Review Article

The Role of Myeloid Lineage Cells on Skin Healing and Skin Regeneration

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Abstract

Skin healing a complex and well-orchestrated process that involves the coordination and activity of many cell types. Myeloid lineage cells are inflammatory cells recruited to the wound site that remove injured tissue and invading pathogens. Besides this role, due to their ability to secrete a variety of growth factors and cytokines, myeloid cells influence each stage of wound healing (primarily inflammation and proliferation phases). Abnormalities in myeloid cell function lead to pathologies such as excessive and deficient healing. Therapies based on modulating myeloid cells may hold therapeutic potential. However, further research is needed to fully elucidate the spatial and temporal mechanisms of myeloid cells in skin healing. The objective of this review is to discuss recent findings on the role of myeloid lineage cells in skin healing and regeneration.

Keywords: Wound healing; Aging; Inflammation; Angiogenesis

Introduction

Wound healing is a well-orchestrated and dynamic process that aims to restore the skin barrier and underlying tissue after damage. Damage to the skin exposes the organism to microbial invasion and loss of function which may result in significant morbidity and mortality. Skin damage can be caused by a variety of sources such as burn injury (chemical, electrical or heat), infection, surgery or morbidities such as diabetes. In healthy individuals, the skin barrier is successfully restored and repair of deeper dermal layers results in minimal scar formation and loss of structure and function. However, in cases of abnormal skin healing such as deficient and excessive healing, patients experience chronic wounds that become difficult to treat. These chronic wounds affect approximately 6.5 million people in the U.S and it costs approximately 25 billion to treat these patients [1]. These numbers will continue to increase due to the aging population and the rise in the incidence of diabetes, two factors correlated with the development of chronic wounds [2]. In this review, we will discuss the role of myeloid lineage cells in normal and abnormal skin healing and whether modulating these cells has therapeutic potential.

Stages of Wound Healing

Skin can be divided into two main layers – the epidermis and dermis. The epidermis forms a barrier to the external environment and is composed of a stratified keratinized epithelium, hair follicles and sebaceous glands [3]. The dermis is composed of connective tissue that provides mechanical stability to the skin. The superficial papillary dermis and deeper reticular dermis consist of collagen, extrafibrillar matrix and elastic fibers [4]. Following injury, the complex process of wound healing aims to restore tissue integrity and homeostasis. This involves the specific coordination of various cellular and molecular cascades which are crucial to successful skin healing and myeloid lineage cells play an important role during these processes. The wound healing response is typically divided into three overlapping phases: inflammation/hemostasis, proliferation and remodeling. The intensity of these responses is dependent on the size of the wound, type of injury, and severity of injury [5,6].

Inflammation and Hemostasis

The inflammation/hemostasis phase aims to prevent blood loss and infection at the wound site. To prevent blood loss there is vascular contraction and clotting cascade. Activated platelets release various chemoattractants and growth factors that initiate the clotting cascade which results in the conversion of fibrinogen into insoluble fibrin [5,7,8]. The result is a fibrin clot that forms a temporary seal at the wound site. Moreover, the fibrin clot facilitates the infiltration of immune cells such as macrophages, neutrophils and lymphocytes [5,7,8]. Macrophages primarily phagocytose debris and dead neutrophils whereas neutrophils remove foreign objects [9]. The infiltration of immune cells triggers the release of cytokines and growth factors such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), tumor necrosis factor (TNF)-α, interleukin (IL) and transforming growth factor (TGF-β) [5]. The migration and proliferation of fibroblasts and keratinocytes into the wound bed is stimulated by the release of these cytokines and growth factors. This event marks the initiation of the proliferation phase of wound healing.

Proliferation

In humans, the proliferation phase begins approximately three days after injury. This phase is characterized by the formation of granulation tissue, angiogenesis and most importantly re-epithelialization of the epidermis [10]. To facilitate this process, matrix metalloproteinases (MMPs) cleave the extracellular matrix (ECM) which allows fibroblasts and keratinocytes to migrate into the wound bed [10]. The formed temporary matrix leads to restoration of the epidermal layer by epithelial cells [11]. Fibroblasts repair the dermis by producing fibronectin, promoting collagen deposition and other ECM components such as proteoglycans and secreting growth factors [6]. This leads to the formation of granulation tissue. Growth

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factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF), and TGF-β are involved in the proliferative phase [5,6,12]. VEGF supports the formation of new capillaries and blood vessels which provides sufficient nutrients and oxygen to meet the demanding metabolic needs of skin repair [12]. The proliferation and mobility of keratinocytes is mediated by EGF and KGF leading to re-epithelialization [5]. Interestingly, re-epithelialization of the epidermis is also aided by hair-follicle stem cells [13,14]. Recently, lineage tracing studies in mice have demonstrated that hair-follicle stem cells transiently contribute to wound re-epithelialization by producing cells that migrate to the epithelial layer and differentiate [15]. Beneath the epidermal layer, wound contraction is mediated by the transformation of fibroblasts into contractile myofibroblasts by mechanical signals and TGF-β [16]. Dermal compartment repair is not just mediated by fibroblasts. Amini-Nik et al. have shown that skeletal muscle progenitors (Pax7+) adopt a myofibroblastic phenotype and contribute to dermal repair in a process regulated by β-catenin signaling in mice [17]. Perhaps these muscle precursors are needed to improve the tensile strength of the scar tissue.
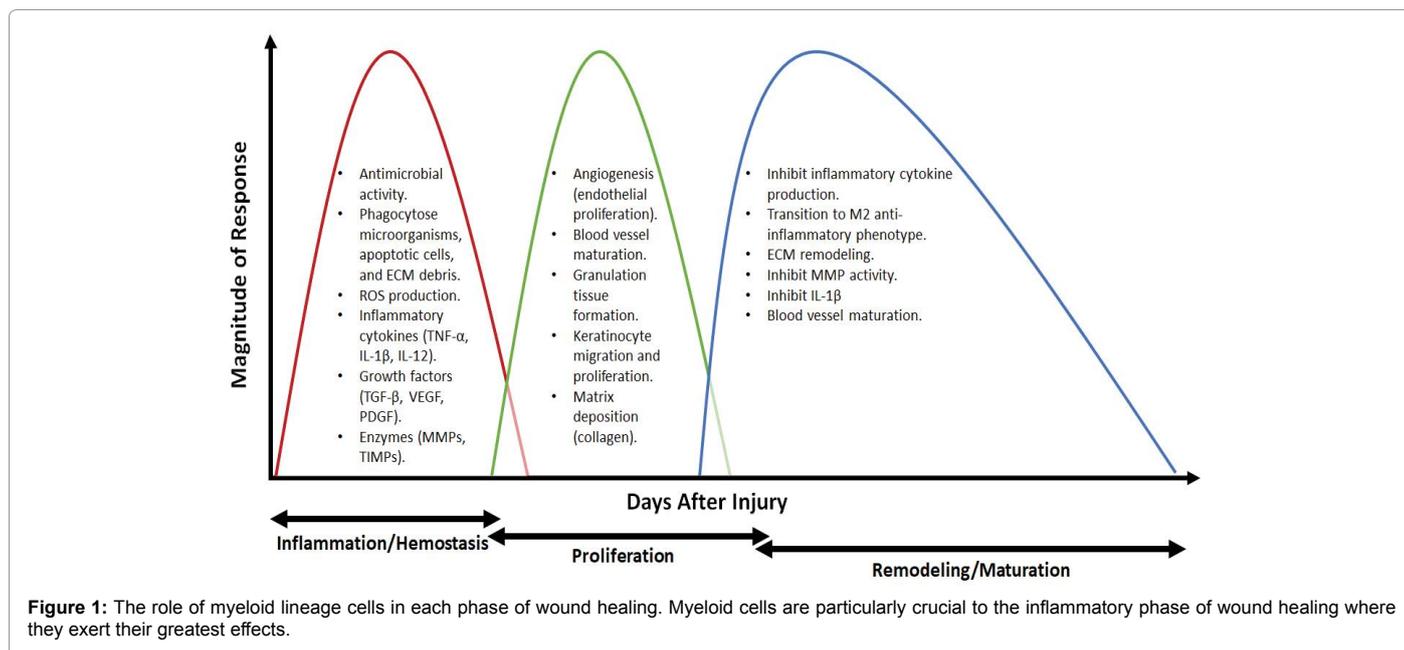
Remodeling and Maturation

The remodeling phase begins once there is satisfactory re-epithelialization and deposition of collagen. This phase is characterized by remodeling of collagen and maturation of the newly formed blood vessel network. Type III collagen and fibronectin are replaced by more organized and mature type I collagen [5]. This transition is needed to improve the tensile strength of the scar; however, the original strength of the uninjured skin is never recovered [10]. This phase can last from weeks to years and results in the formation of mature scar tissue. Unfortunately, wound healing in humans does not result in complete regeneration and the original unwounded dermal structure is never re-capitulated, except in fetus [18]. Overall, wound repair needs to be well-coordinated and timely to prevent infection and restore barrier function. If there are any complications in the wound healing response, deficient or excessive healing may occur (Figure 1).

Abnormal Skin Healing

Most wounds will heal within a few weeks in healthy individuals with scar formation. In contrast, deviations from the normal skin healing process result in pathological conditions such as complex wounds such as deficient and excessive healing. These wounds fail to proceed in an orderly reparative process and as such can persist for months or even years. Deficient healing mainly involves diabetic patients and the elderly, whereas excessive healing is a fibroproliferative response resulting in keloids and hypertrophic scars. A common theory is that the persistence of the inflammatory response prevents the progression to the proliferation and remodeling phases, thus resulting in a chronic wound [19]. This persistent inflammatory response may be a result of alterations in free radical production, growth factor and cytokine production and cellular infiltration [20,21]. Because myeloid cells are fundamental in the inflammation response, it is likely that myeloid cells contribute significantly to chronic wounds.

Deficient Healing

Various predisposing co-morbidities such as diabetes, aging, and obesity contribute to the development of chronic wounds that fail to heal. In diabetes, 25% of patients develop foot ulcers that fail to heal leading to incredible pain and debilitation [1,22]. The failure to progress through the phases of wound healing leads to deficient healing and they all certain characteristics such as increased oxidative stress, excessive protease activity and persistent recruitment of myeloid cells [23]. In deficient healing, the prolonged inflammatory response causes insufficient re-epithelialization, a common outcome in diabetic patients and the elderly [24]. In diabetic ulcer wounds, there is a significant decrease in the TGF-β signaling and fibroblasts become unresponsive to growth factors thus reducing their migration to the wound bed [25-28]. The persistence of pro-inflammatory cytokines such as TNF-α and IL-1β further inhibits the proliferation and migration of fibroblasts [29]. Cytokines such as IL-6 which help mobilize fibroblasts and keratinocytes into the wound bed are also decreased [12]. These irregularities are made worse when elevated levels of MMPs cleave ECM components and reduce collagen deposition [30]. These alterations in cytokine production and

Figure 1: The role of myeloid lineage cells in each phase of wound healing. Myeloid cells are particularly crucial to the inflammatory phase of wound healing where they exert their greatest effects.

activity result in hindered wound closure. Wound closure is similarly delayed in elderly patients due to two main factors. Firstly, stem cells from elderly patients are dysfunctional when compared with younger individuals. Aged bone-marrow derived stem cells (BM-MSCs) have significantly reduced proliferation and angiogenesis compared to younger BM-MSCs [31]. In fact, mesenchymal stem cells (MSC) isolated from the skin of elderly burn patient show diminished cell migration and proliferation capabilities [32]. Secondly, the formation of new blood vessels is impaired in the elderly, thus there may not be sufficient delivery of nutrients and blood to the healing wound [33].

Excessive Healing

Unlike deficient healing, excessive healing is marked by increased fibroblast activity which leads to superfluous deposition of collagen and ECM components [24]. The result is a keloid or hypertrophic scar (skin fibrosis) that significantly impairs organ function. Both keloids and hypertrophic scars are characterized by excessive collagen deposition that is oriented in thick bundles [24]. The difference between keloids and hypertrophic scars arises from the arrangement of collagen fibers. In keloids, there are disorganized sheets of type I and type II collagen arranged randomly and these collagen fibers spill beyond the wound margin; occluded blood vessels are also common [24]. In contrast, hypertrophic scars have an abundance of collagen type III fibers oriented parallel to the epidermis [24]. There is evidence linking persistent inflammation and TGF-β with scar formation. In embryonic skin healing, there is a reduced level TGF-β1 and TGF-β2 which is associated with scar less healing [34]. Interestingly, blocking TGF-β1/2 receptors prevents scarring [35] and Smad3 knockout mice (TGF-β signaling deficient) exhibit less scarring, reduced myofibroblasts and less macrophage recruitment [36]. In human hypertrophic scars, there is a correlation between inflammatory cells and β-catenin levels. In fact, in transgenic mouse models, macrophage migration and adhesion is altered when β-catenin is ablated in these cells [37]. Before we discuss the role of myeloid cells in deficient and abnormal healing, it is important to understand their role in each phase of normal wound healing.

Myeloid lineage Cells During Different Stages of Skin Healing

Myeloid cells, especially macrophages and neutrophils are crucial to the inflammatory phase of wound healing because they protect the host against infection. Recently, the role of macrophages and neutrophils has been extensively characterized in skin healing whereas the influence of mast cells, eosinophils and basophils has less clearly defined. In this section, we will provide a summary of our current understanding of myeloid lineage cells during the different stages of skin healing.

Monocytes and Macrophages

The role of macrophages during wound healing is well-established when compared with other myeloid lineage cells. Resident macrophages are activated by pro-inflammatory mediators and damage associated molecular patterns (DAMPS) that are released in response to skin damage [38]. Circulating monocytes in the bloodstream can also differentiate into macrophages and migrate through the microvasculature to the wound site. Once activated, macrophages phagocytose cellular debris and foreign materials, and these cells are vital to the inflammatory response of wound healing. Macrophages in the wound are primarily derived from circulating monocytes [39]. In humans, monocytes are classified based on the expression of CD14/CD16 and Ly-6C/CD43 and are the major population of circulating

	Human	Murine
Circulating Macrophages	CD14/CD16 Ly-6C/CD43 TNF-α MHC II IL-6 CD11b (EMR1)	Ly-6C^{high} CCR2^{high} CX3CR1^{low} CCR2 CX3CR1 IL-1β IL-6
Tissue Resident Macrophages	CD14/CD16 Ly-6C/CD43	Ly-6C^{low} CCR2^{low} CX3CR1^{high}

Table 1: Markers of circulating and resident macrophages in humans and mice.

monocytes [40]. They are also characterized by the expression of high levels of TNF-α and MHC II [41]. In murine models, these inflammatory monocytes are Ly-6C^{high} CCR2^{high} CX3CR1^{low} and migrate to sites of inflammation in the early stages of wound healing [42]. In contrast, precursors of tissue resident macrophages express Ly-6C^{high} CCR2^{high} CX3CR1^{low} [43]. The expression of Ly-6C is useful in differentiating populations of macrophage cell precursors and it is ideal for detecting activated macrophages in inflammatory tissues such as injured skin. There is also evidence showing that CCR2 and CX3CR1 are crucial for the recruitment of macrophage precursors in the wound site [44-47]. CCR2 signaling is essential for the recruitment of Ly-6C monocytes to skin wounds, which ultimately gives rise to VEGF-expressing macrophages [48]. Moreover, CCR2 deletion in myeloid lineage cells attenuates angiogenesis in skin healing. While Ly-6C, CCR2, and CX3CR1 are useful markers, genetic manipulations involving TNF-α receptor p55, intracellular adhesion molecule-1 and β-1,4 galactosyltransferase show reduced recruitment of macrophages to the wound site [49]. Overall, a complex signaling cascade is needed for the recruitment of macrophages to the wound site (Table 1).

The role of macrophages was first demonstrated in the 1970's by several studies [50-52]. Since then, several studies have illustrated that depletion of macrophages during the inflammatory phase leads to delayed wound closure and granulation tissue formation [53-55]. One of the main roles of macrophages is to promote inflammation. When resident macrophages are exposed to pro-inflammatory cytokines, interferons, DAMPS, and heat shock proteins, they acquire proinflammatory phenotype [38]. Once activated, macrophages begin producing numerous pro-inflammatory cytokines in the wound bed such as IL-1, IL-6 and TNF-α [39]. Furthermore, macrophages produce chemoattractants that recruit additional myeloid cells like neutrophils [39]. Studies have suggested that macrophages are also important for the anti-inflammatory response during the transition to the proliferation phase of wound healing. It has been proposed that upregulation of IL-4 and IL-13 drives the transition to this anti-inflammatory phenotype [56], however recent studies show that this transition can occur independently of IL-4/IL-13 [42,57]. These alternatively activated M2 macrophages also express anti-inflammatory substances like VEGF, insulin growth factor (IGF)-1 and IL-10 [39]. Studies in skin wounds show that the early stages of wound healing characterized by IL-1, IL-6 and TNF-α whereas markers of alternative activation such as arginase 1 (Arg1) and CD206 are increased at later stages [42]. While *in vitro* studies show a clear difference between M1 and M2 macrophages, *in vivo* studies show that the phenotype of macrophages in complex and dynamic as the wound heals. Some populations of macrophages are shown to exhibit both markers of classically and alternatively activated macrophages. For instance, simultaneous expression of TNF-α and mannose receptor has been observed in macrophages in wound suggesting hybrid macrophage phenotypes are important [42] (Table 2).

	Growth Factors, Cytokines and Markers	*In vitro*	*In vivo*
M1 Macrophages	TNF-α IFN-γ TNF-α IL-1β IL-6 VEGF iNOS	• Production of pro-inflammatory cytokines (innate inflammatory response) • Engulf apoptotic cells • Angiogenesis (VEGF production) • TGF-β production • Depletion of macrophages impairs re-epithelialization, angiogenesis, and cell proliferation	
M2 Macrophages	IL-4 and IL-13 IL-10 TGF-β VEGF Arg-1	• Resolution of inflammation • Matrix remodeling	

Table 2: Growth factors, cytokines and markers.

Macrophages influence angiogenesis via the production of VEGF, a growth factor that causes the formation of new blood vessels [58]. Macrophages produce large amounts of VEGF [52,59], but their relative contribution is difficult to assess as VEGF is also produced by other cells like keratinocytes [58]. In a study that ablated VEGF from myeloid lineage cells, myeloid cells were shown to contribute significantly to angiogenesis and dermal VEGF levels [60]. Moreover, excisional wounds lacking myeloid cell-derived VEGF showed delayed healing, whereas incisional were unaffected [60]. Macrophages are also able to influence the production of growth factors that promote cell proliferation during wound healing. Various growth factors such as TGF-α, TGF-β1, and VEGF have been shown to be produced by wound macrophages [61]. In fact, when macrophage adhesion and migration is retarded through CX3CR1 knockout animals, impaired wound healing is observed [44]. Moreover, reduced levels of TGF-β1 and VEGF is observed and fewer myofibroblasts present in the wound [44]. It is important to note that wound macrophages are not the only source of these growth factors as many other cell types in the wound such as fibroblasts, keratinocytes and endothelial cells produce the same factors.

To understand the mechanisms by which macrophage influence different stages of wound healing, specific and conditional macrophage knockouts have been utilized. Mirza et al. used mice expressing diphtheria toxin receptor (DTR) under control of a macrophage-specific promoter to examine the effect of temporal macrophage deletion in the different phases cutaneous wound healing [54]. Macrophage depletion during early repair resulted in impaired granulation tissue formation, delayed re-epithelialization and wound contraction [54]. Deletion of macrophages during the proliferative phase lead to impaired tissue maturation, impaired angiogenesis and reduced cell proliferation [54]. Lastly, macrophage deletion was associated with increased TNF-α and reduced TGF-α and VEGF in the wound [54]. This study suggests that macrophages promote re-epithelialization and dermal repair through regulation of cytokines and growth factors. Other models of wound healing such as corneal healing have also shown that macrophage depletion via clodronate liposomes reduces angiogenesis and ECM deposition during healing [62]. In summary, macrophages have an important temporal effect on skin healing, and further research is needed to elucidate the mechanism by which macrophages influence wound healing. In particular, what drives macrophages to switch from the inflammatory M1 phenotype to anti-inflammatory M2 phenotype, and how this influences the proliferative and remodeling phases of wound healing. While macrophages produce growth factors and cytokines important for wound healing, they are also regulated by signaling pathways such as Wnt/β-catenin. In mice, with a macrophage-specific deletion of β-catenin, there is impaired wound healing and no cells were observed in the region normally contain granulation tissue [37]. Furthermore, these macrophages lacking β-catenin were deficient in migration and adhesion to fibroblasts and could not produce TGF-β1 [37].

Neutrophils

Neutrophils are quickly recruited to the wound site within minutes and are primarily involved in killing invading microorganisms through phagocytosis and degranulation. Neutrophils reach the wound site through a process termed chemotaxis mediated by inflammatory cytokines and H_2O_2 [63]. In mice, burn injuries trigger NLRP3 inflammasome activation in necrotic cells which leads to neutrophils adhesion to the endothelium and intravascular migration to the wound site [64]. Moreover, neutrophil migration is dependent on macrophage inflammatory protein 2 (MIP-2) activities and chemotaxis by formyl peptide receptor 1 (FPR1) [64]. The response of neutrophils is further amplified by neutrophil death, a process mediated by integrin signaling [65]. In neutrophil-depleted mice, wound healing still functions normally with only re-epithelialization being affected [66]. While the main role of neutrophils is to produce protease and substances that kill and degrade pathogens, neutrophils also influence the activity of numerous processes in skin healing. For instance, the expression of various genes involved in angiogenesis, stimulate fibroblast and keratinocyte proliferation and inflammatory cytokines are upregulated in neutrophils at the wound compared to neutrophils in the bloodstream [63], implying that their products serve as essential factors during healing. Animal models have shown that neutrophils are crucial for skin healing due to their role in preventing infection [8]. However, the way in which neutrophils kill pathogens may also cause significant collateral damage to adjacent cellular molecules such as DNA [67]. This is because the proteases and antimicrobial substances produced by neutrophils are not specific to invading pathogens. In some tissues, such as the oral mucosa, there is significantly reduced neutrophil recruitment which results in a wound that heals rapidly and with minimal scar formation [68,69]. Therefore, neutrophils activity may be a double-edged sword: one hand it is crucial to destroy pathogens, but on the other hand it may cause damage to host tissue which is detrimental to wound healing. Indeed, in human wounds that are deficient in healing there is excess neutrophil activity [70]. Neutrophils may also influence wound healing by reducing the inflammatory response at later stages. In normal skin healing, neutrophils undergo apoptosis and are engulfed by macrophages. Interestingly, this engulfment of neutrophils by macrophages causes macrophages to reprogram into an anti-inflammatory phenotype [71,72]. Hence, persistent neutrophil activity leads to an accumulation of apoptotic neutrophils and macrophages may not be able to clear these excessive amounts of neutrophils leading to prolonged inflammation and deficient or excessive healing.

Mast Cells

The role of mast cells in skin healing is not as well-established as macrophages or neutrophils. Despite this, it has been well-documented that mast cells accumulate in the injured and fibrotic skin of humans and mice [73-76]. In mast cell-deficient mice there is accelerated wound healing, however, these mice have other immune alterations such as deficient Gamma Delta T cells (γδ T cells) which may impact wound healing [77]. In another study using the same mice, delayed wound healing was observed; hence there are contradicting findings [75]. Two recent studies utilizing a specific and inducible ablation of mast cells (Mcpt5-Cre mice) showed in no change in dermal wound repair, inflammatory cell recruitment, or fibrosis [78,79]. Studies thus far suggest mast cells do not play an important role in skin healing, but further studies are needed to confirm this notion.

Eosinophils

Eosinophils are granulocyte leukocytes that are specifically regulated by interlukin-5 (IL-5) and are predominately involved in the inflammatory stage of wound healing. Mice overexpressing IL-5 exhibit greater eosinophil infiltration in the wound site and delayed re-epithelization [80], whereas depleting eosinophil depletion accelerated wound closure [81]. Eosinophils are a source of TGF-α [82], a growth factor that promotes keratinocyte migration *in vitro* [83]. Lastly, eosinophils increase expression of collagen and α-smooth muscle actin (α-SMA) in lung fibroblasts during bleomycin-induced lung fibrosis [84]. Like mast cells, the role of eosinophils needs to be better characterized in normal healing and excessive healing like keloids and hypertrophic scars.

Myeloid Lineage Cells in Excessive Skin Healing

Macrophages have been implicated in the development of fibrosis in humans. There is a correlation between the number of macrophages and cellularity in the scar tissue of hypertrophic scar patients. The scar tissue of hypertrophic scar patients [37]. In systemic sclerosis (SSc) patients with associated lung fibrosis, there is increased expression of TGF-β, INF-regulated genes, chemokines and several macrophage markers such as CCL18 and CD163 [85]. Moreover, the skin of SSc patients is enriched with chemokines (CCL18, CCL19 and CXCL13) that are involved with macrophage recruitment, and immunofluorescence showed co-localization of CCL19 with CD163 macrophages [86]. The expression of these chemokines correlated with vascular damage in these patients as well [86]. Like SSc patients, there are increased mast cells and macrophages in the skin of keloid patients, but these are alternative M2 macrophages [87]. This suggests the transition to the M2 phenotype may play role in causing fibrosis. The chemokine receptors, CX3CR1, are also related to fibrosis. Moreover, the higher the expression of CX3CR1, the more severe the pathology is murine heart and kidney fibrosis [88,89]. Similarly, in humans, there is increased number of macrophages expressing CX3CR1 in patients with systemic sclerosis and pulmonary fibrosis [90]. There is a delicate balance of inflammation during wound healing that promotes resolution of fibrosis without compromising the host defence against infection. Dampening the inflammatory response may have therapeutic potential in treating excessive healing. Chemerin15 is a mediator that controls the duration and intensity of the inflammatory response during skin healing [91]. Topical application of chemerin15 during murine cutaneous wound healing reduces inflammation, reduces TNF-α expression, accelerates healing and reduces scarring [91].

In pathologies of excessive healing, such as keloids and hypertrophic scars, prolonged inflammation and TGF-β1 is common [92]. As such, there may be a link between macrophages producing TGF-β1 and development of skin fibrosis. Interestingly, fetal wound healing does not require a response from myeloid cells or TGF-β1 which leads to scar less healing [18]. In murine models, TGF-β production is positively correlated with the number of Ly-6C^{low} macrophages [42,88]. Transgenic mouse models have shed light on the role of macrophages in driving fibrosis. In Smad3 knockout mice with impaired TGF-β signaling, fewer macrophages are recruited to the wound and healing occurs without scarring [93]. Wound macrophages have been shown to produce and release TGF-β1, thus it appears that macrophages contribute to fibrosis. This idea is supported by the finding that in the macrophage-depleted mice, there is reduced collagen accumulation and TGF-β1 mRNA and protein [54]. Another study confirmed that macrophage-depletion reduces TGF-β1 and impairs granulation tissue formation [55]. There is some discrepancy in the literature with regards to macrophage depletion and TGF-β1. Goren et al. found reduced number of myofibroblasts, impaired neovascularization and less wound contraction in macrophage-depleted mice even though TGF-β1 bioactivity was unaltered [53]. The aforementioned studies suggest that macrophages are required for the initiation of fibrosis, but their activity may also influence the remodeling phase of skin repair. Depleting macrophages with carbon tetrachloride during the progression of fibrosis results in decreased myofibroblasts and collagen deposition in the liver [94]. In contrast, macrophage depletion during recovery of fibrosis led to reduced matrix degradation and failure to resolve fibrosis [94]. Macrophages are likely needed for the resolution of fibrosis due to their ability to induce apoptosis and engulf cells like myofibroblasts that accumulate excessively during fibrosis, therefore this raises the possibility for the existence of a different lineage of macrophages besides what has been reported so far.

A growth factor like TGF-β1 that may influence wound remodeling by macrophages is milk fat globule epidermal growth factor 8 (Mfge8) [95]. This growth factor is expressed by macrophages and binds to collagen thus stimulating collagen uptake in the tissue [95]. Resolution of fibrosis is impaired in mice lacking Mfge8 due to decreased collagen turnover, and this defect was rescued after administration of recombinant Mfge8 [95]. This study suggests that Mfge8 secreted by macrophages is crucial for the resolution of fibrosis through its regulation of collagen removal. M1 and M2 macrophages have distinct roles in the progression of excessive healing. M2 macrophages express arginine, an amino acid crucial for the synthesis of glutamate and proline which lead to collagen synthesis [96]. The progression of interstitial fibrosis and hepatic fibrosis is worsened in collagen 4A3-deficient mice because there is significant M2 macrophage infiltration [97-100]. Treatment with antagonist of CCR1, a receptor expressed by M2 macrophages, reduces macrophages, attenuates fibrosis, and prolongs survival in collagen 4A3-deficient mice [101].

Another important myeloid lineage cell that contributes to wound healing and fibrosis are circulating monocyte-derived fibrocytes. Monocyte-derived fibrocytes contribute to the remodeling phase of skin repair by secreting MMPs which degrade components [102]. These fibrocytes express the hematopoietic marker CD45 and express a phenotype intermediate between monocytes and fibroblasts [102]. Fibrocytes enter the wound site via CXCR4 and CXCL12 receptors and influence skin repair by secreting large amounts of collagen-1 [103]. In addition to secreting collagen-1, fibrocytes can also differentiate into fibroblasts and myofibroblasts after TGF-β1 stimulation [104,105]. In patients with lung fibrosis, there is an increased CXCL12 level which is correlated with fibrocyte accumulation and early mortality [106].

Similarly, in scleroderma patients, the severity of dermal fibrosis is positively correlated with the number of dermal fibrocytes [107]. It seems that excessive monocyte-derived fibrocyte activity may contribute to fibrosis through excessive collagen-1 deposition and myofibroblastic differentiation.

Inflammation is a key factor in the development of scarring, and since macrophages are the primary mediators of this response it stands to reason that they may be associated with skin fibrosis. Indeed, excessive macrophage activity is associated with persistent inflammation and skin fibrosis in humans and mice. Macrophages likely contribute to excessive healing through their persistent secretion of pro-inflammatory cytokines and TGF-β1, a growth factor known to cause fibrosis. The switch from a pro-inflammatory M1 phenotype to an M2 anti-inflammatory may also be involved in the resolution of inflammation and matrix remodeling. Whether the observed alteration in macrophages in excessive healing is a secondary event to the main pathology or is the main underlaying mechanism for initiation of excessive healing is a subject to explore further. Modulating macrophage activity (e.g. decrease macrophages or promote the switch to M2 phenotype) to decrease inflammation may have therapeutic potential in attenuating excessive healing.

Myeloid Lineage Cells in Deficient Healing

As we have discussed, myeloid lineage cells have significant effects on wound inflammation due to their production of inflammatory cytokines and growth factors they secrete. This is important in the context of deficient healing which is characterized by excessive inflammation, myeloid cells likely play an important role. Macrophages isolated from diabetic human and mouse wounds have sustained NLRP3 inflammasome activity and IL-1β expression [108,109]. Treating diabetic mouse wounds with a topical pharmacological agent that inhibits NLRP3 inflammasome activity rescues deficient healing [109]. These studies suggest that macrophages contribute to deficient healing by increasing inflammasome activity within the skin. Blocking inflammatory cytokines such as IL-1β, TNFα or IL-17 in diabetic mice reduces pro-inflammatory macrophage activation and accelerates skin wound healing [108,110,111]. Like diabetes, elderly patients experience deficient wound healing. Excisional wounds from aged mice heal more slowly when compared with younger mice due to increased macrophage infiltration during the early stages of skin healing [112]. Other age-related alterations during skin healing include delayed vascularization and decreased collagen deposition and remodeling [113]. In mouse models of diabetic wound healing, older mice show delayed healing, reduced hypoxia-inducible factor 1 expression, and fewer bone-marrow derived cells in the wound [114,115].

In deficient healing, there is an imbalance of matrix metalloproteinases (MMPs) which degrade the ECM and tissue inhibitors of metalloproteinases (TIMPs) which neutralize MMPs [23]. The persistence of MMPs within the wound results in excessive destruction of ECM components, growth factors and receptors [23]. Indeed, chronic wounds contain increased amounts of proteases which have been shown to degrade growth factors like VEGF and PDGF *in vitro* [116,117]. In aged rat skin, there is an overexpression MMP2 [118], a finding that in consistent with studies showing increased protease activity (i.e., MMP2) in the skin of elderly patients, particularly postmenopausal woman [119]. Alveolar macrophages can produce large amounts of MMP 1, MMP2, MMP7 and MMP12. In skin healing, macrophage-derived MMP-10 is crucial to wound healing and collagen breakdown [120]. Moreover, deletion of macrophage-derived MMP-

10 in mice results in decreased expression of other metalloproteinases like MMP-8 and MMP-13 illustrating the importance of macrophages in tissue remodeling [120]. Overall, the breakdown of ECM components through phagocytic uptake and intracellular degradation by macrophages likely plays an important role in deficient healing. Inflammatory cytokines produced by macrophages promote MMP production; therefore macrophages may indirectly hinder the repair process. Macrophages in diabetic mice show both M1 and M2 phenotypes during the early stages of wound healing. At later stages of wound healing, macrophages from these diabetic mice fail to transition to an M2 phenotype and continue to exhibit the inflammatory M1 phenotype [116]. Interestingly, injection of M2 polarized macrophages in full-thickness excisional wounds in wild-type (C57BL/6) and diabetic mice did not improve wound closure or re-epithelialization in either group [109]. The anti-inflammatory properties of M2 macrophages do not appear to be beneficial to wound healing in wild-type or diabetic mice. Further studies are needed to fully elucidate the mechanisms of macrophage polarization and how this influence normal and aberrant skin healing.

Excessive myeloid cell recruitment and pathological inflammation is key in the progression of deficient healing. Indeed, gene profiling of patients with chronic diabetic foot ulcers showed increased M1 macrophage gene expression relative to M2 macrophages during the early stages of healing and non-healing ulcers [121]. Four weeks later, M1 gene expression was 90 times higher in non-healing wounds compared to healing wounds suggesting that persistent M1 activity is associated with deficient healing [121]. In some patients with chronic venous leg ulcers, iron deposits are visible on their legs which are associated with lesions [122]. It is hypothesized that accumulation of iron increases the activity of MMPs which worsens the pathology observed [122]. In a murine wound model, overloading macrophages with iron create a persistent proinflammatory M1 phenotype that impairs wound healing. Indeed, iron overloaded macrophages have been observed in patients with venous ulcers [123] suggesting that excess iron deposition may support a persistent pro-inflammatory macrophage phenotype that promotes deficient healing. The efficacy of treating chronic wounds with exogenous macrophages is currently being explored. These activated macrophages do not correspond to M1 or M2 phenotype and have increased expression of IL-1β, IL-6, TGFβ and IL-4 [124,125]. The use of exogenous activated macrophages in treating chronic wounds has been attempted in hairless guinea pigs [126]. Researchers found that repeated injections of macrophages after chronic sulfur mustard wounds found significantly decreased wound area and improved barrier function at 10 days post-injury [126]. In a two-arm, non-parallel, open controlled trial tested the efficacy of macrophage injection in treating stage III and stage IV pressure ulcers in 100 elderly patients [127], injection of human activated macrophages in suspension was safe and showed efficacy by improving wound closure rates [127]. Topical application of the macrophage activator, glucan, improves wound healing by increasing macrophage numbers and improving re-epithelialization [128,129]. Attracting more macrophages to the wound site via application of the chemoattractant, MCP-1, has also been shown to improve wound healing [47]. There seems to be a trend in macrophage-based therapy to control macrophage recruitment rather than inhibit macrophage function. There is limited evidence to support the use of macrophage-based therapies in clinical settings of deficient healing; nonetheless, this remains a promising area of research.

Neutrophil accumulation has also been implicated in chronic wounds that fail to heal. In humans, chronic wounds show elevated inflammation and prolonged neutrophil infiltration [70,130,131]. This

Myeloid Lineage Cell	Excessive Healing	Deficient Healing
Monocytes and Macrophages	• Increased macrophage recruitment • Increased expression of chemokines • Secretion of TGF-β1 • Altered MMP activity • Increase INF-regulated genes and several macrophage markers (e.g. CCL18 and CD163) • Macrophage-depletion attenuates fibrosis	• Macrophages from diabetic humans and mice have persistent NLRP3 inflammasome activity and IL-1β expression • Macrophage gene expression higher in humans and mice with non-healing wounds • Increased MMP production • Delayed re-epithelialization • Delayed vascularization and collagen deposition
Neutrophils	• N/A	• Increased neutrophil infiltration and activity (e.g. NETs) • Delayed re-epithelialization • Neutrophil depletion rescues diabetic wound healing • ROS generation

Table 3: Summary of the role myeloid cells play in excessive (e.g. fibrosis) and deficient (e.g. diabetes, aging) skin healing.

is important because increased activity of anti-microbial mechanisms such as the neutrophil extracellular trap (NET) may induce damage in the wrong tissues. In mouse diabetic wounds, digesting NETs with DNase1 improves wound repair suggesting that superfluous NET activity by neutrophils contributes to deficient healing [132]. Neutrophil depletion studies in mice further support the notion that increased neutrophil activity leads to deficient healing. Diabetic mice show delayed re-epithelialization with correlates with increased neutrophil infiltration, and neutrophil depletion rescues wound healing in these mice [133]. Adenosine is a molecule with anti-inflammatory and angiogenic properties that may modulate the resolution of fibrosis. In neutrophils, Adenosine signals through the G-protein coupled receptors, A2A and A2B, which inhibits neutrophil recruitment, reduces neutrophil ROS generation, and promotes the transition towards an M2 phenotype [134]. A recent clinical trial found improved healing in patients with diabetic foot ulcers after administration of the adenosine receptor A2A agonist, polydeoxyribonucleotide [135]. Therefore, attenuating neutrophil activity may be a promising avenue of improving deficient healing (Table 3).

Myeloid Lineage Cells for Skin Regeneration: The Past, Present and Future

While myeloid lineage cells are important in skin injury and repair, they might also be useful in regenerating skin. Tissue-resident macrophages control homeostasis and organ regeneration in many tissues [136]. In fact, a study showed that macrophage-depletion in adult salamanders impairs full limb regeneration but has a limited effect on wound closure [136]. Another macrophage-depletion study showed that macrophages are needed for angiogenesis and complete tissue regeneration in neonatal hearts [137]. In the absence of macrophages, neonatal hearts lose regenerative capacity and form fibrotic scars like those seen in adult animals [137]. Monocytes and macrophages may modulate skin regeneration because of their ability to regenerate peripheral nerves. Tissue-resident macrophages secrete VEGF, producing new vasculature that allows proliferating Schwann cells to reconnect nerves [138]. Therefore, macrophages producing VEGF in the skin injury may be crucial for nerve regeneration which is an often-overlooked aspect of skin repair.

In addition to improving angiogenesis, macrophages produce a variety of factors that influence progenitor cells that are vital for complete regeneration. Macrophages secreting IL-10 are crucial for stem cell renewal in the murine retina and a switch to an M2 phenotype during muscle regeneration [139,140]. The transition of macrophages to an anti-inflammatory phenotype is important in the survival of mesenchymal stem cells [141-143]. In skin regeneration, macrophages have also been implicated in the regeneration of new hair follicles.

Chen et al. showed that removal of hair follicles causes adjacent hair follicles to release CCL2 which leads to the recruitment of macrophages secreting TNF-α [144]. This recruitment of pro-inflammatory macrophages provides key signals that aid in the regeneration of new hair. Macrophages likely promote a regenerative environment; therefore a better understanding of macrophage function may reveal ways to regenerate skin. How to fine tune their role in favor of regeneration and avoid fibrosis will be the subject of future research in skin biology and skin regeneration.

Conclusion

Myeloid lineage cells play a crucial role in wound healing, particularly in the inflammation and proliferative phases. Aberrations in myeloid cell function appear to be important contributors to pathologies such as excessive and deficient healing. The incidence of chronic wounds is rising due to an aging population and increasing prevalence of diseases such as diabetes. Further elucidation of the mechanisms by which myeloid cells influence wound healing is needed. A better understanding of myeloid cell signaling and processes will lead to the development of better treatment options. Overall, macrophage-based therapies hold clinical relevance due to their therapeutic potential.

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