Modulation of macrophage functions by ECM-inspired wound dressings – a promising therapeutic approach for chronic wounds

Abstract: Nonhealing chronic wounds are among the most common skin disorders with increasing incidence worldwide. However, their treatment is still dissatisfying, that is why novel therapeutic concepts targeting the sustained inflammatory process have emerged. Increasing understanding of chronic wound pathologies has put macrophages in the spotlight of such approaches. Herein, we review current concepts and perspectives of therapeutic macrophage control by ECM-inspired wound dressing materials. We provide an overview of the current understanding of macrophage diversity with particular view on their roles in skin and in physiological and disturbed wound healing processes. Based on this we discuss strategies for their modulation in chronic wounds and how such strategies can be tailored in ECM-inspired wound dressing. The latter utilize and mimic general principles of ECM-mediated cell control, such as binding and delivery of signaling molecules and direct signaling to cells specifically adapted for macrophage regulation in wounds. In this review, we present examples of most recent approaches and discuss ideas for their further development.

Keywords: chronic wound therapy; extracellular matrix; glycosaminoglycan; macrophage heterogeneity; macrophage modulation.

Introduction

Skin wound healing is one of the most complex processes in the human body. Injury of the skin affects many structures and cell layers, so their repair requires the coordination of different cells in space and time. Wound healing occurs in four sequential, partially overlapping phases: (i) initiation and hemostasis, (ii) inflammation, (iii) tissue formation with angiogenesis, dermal growth, and re-epithelialization, and (iv) remodeling. If orderly progression through these phases fails, wounds can transition to a difficult to heal or non-healing chronic state. A wound is considered chronic, if it does not restore the structural and functional integrity of the injured skin within a predictable period of time under state-of-the-art therapy.

Non-healing chronic wounds are among the most common skin disorders. Demographic change and comorbidities such as obesity, diabetes and vascular diseases contribute to the increasing incidence of chronic wounds worldwide (Sen et al. 2009). A variety of therapies and wound dressings are available for the treatment and care of chronic wounds, however, their effectiveness is often unsatisfactory and wound healing after treatment is only slow or, in some cases, cannot be induced at all (Han and Ceilley 2017). Hence, considering that a common gold standard for chronic wound care is still missing, new therapeutic strategies are urgently needed.

In recent years, major research efforts have been made to elucidate the underlying pathologic mechanisms in chronic skin wounds. This has identified macrophages as a major driver for the non-healing of the skin tissue. From a pathologic perspective, the healing process in chronic wounds is arrested in a persistent inflammatory phase that prevents progression of the normal tissue repair program and impedes the formation of new skin tissue (Eming et al.)
Macrophages critically contribute to the sustained inflammation and failure of tissue repair due to dysregulated activation states (Mirza et al. 2014, 2015; Sindrilaru et al. 2011). Targeted control of misdirected macrophage activation in chronic wounds, therefore, represents a new promising approach on the way to more effective treatment strategies.

In this review, we discuss the control and modulation of macrophage functions as strategy to promote healing of chronic skin wounds with emphasis on wound dressing materials based on extracellular matrix (ECM) components. In the first part, we focus on macrophages and their role in skin wound healing. We briefly describe the current understanding of macrophage heterogeneity and functional diversity and summarize the existing knowledge on the role of macrophages in physiological skin wound healing and their malfunctions in chronic skin wounds. In the second part, we discuss the modulation of macrophages as a therapeutic means in chronic wounds and present general principles of ECM-mediated macrophage control. Finally, we summarize current developments of ECM-inspired strategies for the targeted control of specific macrophage function in chronic wound healing and provide future perspectives for their advancements.

## Macrophages and their role in physiological and chronic skin wound healing

### Macrophage heterogeneity and functional diversity

Macrophages are important immunoregulatory cells that control tissue homeostasis, innate immune processes during inflammation and wound healing responses. This superior function of macrophages is based on their plasticity, which enables them to sense signals from the environment and to integrate and transduce these signals into an appropriate host response in the tissue (Gordon and Martinez-Pomares 2017).

Macrophages are characterized by a complex heterogeneity based on their ontogeny, tissue-residency and plasticity. Macrophages can derive from three different developmental lineages: primitive yolk sac progenitors, erythro-myeloid precursors (EMPs) or hematopoietic stem cells (HSCs) (Gomez Perdiguero et al. 2015). In adult tissues the sources of resident macrophages vary depending on the tissue they reside. While resident macrophages in the lung, brain or liver are maintained by self-renewal and retain their original embryonic lineage (Hashimoto et al. 2013), resident macrophages in the heart, intestine or dermis are replenished by a constant input of circulating monocytes derived from HSCs in the bone marrow (Bain et al. 2014; Molawi et al. 2014; Tamoutounour et al. 2013). Consequently, fetal macrophage lineages in these tissues gradually fade in favor of bone marrow originating-macrophages. In addition to their ontogeny, macrophage identities are further shaped by the environment of the tissue they reside. Indeed, tissue-specific factors trigger epigenetic and transcriptional signatures that result in distinct macrophage phenotypes specialized for tissue-specific functions to maintain tissue homeostasis (Gosselin et al. 2014; Lavin et al. 2014). Recent studies have defined several distinct skin-resident macrophage subsets in respect of their ontogeny, gene expression profiles, localization and functional specialization including different types of perivascular macrophages and sensory nerve-associated macrophages, but the full picture is still lacking (Abtin et al. 2014; Barreiro et al. 2016; Kolter et al. 2019). Injury, infection, or sterile inflammation interfere with the tissue-specific phenotype of resident macrophages and introduces new signals into the tissue microenvironment that alter their activation states. In addition, bone marrow-derived monocytes are mobilized from the circulation and form the major pool of macrophages in the challenged tissue. It is assumed that the recruited bone marrow-derived macrophages take the crucial part in restoring tissue homeostasis by regulating processes of inflammation and tissue repair, but tissue-resident macrophages have also been shown to contribute to these processes (Abtin et al. 2014; Barreiro et al. 2016; Kolter et al. 2019; Puttur et al. 2019).

During the control of inflammation and tissue repair, macrophages develop different functional phenotypes in response to their microenvironment. Classically these phenotypes have been assigned to pro-inflammatory M1-like macrophages (M1) and pro-regenerative/anti-inflammatory M2-like macrophages (M2) (Mosser and Edwards 2008). This definition has been challenged by new omics technologies that revealed much more complexity in macrophage phenotypes with diverse functions and gene expression profiles, which are only partially reflected in the classical definition of M1 and M2 (Butenko et al. 2020; Lantz et al. 2020; Xue et al. 2014). However, it has been recognized that macrophages in tissues under challenge change from subsets with a pro-inflammatory (M1-like) activation profile to subsets with anti-inflammatory/pro-regenerative (M2-like) activation profiles as inflammation progresses through inflammatory resolution and repair to restoration of homeostasis (Mantovani et al. 2013). Current understanding suggests that in inflamed and
repairing tissues different macrophage subsets appear and coexist and adjust their activation profile in response to altering signals in the microenvironment, however, localization in a specific niche and progeny may commit them to certain functions independent of these signals.

In order to standardize and unify the definitions of macrophages, a common framework for macrophage classification has been proposed, which considers activation signals, signalling pathways and transcription factors, surface and genetic markers and functions in tissue to define macrophage subsets and to assign them with M1-like and M2-like characteristics (Murray et al. 2014). Figure 1 summarizes these characteristics. Although the nomenclature of “M1” and “M2” is somewhat outdated, we will use both terms in this review for simplicity. This is not to define distinct macrophage subsets, but rather to describe specific functions, activation states, and gene signatures of macrophages associated with either inflammation or pathogen defense (M1-like) or repair/regeneration or inflammatory resolution (M2-like). For further interest in this subject we refer to recent excellent reviews that summarize and discuss the current understanding of macrophage diversity in tissues in the steady state and during inflammation and tissue repair (Gautier and Yvan-Charvet 2014; Ginhoux and Guilliams 2016; Gordon and Martinez-Pomares 2017; Murray 2017; Okabe and Medzhitov 2016).

Although macrophages play an important role in normal tissue repair and homeostasis, they have also been linked to pathologies (Li et al. 2019). Many chronic inflammatory or degenerative diseases are the outcome of dysregulated activation of macrophages with uncontrolled pro-inflammatory, immunosuppressive or pro-fibrotic functions (Cochain et al. 2018; Gharib et al. 2019; Yang et al. 2018). This includes chronic skin wounds, where pro-inflammatory activation

![Figure 1: Characteristics to distinguish between M1-like and M2-like activation states of macrophages (adapted from Murray et al. 2014).](image-url)
profiles in macrophages predominate over repair functions (Mirza and Koh 2011; Mirza et al. 2014; Sindrilaru et al. 2011).

**Role of macrophages in physiological skin wound healing**

Wound healing of the skin is a complex, multi-phase process that intends to close the tissue defect and results in the formation of a scar that never reaches the original quality and functionality of the skin before injury, e.g. hair follicles and sweat glands are not restored and elasticity and tensile strength remain reduced. In each phase of the wound healing process, macrophages are instrumental in coordinating the activation of immune cells and skin cells by sensing signals from the injured skin and integrating them into the wound healing process using their plasticity (Novak and Koh 2013). Several studies, in which macrophages were depleted or manipulated during the wound healing process, demonstrate their importance in tissue formation and angiogenesis and the associated transition of subsets with M1-like to subsets with M2-like activation states (Goren et al. 2009; Lucas et al. 2010; Mirza et al. 2009). The process of skin wound healing has already been extensively discussed in excellent review articles, e.g. in Eming et al. (2014) and Wynn and Vannella (2016). In the following part we briefly summarize the role of macrophages, which is also illustrated in Figure 2.

Within seconds after injury, damaged and activated skin cells including tissue resident macrophages release a plethora of chemotactic and inflammatory signals mounting an inflammatory response that is carried out by the immigrating immune cells (Minutti et al. 2017). For example, a subset of perivascular macrophages has been identified.

**Figure 2:** Role of macrophages during the course of an acute wound healing response in skin. Macrophages are instrumental in coordinating the wound healing process, and to this end, they exert pro-inflammatory, inflammation-resolving, and tissue-forming functions. It is assumed that different macrophage subsets (skin resident and bone marrow derived) contribute to the wound healing response, some may adjust their activation profile, some may be committed to certain functions. Although a clear picture on specific macrophage subsets is still missing, it is accepted that macrophage gene signatures shift from M1-like to M2-like activation profiles with progression of the healing response. In the early phases of hemostasis and inflammation, macrophage subsets with M1-like signatures dominate. Via the release of pro-inflammatory factors, they recruit immune cells (polymorphonuclear neutrophilic granulocytes [PMN], monocytes [Mo]) and mount the inflammatory response. As the inflammatory phase progresses, there is a gradual transition to subsets with M2-like signatures, which predominate in the phase of tissue formation. Via the release of anti-inflammatory factors, M2-like macrophage subsets mediate the resolution of inflammation. At the same time, via the release of pro-fibrotic and pro-angiogenic growth factors, they activate fibroblasts (Fb) and endothelial cells (EC) to form the granulation tissue and vascular structures.
that releases high amount of neutrophil chemoattractants, and that is critical for the migration of polymorphonuclear neutrophil granulocytes (PMN) into infected skin (Abtin et al. 2014). PMN are the first immune cells that arrive to the injured skin, where they fight invading pathogens by phagocytosis, the release of reactive oxygen species (ROS), myeloperoxidases (MPO), and proteases or the formation of so-called neutrophil extracellular traps (NETs) (Dovi et al. 2004). With a short time delay to PMN, circulating monocytes migrate into the injured tissue and immediately mature into macrophages. Macrophages initially develop pro-inflammatory and anti-microbial functions that complement pathogen defense by PMN (Silva 2010). Further, they release ROS, cytokines and chemokines like TNFα, IL-1β, IL-6, MCP-1, MIP-1α, MIP-1β, IL-8 that enhance the inflammatory process, recruit and activate more monocytes, PMN and other immune cells (Daley et al. 2010). In addition to these classical M1-like activities, macrophage subsets in the early inflammatory phase already develop pro-angiogenic functions that are critical for the normal course of wound healing (Gurevich et al. 2018; Willenborg et al. 2012). As the inflammatory phase progresses, macrophage gene expression gradually shifts from M1-like to M2-like signatures, which is critical for entry into the repair phase (Deonarine et al. 2007; Novak and Koh 2013). One signal for the change in gene signature is the phagocytosis of apoptotic PMN that leads to the release of pro-resolving lipid mediators and immunosuppressive factors such as IL-10, TGF-β1, and IL-1RA that terminate the pro-inflammatory M1-like activation profile (Das et al. 2014). This provides the way for the formation of new tissue, which involves processes of granulation, re-epithelialization, angiogenesis and peripheral nerve regeneration (Cañedo-Dorantes and Cañedo-Ayala 2019).

The formation of the various tissue structures is assumed to occur in interaction between endothelial cells, fibroblasts, keratinocytes and the ECM and is coordinated by macrophage subsets with M2-like activation profiles, which now predominate in the wound environment (Wynn and Vannella 2016). Interestingly, Barreiro et al. (2016) demonstrated that in addition to recruited bone-marrow derived macrophages, as shown by Lucas et al. (2010), a specific subset of perivascular skin resident macrophages with a distinctive anti-inflammatory transcriptional profile are indispensable for these repair processes (Barreiro et al. 2016). Granulation tissue is formed by fibroblasts that migrate into the wound in response to growth factors such as PDGF, TGFβ, bFGF that are primarily released by macrophages (Shook et al. 2018). Matured fibroblasts proliferate in the wound and release MMPs to degrade the provisional matrix (Gill and Parks 2008). At the same time, they differentiate into myofibroblasts, which contract the wound tissue and produce new ECM that is deposited to form scar tissue (Hinz 2016). Fibroblast activation is controlled by distinct macrophage populations with M2-like characteristics. A population of CD301b-expressing macrophages that selectively induces fibroblast proliferation from adipocyte progenitor cells in the wound bed via PDGF-C and IGF1 signaling has been identified (Shook et al. 2016, 2018). Macrophages activated via the IL-4R pathway mediate pro-fibrotic collagen cross-linking within the granulation tissue. IL-4 signaling promotes the release of Relma from macrophages that induces the enzyme lysyl hydroxylase 2 (LH2) in fibroblasts controlling collagen fiber linkage (Knipper et al. 2015). On the other hand, macrophages with activated STAT3 pathway counteract the activation of pro-fibrogenic functions in fibroblasts via the expression of IL-10 (Do et al. 2018). By this way, macrophage subsets with STAT3 signaling contribute to the resolution of tissue formation and regulate scarring (Pakshir and Hinz 2018).

However, formation of the granulation tissue is important for the stabilization of new vessels (Knipper et al. 2015) that have already started to form in the inflammation phase in response to macrophage-derived VEGF (Willenborg et al. 2012). Simultaneously with the migration of fibroblasts into the wound, the process of re-epithelialization begins, which eventually leads to the restoration of the skin barrier. Re-epithelialization occurs in close communication of keratinocytes with fibroblasts (Werner et al. 2007). Although macrophage depletion in wound healing models results in delayed re-epithelialization, this is assumed to be related to the impaired fibroblast activation due to deficient macrophage signals. However, detailed studies on the macrophage epidermal crosstalk during wounding are lacking. These may even highlight a role of skin-resident macrophages, as recently found by Kolter et al. (2019) in dermal nerve regeneration. They describe a specific subset of highly CX3CR1 positive resident macrophages that colocalize with peripheral nerves and critically contribute to nerve regeneration after wounding (Kolter et al. 2019).

**Macrophage malfunction in chronic wounds**

The importance of macrophages in the coordination of the wound healing response becomes apparent when their activation is disturbed, which leads to chronic non-healing wounds. Clinically, chronic wounds are classified based on their underlying cause as chronic venous ulcers (CVU), diabetic foot ulcer (DFU), or pressure ulcers (decubitus). In addition to vascular insufficiency, diabetes mellitus and local pressure effects, other factors such as advanced age,
nutritional status or immune status may contribute to the development of chronic wounds. Despite their different etiologies, the various chronic wounds share certain common features, such as a persistent inflammatory response with increased numbers of PMN and macrophages, deficient local angiogenesis and tissue formation due to lacking or proteolytically inactivated growth factors, increased oxidative damage, senescence, and a lack of stem cells (Fivenson et al. 1997; Krisp et al. 2013; Lauer et al. 2000; Sindrilaru et al. 2011; Wetzler et al. 2000; Yager et al. 1996). Macrophages play a central role in these pathologic processes (Figure 3). Uncontrolled activation of pro-inflammatory functions in macrophages drive the chronic inflammatory process particularly in diabetic ulcer and chronic venous wounds (Khanna et al. 2010; Mirza and Koh 2011; Mirza et al. 2014; Sindrilaru et al. 2011). As illustrated in Figure 3B, dysregulated macrophage activation differs between these types of chronic wounds.

Macrophages in human diabetic foot ulcers present a decreased expression of M2-like genes including CD206, CD36 and PPARγ, while M1-like factors such as IL-1β, IL-18, iNOS are upregulated (Bannon et al. 2013; Gallagher et al. 2015; Mirza and Koh 2011; Mirza et al. 2013, 2015). Wound healing studies in well-established mouse models of type 1 diabetes (T1D) (streptozotocin [STZ]) and type 2 diabetes (T2D) (ob/ob; db/db; diet-induced obesity [DIO]) revealed dysbalanced macrophage activation states in diabetic wounds (Kimball et al. 2017; Mirza and Koh 2011; Okizaki et al. 2015; Ramalho et al. 2018). Wounds in diabetic mice present increased levels of macrophages particularly in the late phase of repair (Gallagher et al. 2015; Kimball et al. 2018; Mirza and Koh 2011). These macrophages are predominantly derived from infiltrating monocytes. In fact, it was found that the number of distinct high CX3CR1-expressing tissue-resident macrophages subsets was not affected (Burgess et al. 2019). It is assumed that diabetes-induced increased

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Figure 3: Dysregulated activation of macrophages in chronic wounds.
(A) Dysregulated macrophage activation results in persistent M1-like macrophage signatures that mount an chronic inflammatory wound environment with excessive pro-inflammatory factors and proteases, which destroy tissue and growth factors (GF), induce senescence programs in tissue cells (fibroblasts [Fb], endothelial cells [EC], keratinocytes [KC]) and prevent the inflammatory resolution. In addition, macrophage M2-like signatures are impaired resulting in a lack of anti-inflammatory and pro-healing signals. (B) In chronic venous ulcer (CVU) macrophages accumulate iron due to the uptake and processing of erythrocytes (ery), which escape from defective vessels into the tissue. Excessive iron catalyzes fenton reactions producing highly reactive radicals and oxygen species (ROS) and turns macrophages in a pro-inflammatory activation state with high TNF release while M2-related activation (IL-4Ra, IL-10) is impaired. In diabetic wounds (diabetic foot ulcer = DFU) increased infiltration of pro-inflammatory Ly6C<sup>high</sup> monocytes give rise to macrophages that present increased activation of pro-inflammatory signaling pathways, like MAPK, NOTCH. Increased inflammasome activity and IL-1β release hamper M2 signaling pathways, like PPARγ activation. Macrophages present impaired M2-like functions such as the uptake of apoptotic PMN and the release of pro-resolution and pro-healing factors like IL-10, TGF-β, VEGF, IGF1. M1- and M2- related features are marked with red and green arrows, respectively. (Clinical pictures provided by the Department of Dermatology, Venereology and Allergology, University of Leipzig).
myelopoiesis in hematopoietic stem cells, which results in elevated levels of circulating CCR2^hi^Ly6C^hi^ monocytes contributes to the accumulation of macrophages in diabetic wounds (Barman et al. 2019; Singer et al. 2014). This is supported by recent studies, which revealed sustained infiltration of Ly6C^hi^ monocytes with characteristic M1-like signatures (IL-1β, TNF) while the transition to Ly6C^lo^ monocytes with M2-like profiles (TGF-β, Arg1, IL-10) is impaired (Kimball et al. 2018; Pang et al. 2021). Convincingly, wound macrophages isolated from diabetic mice present elevated M1-like signatures including increased Notch signaling and persistent activation of the Notch receptor (Khanna et al. 2010). In addition, they express less factors important for tissue formation including IGF1 and VEGF (Mirza and Koh 2011; Mirza et al. 2013). Although it is apparent that macrophages are affected from changes in the signaling environment in the diabetic wounds, it has also been noted that epigenetic modifications in hematopoietic stem cells that manifest macrophages in a preprogrammed pro-inflammatory polarization state also contribute to dysregulated phenotypes in chronic wounds (Gallagher et al. 2015).

In contrast to the situation in diabetic wound healing, much less is known on the dysregulation of macrophages in chronic venous wounds. Biopsies from human venous ulcers show macrophages that contain high amounts of iron (Caggiati et al. 2010; Sindrilaru et al. 2011). Patients with chronic venous wounds suffer from chronic venous insufficiency with pathologic hypertension in the leg veins that result in the leakage of erythrocytes into the tissue (Santler and Goerge 2017). Dermal macrophages phagocytose erythrocytes from the tissue, recycle the iron and accumulate it. Using a mouse model of systemic iron overload (hemochromatosis model), Sindrilaru et al. (2011) showed a causative link between iron excess, sustained pro-inflammatory macrophage activation and the non-healing of chronic venous ulcers. Macrophages in wound tissue of iron overloaded mice presented uncontrolled pro-oxidant and pro-inflammatory M1-like signatures with increased expression of TNF, IL-12, CCR2 and Ly6C. At the same time, M2-like features were impaired in the macrophages, with some markers such as Dectin-1, IL-4Rα, and CD204 downregulated, while others such as CD206, CD301, CD163 were unaltered or even increased. Consistently to the data from mice, macrophages purified from human chronic venous wounds showed increased expression of TNF, IL-12 and CCR2 and downregulated Dectin-1, IL-4Rα, IL-10 while CD163 was increased. Iron-mediated phenotypic macrophage alteration was associated with the accumulation of ROS (hydroxyl radicals, peroxynitrite) and TNFα in the wound tissue, and the induction of oxidative DNA damage and senescence in surrounding skin tissue cells.

**ECM-mediated modulation of macrophages: a new strategy in chronic wound therapy?**

**Modulation of macrophages as a therapeutic means**

As outlined above, the nonhealing environment of chronic wounds is dominated by macrophages with pro-inflammatory M1-like activation states, whereas functional M2-like macrophage subsets are suppressed, which drives a persistent inflammatory response and impairs critical processes of normal wound healing.

Despite various therapy options, many chronic wounds do not heal suggesting that current measures do not get to the root of the disturbed wound healing response. Strategies that address the impaired transition from inflammation to tissue formation have been considered as new therapeutic option (Landén et al. 2016). Given the importance of macrophages in wound healing process, approaches targeting dysregulated macrophage functions appear most promising. Studies in mice have shown that reversing the imbalance between M1-like and M2-like macrophage activation states improves wound healing. Here, macrophage modulation was achieved by various means, e.g. by blocking IL-1β or TNF, by pharmacological inhibition of inflammasome, by neutralizing MCP-1 or by chelating of iron with desferrioxamine (Gallagher et al. 2015; Mirza et al. 2013, 2014; Sindrilaru et al. 2011). These examples demonstrate the significant effect of the wound microenvironment on macrophage activation states and suggests that latter can be therapeutically targeted through changing the signaling environment in chronic wounds.

Strategies for such targeted control of macrophage activation have been widely explored in diverse fields of non-dermal tissue repair. They comprise measures for **in situ**
modification of endogenous macrophages, e.g. via biophysical cues or the delivery of bioactive (macrophage-regulating) signals or cells, but also cytotherapy approaches, when exogenous pre-activated or “educated” macrophages are delivered into the tissue to replenish needed macrophage subsets and functions. Various excellent reviews describe the general principles of these measures and their use in different settings of tissue repair (Alvarez et al. 2016; Smith et al. 2017; Spiller and Koh 2017). Here we focus on approaches utilizing features and functions of the ECM to target specifically macrophages in chronic wounds. ECM-based materials have been recognized for their favorable pro-repair features in nondermal tissue repair, which is why they have found their way into skin repair applications (Turner and Badylak 2015). They meet the important requirements that wound dressings must fulfill, such as high biocompatibility, elasticity, mechanical protection, protection against infections and microorganisms, moisture control and gas transport. In addition, they can be tailored to control specific cellular functions. Here, natural functions of the ECM as a cell regulator are utilized.

**Principles of ECM-mediated macrophage control**

Two classes of macromolecules form the ECM. These are proteins such as collagen, elastin, fibronectin, or laminin and various glycosaminoglycans (GAGs) such as hyaluronic acid (HA), heparin, and chondroitin sulfate. Each of these are involved in the natural regulation of cellular functions including immune cell functions (Sorokin 2010; Yue 2014). ECM signaling to the cells occurs in two principle ways, directly and indirectly, as illustrated in Figure 4. Both ways of ECM-mediated cell regulation can be targeted and adapted for the development of immunomodulatory ECM-based wound dressings to control macrophage functions in chronic wounds.

Direct signaling involves the recognition of specific proteins or peptide domains and of GAGs by the cells. Some of the recognition sides, so-called matricryptins, are only exposed during the course of structural or conformational changes of ECM molecules (Davis et al. 2000). Cells directly sense the physical and biological properties of their surrounding ECM environment by various receptors including integrins, HA receptors, but also toll-like receptors and adapt their cellular activation and differentiation and functions like adhesion, migration, proliferation (Yue 2014). Hence, physicochemical properties and distinct signals of ECM-based materials have been used to influence cell behavior including macrophage activation (Cramer and Badylak 2020). Developments for specific control on macrophages in wounds are discussed in the next section.

The indirect way of ECM-mediated cell control involves the regulation of growth factors and cytokines that signal

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**Figure 4:** Control of cell functions by the ECM. ECM-mediated signaling to cells occurs directly via receptor-dependent recognition of specific proteins or peptide domains and of glycosaminoglycans (GAG) and indirectly via presentation of growth factors (GF) and cytokines that are bound in the ECM via electrostatic interaction with sulfated GAG.
Concrete strategies for targeted control of macrophage by ECM-inspired wound dressing materials

ECM-based wound dressings are already in clinical use in wound care. Most of these materials are processed biological ECM scaffolds (AlloDerm, OASIS, MatriStem) or engineered scaffolds composed of natural ECM components (Integra, Hyalomatrix) or even constructs loaded with living skin cells (Dermagraft, Apligraf). Biological properties of the ECM are typically preserved in the scaffolds and available to wound-derived cells that encounter the materials. In addition, naturally embedded signaling factors reach the wound tissue via degradation of these materials. Such ECM-derived wound care dressings are mostly used as dermal substitutes in deep partial- and full-thickness skin wounds, as well as in the treatment of chronic wounds, where they were shown to support the wound healing outcome (Rennert et al. 2013). The ECM materials are assumed to provide a favorable wound environment for the migration and growth of skin cells such as keratinocytes, fibroblasts and endothelial cells. Whether they regulate macrophage function and activation in wounds is not well understood. Witherel et al. (2016) analysed the response of human monocyte-derived and THP1-derived macrophages to ECM-based dressings including Integra, PriMatrix, AlloMend and Oasis. The ECM-matrices indeed had an effect on macrophage gene expression with different results. However, only six genes were analyzed in the study, and TNF, CCL22, TIMP3, and CD163 were regulated by the wound matrices. Further studies are needed to understand macrophage regulation by ECM-based dressings, which are already in clinical use, and to assess whether they modulate macrophage activities, when applied on chronic wounds.

In recent years researchers have begun to develop functionalized ECM-based wound dressing materials that specifically modulate cell response to curb inflammatory and repair processes in wounds. In design, these new materials differ from classical ECM-scaffold wound dressings in the way that ECM structures are specifically modified or even synthetically engineered, on the other hand natural ECM functions are mimicked and utilized to promote specific cell functions in wounds. For the purpose of this review, the term “ECM-derived” will refer to biologic materials consisting of natural ECM components and the term “ECM-inspired” will refer to materials consisting of modified and synthetically produced ECM structures. Table 1 provides a comparative overview of both types of ECM-based materials.

Table 1: Comparison of ECM-derived materials and ECM-inspired materials.

| ECM-derived materials | ECM-inspired materials |
|-----------------------|------------------------|
| **Composition**       |                        |
| Acellular ECM scaffolds| Composites of natural and chemically modified ECM components |
| Composites of natural ECM components | Composites of synthetically produced ECM components |
| Biohybrids: Combination with synthetic polymers |
| **Providing of signals** |                        |
| Biomimetics of tissue, recapitulates ECM biology with natural signals: | Mimicking of ECM signaling functions: |
| – GF/cytokines | – Incorporation of GF/cytokines |
| – Biophysical cues | – Adjustment of biophysical cues |
| **Mode of action** |                        |
| Non-specific targeting of variety of cells in the wound healing process | Specific targeting of cells/functions of interest in spatio-temporal manners |
| Delivery of factors via material degradation | Delivery of factors via controlled release strategies |
| GF, growth factor. |                        |
This section discusses concrete strategies and future perspectives of ECM-inspired materials for the control of macrophage functions in chronic wounds. The different approaches are illustrated in Figure 5.

**Biophysical signaling from ECM scaffolds**

A recent study investigating macrophage behavior in engineered ECM networks with adjustable physicochemical properties demonstrated that the functional activation of macrophages can be regulated by dimensionality and by dynamic changes in the composition and mechanics of the ECM (Friedemann et al. 2017). For example, macrophages embedded in stiffer three-dimensional artificial ECM networks reduce their pro-inflammatory cytokine response. This effect was abrogated by introduction of GAG components into the network. In contrast, macrophages cultured on two-dimensional artificial ECM networks with similar properties retain their less inflammatory phenotype in the presence of the GAG component. This indicates that by manipulating structure, stiffness and GAG content compositions of ECM-based materials may be found to direct macrophages towards a pro-healing mode.

Stabilized acellular equine pericardial collagen matrices (sPCM) may represent such composition (El Masry et al. 2018). The biomechanics of this ECM-derived material are maintained by a bridge stabilization process that additionally confers resistance to enzymatic degradation by MMPs and other proteases. Immunomodulatory effects of sPMC were tested in comparison to a polycarbonate membrane (PC) in a subcutaneous implantation model in mice. In the early inflammatory response, macrophages around the sPMC implant showed higher levels of pro-inflammatory markers such as IL-1β, iNOS, and TNF-α, but at the same time an increased efferocytosis (phagocytic cell uptake) activity and up-regulated expression of pro-resolution genes such as IL-10, arginase-1, and VEGF as it also occurs in the early phase of an acute wound healing response. At later stages pro-inflammatory activation in the macrophages around the sPMC implant was resolved, while pro-resolution features were further increased. In contrast, macrophages isolated from late PC implantation site, showed the opposite activation profile. This demonstrates the ability of sPMC to regulate...
macrophage responses in situ and favor pro-healing functions. Application of sPMC to full-thickness skin wounds in mice accelerated wound closure and improved the quality of healing in terms of collagen deposition and maturation compared to wounds treated with Tegaderm, a generic wound covering dressing. However, the inflammatory response and macrophage activation profile were not analyzed in the skin wound model. This leaves an open question of whether the observed macrophage modulation in the subcutaneous tissue is also induced in excisional skin wounds and contributes to the favorable healing response there. Furthermore, the mechanism by which sPMC regulate macrophage activation was not addressed. Since sPCM is resistant to degradation, the release of bioactive signals from the matrix into the wound environment is rather unlikely, thus macrophage activities might be more controlled by biophysical cues of the sPMC. Interestingly, sPMC resemble properties of healthy skin in terms of stiffness, height map and surface topography, which may represent an homeostatic environment that favors immunosuppressive macrophage activation states (Davies et al. 2013). Nevertheless, the structural signals of the ECM that confers macrophages into a specific activation state are far too complex and not well understood or explored to be utilized for targeted macrophage control.

Hyaluronic acid-based signaling

Hyaluronic acid is a non-sulfated GAG that is found in high frequency in the ECM and that possess potent anti-inflammatory properties. It downregulates pro-inflammatory activities of immune cells, especially in macrophages, via direct receptor-mediated signaling to the cells including cell receptors CD44, RHAMM and LYVE (Lim et al. 2018; Misra et al. 2015). The anti-inflammatory activity of HA is determined by its molecular size and only high molecular weight HA promotes pro-resolution functions in macrophages. This is lost when HA is degraded to smaller fragments (Day and La Motte 2005; Jiang et al. 2005) as it may occur in chronic wounds that have enriched hyaluronidase activity (Dechert et al. 2006).

Various chemical modifications of HA have been tested to stabilize HA in tissue applications such as esterification and sulfation. The FDA approved regenerative wound care matrix, Hyalomatrix®, contains fibers of esterified HA. The matrix slowly degrades in contact with the wound site and thereby releases high amounts of the stabilized HA, which promotes re-epithelialization in human chronic wounds independent of their etiology (Caravaggi et al. 2011). However, whether this therapeutic effect involved the regulation of macrophage activation by the esterified HA was not investigated so far.

Sulfation of HA has been demonstrated to uncouple the anti-inflammatory activity of HA from the molecular size, particularly when modified to a high degree of sulfation (Jouy et al. 2017). In addition, sulfation of HA enhances the immunomodulatory capacity of HA as sulfated HA derivatives were shown to exhibit significant anti-inflammatory activities at nanomolar concentrations, where HA is normally ineffective (Zhang et al. 2011). Culture of human M1-like macrophages with highly sulfated HA suppressed their pro-inflammatory activation profile and resulted in decreased release of TNF, IL-12, MCP-1 – pro-inflammatory cytokines that are overexpressed in macrophages in poorly healing wounds in human and mice (Jouy et al. 2017). Mechanistically, sulfated HA is recognized by CD44 and scavenger receptors CD36, LOX-1 that promote its internalization in the macrophages. Most strikingly, it inhibits the phosphorylation of transcription factors related to M1-like activation states and pro-inflammatory gene expression such as pNFkB, pSTAT1, IRF5. Sulfated HA also modulates macrophage activation in vivo, as shown in a murine model of acute skin inflammation (Hauck et al. 2021). Macrophages isolated from inflamed skin injected with sulfated HA showed reduced expression of IL-1β and up-regulation of IL-10 and IL-1RA in comparison to macrophages from inflamed skin injected with PBS. In addition, signs of inflammation including redness, epidermal thickening and immune cell infiltration was clearly reduced in skin treated with sulfated HA. This suggests sulfated HA as a promising immunoregulatory component for the control of macrophage functions in chronic wounds, especially since the anti-inflammatory effect of sulfated HA on macrophages is retained after integration of the GAG into collagen networks that may serve as delivery matrix (Franz et al. 2013). In a recent study Hauck et al. (2021) developed a hydrogel wound dressing composed of an artificial ECM network that was designed to release sulfated HA over a period of time into wounds. They applied the hydrogels onto wounds of diabetic db/db mice, which showed faster wound closure and improved tissue formation in comparison to wounds treated with control hydrogels without sulfated HA. They detected reduced expression of M1-like macrophage-related pro-inflammatory factors IL-1β, S100A9, and NLRP3 in the wounds, determined increased levels of IL-10 in wound lysates and observed increased numbers of macrophages expressing the M2-like marker RELMα in wound tissue sections. Although Hauck et al. did not
isolate macrophages from the diabetic wounds and further analyzed them for alteration in their activation state, their data clearly show a modulatory effect on macrophages in the diabetic wounds by the hydrogels releasing sulfated HA, which is associated with improved healing of those wounds.

Overall, these studies demonstrate the potential of chemically modified HA as immunomodulating factor that provides control over pro-inflammatory activation states in macrophages and thus inflammatory settings as in chronic wounds. However, further studies are required on the way to clinical translation. In this respect, it is of particularly importance to better understand the modulatory effect of modified HA on distinct macrophage subsets given their different ontogeny and plasticity and their different roles in the regulation of the wound healing process.

GAG-based scavenging of macrophage controlling chemokines

Excessive amount of monocyte-attracting chemokines are found in chronic wounds in patients (Fivenson et al. 1997) and have been correlated to the increased influx of Ly6Chigh monocytes, which give rise to pro-inflammatory macrophages in diabetic wounds in mice (Kimball et al. 2018). Chemokines possess a general high binding affinity to sulfated GAGs and therefore represent a promising target for local in situ scavenging by ECM matrices containing GAGs with high sulfation content (Freudenberg et al. 2016). Based on these facts, Lohmann et al. (2017) investigated the idea of binding excessive chemokines from chronic wounds to interrupt the infiltration of inflammatory cells and restore a natural signaling environment. Building on the principle of the GAG sulfation code, they tailored the naturally occurring GAG heparin that has a high anionic charge density, by specific removal of sulfate groups such that it selectively binds and inactivates pro-inflammatory chemokines, and in particular IL-8 and MCP-1, which are primarily responsible for the infiltration of monocytes, but also PMN. Using cell culture models, mouse models of normal (wild-type mouse) and diabetic (db/db mouse) wound healing, and binding studies with wound fluids from chronic wound patients they demonstrate that hydrogels based on the modified heparins selectively bind these chemokines and consequently reduce migration of monocytes and PMN. In response to reduced immune cell infiltration, diabetic wounds in db/db mice treated with the chemokine-binding hydrogels exhibited a decreased pro-inflammatory signaling environment and showed improved healing. Remarkably, the pro-healing effect of the chemokine-binding hydrogels exceeded that of Promogran™, an ECM-based wound dressing in clinical use (Cullen et al. 2002).

Overall, the study results highlight the therapeutic potential of GAG-based scavenging of excessive pro-inflammatory signals from the wound environment to control macrophage functions and inflammation. This effort has focused on the removal of pro-inflammatory chemokines, but expansion of this principle to other pro-inflammatory cytokines produced by macrophages or targeting them is highly desirable. Hence, further research is required for the design of GAGs with customized binding features for specific factors (pro-inflammatory cytokines) by tailoring GAG structure, size, and sulfation pattern.

GAG-based delivery of macrophage signaling factors

Macrophages respond rapidly to a pallet of signaling molecules and adapt their function according to the requirements, which can be exploited to generate desired macrophage phenotypes. In chronic wounds, therapeutic modulation of endogenous macrophages would involve a clear shift to M2-like activation states accompanied with blocking of pro-inflammatory M1-like functions. Suitable cytokines and growth factors for this approach include IL-1-RA, IL-10, IL-4, TGF-β, SDF-1, TSG6. All of these factors have been shown to suppress pro-inflammatory macrophage activities and/or promote anti-inflammatory and pro-repair functions in inflammatory disease models or impaired wound healing models (Casella et al. 2016; Jung et al. 2017; Krieger et al. 2016; Mirza et al. 2013; Qi et al. 2014; Vander Beken et al. 2019). The factors IL-4, SDF-1 and TGF-β were transferred as releasable signaling molecules into ECM-based materials suitable for delivery in wound healing applications. Advantages of such ECM-inspired cytokine delivery include preservation of cytokine activity, stabilization of cytokines with short half-lives through their interaction with GAGs, sustained cytokine release that can be controlled by adjustable binding affinity between GAGs and cytokines (Hudalla and Murphy 2011).

Schirmer et al. (2016) presented a strategy for sustained delivery of stabilized IL-4 through integration in an ECM-inspired material based on star-shaped poly(ethylene glycol) and heparin. Electrostatic binding of IL-4 with heparin protected the cytokine from degradation in proteolytic environments and allowed its release from the hydrogel for time periods of weeks.

Mice with myeloid-cell-restricted IL-4 gene deletion and consequently impaired IL-4 signaling in macrophages
demonstrate impaired wound healing with defective granulation-tissue formation and impaired stabilization of vascular structures (Knipper et al. 2015) as it is found in chronic wounds. Further, macrophages expressed reduced CD163, CD206, IL-10 and Relmα underlining the importance of IL-4 signaling for the induction of M2-like phenotypes and function. Consistent with this, topical administration of IL-4 in wounds of wt mice increased granulation tissue formation and accelerated wound closure, demonstrating that exogenous administration of IL-4 induces a pro-repair effect (Salmon-Ehr et al. 2000). Heparin-based materials were shown to release high amounts of bioactive IL-4 that induced the differentiation of macrophage with M2-like phenotypes in vitro (Bonito et al. 2018; Schirmer et al. 2016). This suggests their suitability to promote IL-4-regulated macrophage phenotypes in protease-rich pro-inflammatory environments such as chronic wounds. However, further tests on their effectiveness in appropriate skin wound models are needed.

The growth factor TGF-β has been shown to induce macrophages with primarily anti-inflammatory functions (Gong et al. 2012). Okizaki et al. (2015) found impaired TGF-β signaling in wounds of STZ-induced diabetic mice. Administration of TGF-β improved healing of the diabetic wounds. This was associated with a normalized M2-like macrophage activation in the granulation tissue suggesting the delivery of TGF-β as promising therapeutic measure. TGF-β binds to sulfated GAGs (Rider 2006), and hydrogel systems containing heparin or artificially sulfated HA for reversible immobilization of TGF-β exist (Rother et al. 2019; Watarai et al. 2015). Bioactivity of released TGF-β was demonstrated by its ability to induce differentiation of fibroblast into myofibroblasts, but its effect on macrophage activation was not analyzed. However, delivery of TGF-β through ECM-inspired hydrogels in chronic wounds appears particularly promising, as it controls macrophages and fibroblasts and activates pro-repair activation states in both cell types. Analysis of this favorable dual effect in wound settings is highly warranted.

The chemokine SDF-1 is a further factor with dual functions in immune regulation and angiogenesis. SDF-1 plays an important role in the recruitment of endothelial progenitor cells from the bone marrow for initiation angiogenesis in repair processes (Zheng et al. 2007). It was also shown to regulate monocyte infiltration and, in particular, to attract anti-inflammatory monocytes. Krieger et al. (2016) engineered PEG-diacrylate hydrogels functionalized with N-desulfated heparin to control binding and release of SDF-1. SDF-1 releasing hydrogels promoted the infiltration of anti-inflammatory Ly6c<sup>down</sup> monocytes in wounds in a dorsal skinfold chamber in mice. Accumulation of the monocytes resulted in a stabilized microvascular network, which is requires the activation of M2-like macrophages (Knipper et al. 2015). Olekson et al. (2015) showed that the delivery of SDF-1 liposomes promotes granulation tissue formation, endothelial cell proliferation and wound closure in diabetic db/db mice. However, wound macrophages were not analyzed in the study that would have revealed whether the pro-repair effect of SDF-1 involved the modulation of macrophage activation states.

**Future perspectives**

The development of strategies to modulate macrophage activity using ECM-inspired hydrogels as a therapeutic agent in chronic wounds has just begun, and there is great potential to refine and expand these initial approaches. Thus, the listed examples of cytokine-induced macrophage control include only single-factor delivery systems. Signals in the wound environment are much more complex and consist of a cocktail of cytokines and growth factors, which give rise to the versatile macrophage subsets that occur in the wound healing process. In this regard, strategies of a combinatorial delivery of macrophage regulating signals should be considered, e.g. of factors suppressing M1-like activation states and of factors promoting M2-like phenotypes. In addition, other macrophage-instructing cytokines with binding affinity to GAGs could be considered for smart delivery strategies by ECM-based materials in chronic wounds, such as IL-10. For example, injectable HA-based hydrogels with sustained release of IL-10 have been shown to reduce the local and systemic pro-inflammatory cascade in a mouse model of acute kidney injury (Soranno et al. 2016). A further interesting GAG binding factor is tumor necrosis factor-stimulated gene-6 (TSG-6), which has been identified as one important factor in therapeutic regulation of macrophage activation by MSCs or fibroblasts in different inflammatory disease models, including diabetic wound healing (Choi et al. 2011; Ferrer et al. 2017; Qi et al. 2014).

Moreover, electrostatic interaction between cytokines and GAGs is not a consistent fact. Some cytokines may not be suitable for GAG-mediated delivery and other strategies may be preferred as an alternative option. For example, administration of the IL-1β-inhibitory cytokine IL-1RA effectively interrupted persistent M1-like macrophage activation in chronic iron-overload wounds and in diabetic wounds in mice (Perrault et al. 2018; Vander Beken et al. 2019). In the studies IL-1RA was injected in the wound margin or delivered indirectly via administration of IL-1RA releasing dermal MSCs. Qiu et al. (2016), have
demonstrated a method for controlled release of bioactive IL-1RA by embedding the cytokine in HA-chitosan microspheres, which may be employed for delivering IL-1RA in wound healing applications. The use of nanocarriers as delivery system of growth factors has become an exciting research area in recent years. Combination of nanocarriers with ECM-based materials may advance smart strategies for controlled and sustained cytokine delivery (Wang et al. 2017). In this regard worthy to mention the control of macrophage functions via nanotherapeutics (reviewed in Su et al. 2020), involving the delivery of siRNA or miRNA via nanocarrier. Leuschner et al. (2011) developed optimized lipid nanoparticles carrying a CCR2-silencing short-interfering RNA that specifically degrades CCR2 mRNA in monocytes. Injection of the nanoparticles prevented the accumulation of CCR2+/Ly6Chigh pro-inflammatory monocytes at inflammation sites and thereby reduced inflammatory response in various disease conditions in mice.

Mesenchymal stromal cells (MSCs) have been recognized for their powerful immunomodulatory activity on macrophages. Exogenous administration of MSC in impaired healing wounds showed therapeutic effects in human and in mice, which was attributed to the interaction and paracrine signaling between macrophages and MSC (Jiang and Scharffetter-Kochanek 2020). Clinical translation of the immunomodulatory effect of MSCs requires efficient and safe topical delivery of the cells in a carrier system that maintains their immunoregulatory capacities. ECM-based materials have been successful as embedding scaffold in living cell treatment in chronic wound therapy (Zaulyanov and Kirsner 2007) and may represent a suitable carrier for immunoregulating MSCs (Capella-Monsonis et al. 2020). With regard to the influence of ECM on cell behavior and function, and in particular on the fate of stem cells (Guilak et al. 2009), artificial ECM matrices in which biochemical and structural cues can be adjusted and fine-tuned have great potential for the design of scaffolds that provide an optimal environment for delivery of MSCs in an immunomodulating living cell wound care dressing (Nicolas et al. 2020). Smart material design may be capable to even enhance their immunoregulating effect (Wong et al. 2020).

Conclusion

Uncontrolled inflammatory activities and impaired activation of pro-repair functions of macrophages vitally contribute to the highly inflamed environment in chronic wounds eventually leading to the failure of tissue formation and healing. Hence, control of macrophage functions and a shift towards pro-repair activation profiles are desirable for efficient healing. ECM-based biomaterials offer several strategies for modulating macrophages in tissues in a spatiotemporal manner that can be implemented in the design of novel immunomodulatory wound dressings, functionalized with macrophage-controlling signals, or in the development of scaffolds for the delivery of functional cells and factors with immunoregulatory impact on macrophages. In addition, they provide a delivery platform for emerging methods of macrophage control such as nanoparticle carrying siRNA for transcriptional modulation of macrophages.

Although macrophage modulation in impaired wound healing has been successful in mice, their therapeutic effect in chronic wounds in patients remains to be investigated. It also needs to be shown, if in the complex setting of chronic wounds, a modulation of macrophages alone is sufficient to resolve inflammation and push the wound environment into a pro-repair state. Additional anti-inflammatory interventions, control of infection and support of tissue formation may be needed to promote the wound healing process. In fact, ECM-based materials that are already explored for the delivery of anti-microbials (Fischer et al. 2015) and sustained release of angiogenic and pro-fibrotic growth factors in wound healing (Freudenberg et al. 2015), opens up more possibilities for the combination of therapeutic strategies in multifunctional ECM-based wound dressings.

Nonetheless, to develop these experimental approaches into successful therapies, a better understanding of the complex cellular processes during acute and chronic wound healing is needed. In addition, deciphering plastic macrophage phenotypes and the evolution of their functions in response to spatio-temporal changes in their signaling environment in general and during the complex phases of wound healing in particular, is required. In this context comorbidities and immune states of patients should also be considered. New technologies, especially gene sequencing, single cell laser capture, proteomic and microbiome analyses, and high-resolution imaging may provide comprehensive information that aid to define macrophage subtypes with specific activation profiles in physiological healing wounds and to recognize their dysregulation in chronic wounds. At the end, this allows to identify key regulators as therapeutic targets to treat nonhealing wounds.

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