Table 4. *Summary of strategies utilizing controlled delivery of drugs and small molecules to modulate macrophage polarization in tissue regeneration.*

Bioactive substance	**biomaterial composition**	**effect on macrophages**	**source**
drugs and small molecules	7,8- Dihydroxyflavone loaded PLLA electrospun fibers	Promotion of macrophage polarization to M2 phenotype	(Xiao et al., 2021)
	silibilin implemented into GelMA hydrogel	silibilin induces M2 polarization	(Xu et al., 2022)
	Polydimethylsiloxane -based microporous 3D scaffold platform loaded with dexamethasone	promotion of polarization to M2 phenotype	(Shen et al., 2021)
	Resveratrol delivered in thermosensitive poly(lactic-co-glycolic acid) (PLGA) vesicles with quadrilateral ruthenium nanoparticle core	Promotion of M2 polarization	(Ye et al., 2021)

	Artemisinin loaded electrospun PLGA/silk fibroin membrane	Promotion of macrophage polarization towards anti-imflammatory phenotype	(Mao et al., 2022)
	Minocycline loaded polycaprolactone nanoparticles	Faster change into M2 phenotype and pro-reparative stages	(Dervan et al., 2021)
	Composite consisting of Dimethyloxalyl glycine loaded hydroxypropyl chitin hydrogel and kartogenin conjucated chitosan porous microspheres	Due to Dimethyloxalyl glycine, macrophages polarize towards M2 phenotype	(Ji et al., 2022)
	APY29 and engineered endothelial cell-derived exomes loaded into hyaluronic acid hydrogel	Due to APY29, macrophages polarize towards M2 phenotype	(Mi et al., 2022)
	Telmisartan loaded electrospun polycaprolactone/polyvinylpyrrolidone scaffold	Promotion of macrophage polarization to M2-like phenotype	(S. Wu et al., 2022)
drug + cytokine	Injectable silk hydrogel loaded with IL-4 and dexamethasone.	Polarization of M1 macrophages to M2 macrophages increased.	(Shen et al., 2021)
Drug + hormone	Drug dexamethasone phosphate loaded liposomes surface-modified with phosphatidylserine	Both Both dexamethasone and phosphatidylserine induced polarization to M2 phenotype	(Mao et al., 2022)

6. CONCLUSIONS

Macrophages are one of the first responders to inflammation and can be found in various tissues in our bodies. Due to their heterogenous nature and their ability to regulate other immune cells, they have shown to be a promising target for strategies of immunomodulation via biomaterials. Furthermore, macrophage phenotype has shown to affect tissue regeneration processes and the severity of foreign body reaction, thus making modulation of macrophage polarization an interesting strategy for regenerative medicine. It has been shown that the swich from pro-inflammatory M1 phenotype to pro-regenerative M2 phenotype is a necessary process for resolving inflammation and initiating the tissue regeneration process. Although, recent studies have demonstrated,

that the matter is not that simple, and the timing of that switch impacts the healthy tissue healing process. Moreover, as stated before macrophage phenotype is not as black and white as just M1 and M2 types, but there are at least four subtypes of M2 phenotype. These are all activated by different cues and secrete varying cytokines and chemokines, thus affecting the microenvironment in contrasting ways (Martin & García, 2021). With recent information gathered from macrophages, the phenotype seems to be more fluid and not restricted to these different subtypes. Due to the complex nature of macrophage phenotype, more research needs to be done in order to design better and safer immunomodulatory biomaterials for tissue regeneration.

There are various strategies for modulating macrophage polarization via biomaterials. The biomaterial physiochemical properties, such as stiffness, topography, pore size, particle size, wettability, surface chemistry, and composition, have impact on macrophage phenotype and can be utilized in regenerative biomaterials. Furthermore, Biomaterials can be loaded with bioactive substances, which are released in controlled matter in order to make the M1/M2 switch happen in a right time and make the process more precise. For instance, cytokines, peptides, ions, genes, and drugs can be used to load biomaterials. Recently nanoparticles have shown to be suitable delivery systems for bioactive substances, but also pristine nanoparticles can regulate macrophage polarization.

Aim of this thesis is to discuss the recent advances of immunomodulatory biomaterials from the regenerative medicine point of view and more specifically, how to modulate macrophage polarization with biomaterials. More research is to be done in this field and finding of more accurate biomaterial-immune models will help in design of new materials. In conclusion, biomaterial mediated regulation of macrophage polarization presents promising new strategy for regenerative medicine, which can be utilized in various tissue injuries and non-healing wounds.

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