Disclosure of the Culprits: Macrophages—Versatile Regulators of Wound Healing

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Significance: Macrophages are invariably present and tightly regulate all phases of adult wound healing, including inflammation, granulation tissue formation, and matrix deposition with the unavoidable outcome of scar formation. In response to environmental cues, macrophages mount a “classical” pro-inflammatory M1 activation as opposed to the “alternative” M2 phenotype, with wound macrophages having long been viewed as M2 macrophages.

Recent Advances: Recent studies rather point to large temporal and phenotypic variations of wound macrophages subsets. Therefore, a functional classification of macrophages according to wound-healing phases appears to better meet the *in vivo* complexity. In an ideal but simplistic scenario grossly reflecting normal wound healing, initial tissue injury induces inflammatory M1-like macrophages, which, upon engulfment of apoptotic neutrophils or in response to other inflammation dampening stimuli, switch toward anti-inflammatory M2-like macrophages and further toward growth factor-producing pro-fibrotic M2a-like macrophages. Although not yet documented for skin wounds, a subset of metalloproteinase-producing fibrolytic M2c-like macrophages may contribute to fibrosis resolution. Recent work identified a diversity of novel macrophage phenotypes associated with normal and pathologic wound healing, most of them ranging out of the M1/M2 paradigm. Iron-overloaded M1-like macrophages represent such a novel phenotypic subset driving the non-healing state of chronic venous leg ulcers.

Critical Issues: Despite growing evidence that macrophage dysfunctions are, at least in part, responsible for pathologic wound healing, including non-healing wounds and excessive scar formation, these are hardly specifically addressed even by modern therapeutic strategies.

Future Directions: If characterized in sufficient detail, distinct macrophage subsets and their impaired functions provide ideal targets for improving wound healing.

SCOPE AND SIGNIFICANCE

RESEARCH FROM THE past years has uncovered the unique plasticity of macrophages to rapidly adapt their functions to molecular environmental cues and to reshape most differing inflammatory responses. Some of these advances will be discussed later in the light of physiological and pathological wound healing as a

model of macrophage-driven inflammatory responses.

TRANSLATIONAL RELEVANCE

The use of transgenic mouse models enabling specific macrophage depletion at any wound-healing phase revealed that wound macrophages regulate all phases of adult healing. This is made possible by the

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Abbreviations and Acronyms

CCL2 = C-C chemokine ligand 2
CCR2 = C-C chemokine receptor type 2
CVUs = chronic venous leg ulcers

(continued)

Abbreviations and Acronyms (*continued*)

CX3CR1 = fractalkine receptor
CX3CL1 = fractalkine ligand 1
DAMPs = damage-associated molecular patterns
DTR = diphtheria toxin receptor
ECM = extracellular matrix
GM-CSF = granulocyte macrophage-colony stimulating factor
ICOS = inducible costimulator
ICOSL = ICOS ligand
IL = interleukin
iNOS = inducible nitric oxide synthase
IFN = interferon
LAD1 = leukocyte adhesion deficiency syndrome 1
LPS = lipopolysaccharide
MCP-1 = macrophage chemoattractant protein 1
MIP-2 = macrophage inflammatory protein 2
PAMPs = pathogen-associated molecular patterns
PDGF = platelet-derived growth factor
ROS = reactive oxygen species
TGF- β_1 = transforming growth factor beta-1
TLR = Toll-like receptor
TNF α = tumor necrosis factor alpha
VEGF = vascular endothelial growth factor

unique capacity of macrophages to dynamically adapt their functional activation to changing stimuli in the wound environment. The resulting macrophage phenotypes range beyond the classical M1/M2 classification, and their characterization has just begun. The most favorable wound macrophage functional dynamics is met during acute wound healing in wild-type mice as a model of physiologic adult wound healing. Animal models with delayed wound closure have been generated to serve as models for chronic wounds as occurring in diabetic, pressure, or chronic venous leg ulcers (CVUs). Thus, persistence of pro-inflammatory tumor necrosis factor alpha (TNF α)-producing M1-like macrophages in diabetic wounds and CVUs amplifies tissue break-down and impairs wound healing. On the other hand, over-activation of pro-fibrotic M2a-like macrophages promotes scar formation. The role of fibrolytic M2c/M_{reg}-like macrophages in the resolution of skin fibrosis still needs to be deciphered.

CLINICAL RELEVANCE

Identification of macrophage functional subsets with a causal role in pathologic wound healing may revolutionize current therapeutic strategies. In CVUs, the nonhealing state is driven by iron-overloaded macrophages that release pro-inflammatory mediators and reactive oxygen species (ROS). These could be targeted by TNF α inhibitors, iron chelators, and ROS scavengers that improve wound healing. Impaired phagocytic macrophage functions are associated with diabetes and accumulate with senescence, most likely contributing to impaired wound healing in diabetic or elderly patients. Boosting macrophage functions may improve overall wound healing in these patients, as previously demonstrated with granulocyte

macrophage-colony stimulating factor (GM-CSF). Increasing the phagocytic behavior of macrophages populating mature wound tissues may reduce fibrosis and scar formation.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

The phases of adult wound healing

Directly after skin injury, a series of biomolecules that are released either by invading pathogens (pathogen-associated molecular patterns [PAMPs]) or by injured tissue (damage-associated molecular patterns [DAMPs]) act as exogenous or endogenous signals to initiate an inflammatory response which is orchestrated by macrophages.^{1,2} Sterile fetal dermal wounds offer the ideal model of fast and scarless healing, as they are virtually free from PAMPs and DAMPs and infiltrated with negligible amounts of macrophages.³ In contrast, adult wound healing depends on inflammation and growth factors that promote rapid healing, at the expense of fibrosis and scarring.⁴

Therefore, adult wound healing is an evolutionary optimized process that follows a sequence of events comprising three interdependent overlapping phases: (1) inflammation; (2) granulation tissue formation and myofibroblast-driven wound contraction, and, eventually; (3) matrix deposition and remodeling.^{4,5}

Inflammation. Polymorphonuclear neutrophils are the first leukocytes to infiltrate the injured tissue, tightly followed by monocytes that are recruited and differentiate at wound sites toward wound macrophages. The early wound macrophages are highly inflammatory, producing increased amounts of pro-inflammatory cytokines, proteases, and ROS that combat contaminating pathogens, and highly phagocytic, to remove necrotic cells and tissue debris

and thus protect from further tissue damage. On phagocytosis of apoptotic neutrophils that accumulate with high numbers as inflammation resolves, macrophages start producing anti-inflammatory cytokines and growth factors.⁶

From the functional perspective of macrophage activation, the inflammatory phase of wound healing may, thus, be sub-divided in an early inflammatory response (days 1–4 after wounding) that is dominated by DAMPs and PAMPs which activate pro-inflammatory macrophages, and a late inflammatory response (days 5–7 after wounding) that is marked by the accumulation of apoptotic cells, where anti-inflammatory, tissue repair-promoting macrophages prevail.

Granulation tissue formation and wound contraction. Anti-inflammatory macrophages produce growth factors to recruit fibroblasts and activate them to differentiate toward myofibroblasts in the wound tissue. Myofibroblasts develop important contractile forces and efficiently produce extracellular matrix (ECM) components to bridge the gap between the wound margins.⁷ In parallel, macrophages and activated fibroblasts release pro-angiogenic factors to recruit endothelial progenitor cells and enable new vessel formation. Macrophages, myofibroblasts, and neovessels build the granulation tissue with a further release of growth factors that stimulate keratinocyte proliferation and re-epithelialization.^{4,5}

Matrix deposition and remodelling. With continuous presence of growth factors, granulation tissue formation, and matrix deposition, the skin defect is temporarily repaired with an abundantly cellular and fibrous scar tissue. This physiologic fibrosis, providing tensile strength to the newly formed tissue, thereafter is reshaped mainly by metalloproteases that are released by keratinocytes, fibroblasts, endothelial cells, and macrophages; most of these cells will eventually undergo apoptosis.

Macrophages control adult wound healing

Observations from fetal wounds which heal without fibrosis in the presence of only a few wound infiltrating macrophages fuelled for a long time the hypothesis that inflammation is not required for wound closure.³ This was strengthened by the findings that mice deficient for the spleen focus-forming virus (SFFV) proviral integration 1 (PU.1) transcription factor lacking macrophages and functional neutrophils due to impaired myelopoiesis healed with a normal healing rate but without scar formation. Of notice, in these experiments, wounds

were inflicted in newborns treated with antibiotics.⁸ Thus, a markedly reduced inflammatory response and asepsis are two conditions that are essentially required for scarless wound healing.

Meantime, the requirement of macrophages for physiologic adult tissue repair, as suggested by classical studies of Leibovich and Ross,⁹ is well understood. In the past years, the use of transgenic mouse models with human diphtheria toxin receptor (DTR) expression restricted to monocyte/macrophage populations enabled the induction of specific macrophage depletion at any wound-healing phase. Macrophage depletion before wounding resulted in delayed re-epithelialization, reduced collagen deposition, impaired angiogenesis, and decreased cell proliferation associated with increased levels of TNF α and reduced transforming growth factor beta-1 (TGF- β_1) and vascular endothelial growth factor (VEGF) expression in the healing wounds.¹⁰ In a LysM-Cre DTR model, macrophage-devoid wounds exhibited increased and prolonged persistence of pro-inflammatory cytokine interleukin (IL)-1 β , neutrophil- (macrophage inflammatory protein 2 [MIP-2]), and macrophage- (macrophage chemoattractant protein 1 [MCP-1]) chemotactic factors and reduced expression of TGF- β_1 and VEGF, resulting in impaired wound contraction by loss of myofibroblast differentiation and defective wound neo-vascularization.¹¹ In both studies, the persistence of pro-inflammatory markers was attributed to the uncontrolled activation of Ly6C⁺ and Ly6G⁺ circulating precursors and of wound infiltrating neutrophils.

Valuable information for macrophage functions in coordinating different phases of wound healing was provided by a study using the inducible LysM-Cre DTR system to deplete wound macrophages at sequential time points after injury. Macrophage depletion in the early inflammatory phase severely reduced granulation tissue formation and re-epithelialization; whereas macrophage ablation later on, during granulation tissue formation, resulted in severely disturbed neoangiogenesis and wound closure due to insufficient TGF- β_1 and VEGF concentrations in the macrophage-depleted wounds.¹² Of notice, depletion of macrophages in the remodeling phase did not further affect wound healing. Thus, macrophages fulfil different functions at various stages of wound healing.

Origins of wound macrophages

Wound macrophages mainly originate from peripheral blood monocytes that are recruited to the wounded tissue and differentiate into macrophages

of diverse phenotypes and specializations. In mice, circulating monocytes present with at least two distinct phenotypes according to their surface marker expression and fate.¹³ Monocytes defined by high expression of the myeloid lymphocyte antigen Ly6C and of the chemokine receptor-2 (CCR2) (Ly6C^{high}CCR2^{high}) are rapidly mobilized during inflammation and, depending on local and systemic conditions, exert both activating and inhibitory immune responses.¹⁴ The second monocyte pool with a high expression of the fractalkine receptor CX₃CR1 and low Ly6C and CCR2 levels (Ly6C^{low}CCR2^{low}CX₃CR1^{high}) that mainly patrol vascular endothelia were proposed to promote healing by myofibroblast accumulation, angiogenesis, and collagen deposition.^{14,15}

An emerging view supports the sequential recruitment of Ly6C^{high} and Ly6C^{low} monocytes to wounded tissue in response to CCR2/CCL2 and CX₃CR1/CX₃CL1 interactions, respectively.¹⁶ Early recruited monocytes are pro-inflammatory and infiltrate the wound site in response to CCL2 that is most likely released by activated resident macrophages and fibroblasts in the injured skin.^{17,18} Patrolling Ly6C^{low}CX₃CR1^{high} monocytes lack CCR2 expression and cannot respond to CCL2 in early inflammatory wound tissue. Instead, they infiltrate the wound area in the late inflammatory phase in response to CX₃CL1 that is expressed by wound macrophages and endothelial cells. Interestingly, CX₃CR1-deficient mice exhibited severely delayed wound healing with reduced macrophage and myofibroblast recruitment and low concentrations of TGF- β ₁ and VEGF in the granulation tissue.¹⁹ In contrast, the absence of Ly6C^{high}CCR2^{high} inflammatory monocytes/macrophages in myeloid-restricted CCR2 deficient mice did not affect overall wound closure despite impairing the early phases of neovascularisation and myofibroblast differentiation.²⁰ Thus, the early recruitment of Ly6C^{high}CCR2^{high} monocytes may reflect an evolutionary conserved response to injury in pathogen-rich milieus, which, in experimental/surgical aseptic conditions or in the pathogen-reduced environment nowadays, may not be required in its full amplitude. In this case, Ly6C^{high}CCR2^{high} monocyte recruitment may become detrimental for tissue repair and/or set the stage for scar formation.

In the human myeloid compartment, a subset of CD16⁻ monocytes have high CCR2 and low CX₃CR1 expression, resembling murine Ly6C^{high}CCR2^{high} inflammatory monocytes, while CD16⁺ monocytes express CX₃CR1 at high levels, similar to murine Ly6C^{low}CX₃CR1^{high} cells. Whether these subpop-

ulations are counterparts of the murine monocyte/macrophages subsets performing sequential infiltration of wound margins and differentiating toward M1- or M2-like wound macrophages is less investigated. We found both CCR2 and CX₃CR1 to be highly expressed by early recruited macrophages in acute wound margins of healthy volunteers and to be downregulated in late inflammatory macrophages,²¹ but the detailed kinetics of human wound macrophage polarization still needs to be studied.

Macrophages adopt a variety of phenotypes at wound sites

Depending on the stimuli encountered in the injured skin, wound macrophages may adopt a variety of phenotypic and functional profiles.^{22,23} The current paradigm postulates that macrophages, mirroring the Th1/Th2 polarization of helper T cells, polarize *in vitro* toward classically activated M1 macrophages in response to interferon (IFN) γ and lipopolysaccharide (LPS) or toward alternatively activated M2 macrophages after stimulation with IL-4 and IL-13.²⁴ M1 macrophages support microbicidal and cytotoxic host defence functions by releasing high concentrations of pro-inflammatory cytokines (TNF α , IL-1 β , IL-6, and IL-12) and ROS.²⁵ In contrast, M2 macrophages produce anti-inflammatory cytokines (IL-10), growth factors (TGF- β ₁, VEGF, and platelet-derived growth factor [PDGF]) and ECM, and express markers associated with tissue repair (arginase 1, chitinase 3-like 3 lectin, FIZZ1 [found in inflammatory zone 1], and factor XIII-A).^{26–28} Therefore, wound macrophages have long been viewed as alternatively activated M2 macrophages.²⁵

However, an improvement of cell isolation techniques from the skin in recent years enabled the observation of functional macrophage polarization *in vivo* during physiologic and pathologic wound healing. Using mechanical disruption combined with enzymatic digestion, usually with collagenase and hyaluronidase, wound cells could be successfully isolated and further characterized by flowcytometry or genomic analysis.^{20,21,29} An alternative, more laborious, method using subcutaneously implanted polyvinyl alcohol sponges enabled the recovery of relatively pure populations of wound inflammatory cells and even further functional analyses with purified wound macrophages.¹⁸

Recent *in vivo* studies on macrophages freshly isolated from human and murine acute wounds by enzymatic digestion²¹ or from sponges implanted in murine aseptic wounds¹⁸ demonstrate that

wound macrophages adopt a diversity of phenotypes in response to different environmental cues during the tissue repair progression. In both studies, macrophages displayed both M1 and M2 activation markers throughout wound healing,^{18,21} with pro-inflammatory M1 markers (TNF α , IL-12, and IL-6) prevailing in the early inflammatory phases, and M2 markers (CD206, arginase 1, Dectin-1, IL-10, IL-4R α , and TGF- β ₁) increasing in late inflammatory and granulation tissue phases. These findings may suggest that, at any time point of healing, wound macrophages display “hybrid” M1/M2 activation phenotypes which may enable their versatility to rapidly switch between different functions. Therefore, instead of classifying *in vivo* wound macrophages into M1 or M2 phenotypes, we prefer to designate as M1-like pro-inflammatory macrophages the population occurring early during wound repair, elicited by classical pro-inflammatory stimuli such as IFN γ and bacterial LPS, with high phagocytic activity for non-opsonized particles and predominantly, but not exclusively, expressing pro-inflammatory M1 markers, carrying out host defence as a major task. Likewise, M2-like macrophages will designate the population mainly expressing anti-inflammatory M2 and tissue remodeling markers during later phases of wound healing, with anti-inflammatory and tissue regenerative functions. In fact, even this classification has its limitation for certain macro-

phage subpopulations occurring in both pathologic and physiologic conditions, that will be detailed next.

To date, at least four different macrophage phenotypes have been proposed to be induced at different stages of normal tissue repair: (1) pro-inflammatory M1-like macrophages, induced by necrotic cells and/or pathogens in early inflammation; (2) anti-inflammatory M2-like macrophages, induced in late inflammation that synthesize mediators which dampen inflammation (IL-10) and promote tissue repair (TGF- β ₁); (3) pro-fibrotic M2-like macrophages, induced in the phase of new tissue formation which produce growth factors and ECM; and (4) fibrolytic M2-like macrophages, induced in the ischemic scar milieu which secrete proteases and promote tissue remodeling (Fig. 1).³⁰ Recent studies suggest this phenotypic/functional macrophage classification to be applied to cutaneous wound healing as well, and that dysfunctions of any macrophage phenotype can result in disturbed healing.

Pro-inflammatory functions of wound macrophages. Since early M1-like wound macrophages highly express CCR2,²¹ they most likely differentiate from Ly6C^{high}CCR2^{high} inflammatory monocytes. Dermal resident macrophages may as well rapidly mount pro-inflammatory activation on injury, in response to PAMPs or DAMPs in the fresh wound.³¹ Both resident and recruited M1-like

Figure 1. Functional macrophage phenotypes proposed to be induced at different stages of normal tissue repair: (1) pro-inflammatory M1-like macrophages are induced by necrotic cells (damage-associated molecular patterns) and/or infection (pathogen-associated molecular patterns) in early inflammation (days 1–5 after wounding) and produce pro-inflammatory cytokines, proteases, and reactive oxygen species (ROS) to support host defence; (2) anti-inflammatory M2-like macrophages are induced on phagocytosis of apoptotic cells, which abundantly populate the wound tissue in the late inflammatory phase, around day 5 after wounding. They synthesize mediators that dampen inflammation (interleukin [IL]-10) and lay the foundation for new tissue formation (vascular endothelial growth factor [VEGF], transforming growth factor beta-1 [TGF- β ₁]); (3) pro-fibrotic M2-like macrophages promote the granulation tissue formation and wound contraction by producing growth factors (TGF- β ₁, platelet-derived growth factor) and extracellular matrix persisting in the wound milieu to day 10 after wounding; and (4) fibrolytic M2c/M_{reg}-like macrophages may be induced in the ischemic scar milieu to secrete proteases and promote tissue remodeling without re-inducing inflammation due to simultaneous secretion of regulatory IL-10. The occurrence of M2c-like fibrolytic macrophages in cutaneous scars still needs to be proved.

macrophages further amplify inflammation, especially *via* an autocrine loop driven by $\text{TNF}\alpha$ ²¹ until tissue damage and infection are controlled (Fig. 1).⁶ M1-like macrophage depletion¹² or the genetic deletion of their inflammatory mediators osteopontin, $\text{TNF}\alpha$, MCP-1, or MIP^{32–34} is systematically associated with reduced scar formation, suggesting that M1-like macrophages link early inflammation with late-stage fibrosis. On the other hand, reduced wound infiltration with early-phase M1-like macrophages either by conditional depletion¹² or due to impaired recruitment (in P/E selectin, ICAM1, or CCR2 deficiency) severely delayed granulation tissue formation and wound closure.¹⁶ Notably, an identical outcome of impaired wound healing was induced when early wound macrophages were over-activated by injection of $\text{TNF}\alpha$ around wounds.²¹ Taken together, these findings underpin the requirement for proper recruitment and function of the pro-inflammatory M1-like macrophages for the control of all wound-healing phases.

Consistent with these studies, we recently found the persistent activation of M1-like macrophages to be clinically highly relevant for the pathogenesis of CVUs. Here, iron accumulating in ulcer infiltrating macrophages upon phagocytosis of extravasated erythrocytes induces DAMPs, which perpetuate an unrestrained M1-like activation with high release

of ROS and inflammatory cytokines and deleterious effects on wound repair (Fig. 2).²¹ Using a newly established iron overload mouse model, we could demonstrate that this iron-loaded, $\text{TNF}\alpha$ -producing macrophage population was causal for oxidative tissue damage, impaired functions with premature senescence of wound-associated fibroblasts, and, eventually, severely impaired wound healing.²¹ A similar scenario emerges for the pathogenesis of chronic wounds associated with diabetes; a population of macrophages exhibiting M1-like features with high expression of $\text{IL-1}\beta$, inducible nitric oxide synthase (iNOS), and matrix metalloproteinase 9 was shown to persist through the late phases of wound healing and was associated with impaired granulation tissue formation, angiogenesis, collagen deposition, and wound healing due to reduced levels of anti-inflammatory IL-10 and growth factors (insulin-like growth factor [IGF]1, $\text{TGF-}\beta_1$, and VEGF) in diabetic db/db mice.²⁹ The incapacity of macrophages to switch toward the tissue repair-promoting M2-like activation was, at least in part, attributed to their impaired phagocytosis of apoptotic cells in the diabetic microenvironment.³⁵

Anti-inflammatory functions of wound macrophages. Anti-inflammatory macrophages are

Figure 2. Schematic showing iron-dependent activation of the novel hybrid M1/M2 macrophage population leading to chronic inflammation, tissue breakdown, and impaired wound healing in chronic venous leg ulcers. Chronic venous valve insufficiency leads to hypertension in the lower limb veins with persistent erythrocyte extravasation. Engulfment of red blood cells by tissue macrophages (erythrophagocytosis) and release of hemoglobin-bound iron activates a pro-inflammatory hybrid M1/M2 macrophages subset with enhanced release of $\text{TNF}\alpha$, peroxynitrite (ONOO^*), and hydroxyl radicals (OH^*). $\text{TNF}\alpha$ leads to the perpetuation of the hybrid M1/M2 activation. Peroxynitrite and hydroxyl radicals result in oxidative and nitrative damage and the induction of a $\text{p16}^{\text{INK4a}}$ -induced senescent program in wound resident fibroblasts, which, thus, cannot contribute to efficient tissue restoration. (Reprinted by granted permission from Sindrilaru *et al.*²¹)

induced in the late inflammatory stage of wound healing, which is dominated by apoptotic neutrophils (Fig. 1). These suppressive macrophages terminate inflammation by producing type 2 cytokines (IL-10) and lay the foundation for new tissue formation by secreting growth factors such as VEGF or TGF- β_1 .³⁶ Consistently, their depletion resulted in enhanced and prolonged TNF α expression in wound margins¹⁰ along with impaired neo-angiogenesis due to endothelial cell apoptosis, immature granulation tissue formation, and delayed wound healing.¹²

Since neither IL4 nor IL13 could be detected in aseptic wounds,¹⁸ it remains unclear whether these classical stimuli for *in vitro* M2 polarization control wound macrophage polarization *in vivo*. Alternatively, other type 2 cytokines (IL5, IL10), steroids, or TGF- β_1 have been suggested to skew wound macrophages toward anti-inflammatory activation, but the *in vivo* relevance of these activation mechanisms still needs to be studied.^{22,37} Therefore, these anti-inflammatory macrophages should be designated as M2-like wound macrophages. Moreover, sequential *in vitro* stimulation of macrophages with Toll-like receptor (TLR) agonists and adenosine was shown to shift an initial M1-like phenotype toward a new, VEGF-expressing pro-angiogenic M2d-like activation, independent of type 2 cytokines.³⁸ This macrophage subtype induced by co-stimulation of TLR and the adenosine receptor A_{2a}R has been proposed to control the transition from inflammation toward granulation tissue formation during wound healing, which has not yet been proved *in vivo*.³⁹

Therefore, the nonphlogistic removal of apoptotic neutrophils by macrophages remains the most potent stimulus to initiate the phenotypic switch from pro-inflammatory M1-like toward anti-inflammatory M2-like macrophages.^{36,40} This process involves the sequential steps of tight adhesion followed by formation of phagocytic synapses between macrophages and apoptotic neutrophils and subsequent phagocytosis, and essentially depends on the proper function of β_2 integrin adhesion molecules on macrophages.⁴¹ Macrophages derived from CD18-deficient mice and from patients suffering from Leukocyte Adhesion Deficiency syndrome type 1 (LAD1) lacking functional β_2 integrins due to mutations in the *CD18* gene (encoding the common β chain of β_2 integrins) revealed severely reduced adhesion to and phagocytosis of apoptotic neutrophils with subsequent insufficient TGF- β_1 activation. Consistently, LAD1 patients spontaneously develop difficult-to-heal skin ulcerations, and CD18-deficient mice present with

severely delayed wound closure due to impaired TGF- β_1 -dependent granulation tissue formation.⁴¹ Mechanistically, engagement of β_2 integrins on macrophages by apoptotic neutrophils activates a downstream signaling pathway via the guanine nucleotide exchange factor Vav3 and the small Rac2GTPase, directly leading to oxidative TGF- β_1 activation. This TGF- β_1 activation is most likely due to enhanced ROS release driven by CD18-Vav3-Rac2-dependent assembly of the phagocyte NADPH oxidase.^{42,43} Other mechanisms involving opsonization of apoptotic neutrophils by serum amyloid proteins (pentraxins),⁴⁴ components of the complement system,⁴⁵ or thrombospondin-mediated uptake of apoptotic neutrophils by β_3 integrins/CD36 receptor complexes⁴⁶ have been shown to mediate the phagocytosis of apoptotic cells by macrophages in different models of autoimmune inflammation, but their role for apoptotic corps clearance in wounds has not been deciphered so far.

Besides β_2 integrin deficient conditions, impaired phagocytic removal of apoptotic neutrophils by macrophages emerges to drive wound healing disturbances associated with diabetes.³⁵ Wound margins from diabetic patients and diabetic db/db mice accumulate apoptotic cells with significantly higher numbers than healthy or wild-type controls,³⁵ in parallel with persistently increased levels of pro-inflammatory IL-1 β and IFN γ .²⁹ Finally, a decline in phagocytic macrophage functions characterizes immune senescence and may be responsible for wound-healing disturbances associated with aging.⁴⁷⁻⁴⁹ Although the molecular mechanisms underlying these defects have not been deciphered so far, adoptive transfer of macrophages derived from young, but not from old mice significantly accelerated wound healing of aged mice.⁵⁰

Curiously enough, despite a wealth of evidence for the critical role of nonphlogistic removal of apoptotic neutrophils by macrophages for dampening inflammation *in vitro*⁵¹ and promotion of tissue repair in wound healing,^{41,42} apoptotic cells are seldom regarded as prototypical stimuli for M2-like macrophage activation.

Pro-fibrotic macrophages. The finding that macrophage-deprived wounds heal with reduced scar formation provides a strong link between wound infiltrating macrophages and fibrosis. Macrophages contribute to fibroblasts recruitment to wound sites, their activation and differentiation to myofibroblasts, and the deposition of ECM. Various growth factors, including TGF- β_1 , PDGF,

fibroblast growth factor 2, or insulin-like growth factor 1, are responsible for different macrophage-dependent tissue repair-promoting functions.⁵² Among them, TGF- β_1 is the prototypic pro-fibrotic cytokine that is directly produced by macrophages and regulates fibroblasts functions during wound healing.^{18,41} Accordingly, depletion of macrophages from wounded skin during granulation tissue formation resulted in decreased TGF- β_1 -dependent collagen deposition and reduced scar formation.^{10,12}

According to the M1/M2 dichotomy, TGF- β_1 -producing pro-fibrotic macrophages are expected to mount M2-like phenotypes. An opposing observation was made in CVU margins, where pro-inflammatory M1 macrophages persist and are associated with progressive fibrosis, eventually leading to irreversible lipodermatosclerosis.²¹ Furthermore, administration of GM-CSF *via* the induction of IL-6 and MCP-1-producing M1-like macrophages almost completely restored impaired wound healing and scar formation due to dysfunctional macrophage in diabetic mice.⁵³ We cannot exclude the fact that M1-like macrophages undergo a so-far-undetected switch toward M2-like activation in these cases; however, it is more likely that in complex environments such as chronic wounds, at least some macrophage subpopulations acquire phenotypic constellations beyond the M1/M2 paradigm. Unexpectedly, local treatment of wounds with M2-prone macrophages generated *ex vivo* from embryonic stem cells severely delayed the healing of deep dermal wounds when compared with application of M1-like bone marrow-derived macrophages,⁵⁴ supporting a much more complex spatial and temporal regulation of macrophage functions even during normal wound healing.

Another open issue concerns the delimitation of *bona fide* pro-fibrotic from anti-inflammatory macrophages identified in the late inflammatory healing phase. Possibly, the two populations only represent snapshots of a continuous adaptation of macrophages to dynamically changing conditions in the wound environment. Essentially, pro-fibrotic macrophages can be viewed as anti-inflammatory macrophages that amplify their secretion of growth factors and ECM, possibly depending on the overlying re-epithelialization. Such macrophages producing collagen, fibronectin, and other matrix components were generated *in vitro* on exposure to IL-4 and IL-13 and STAT6 signaling activation and were functionally classified as M2a-like macrophages.^{22,55} Recently, it has been suggested that such type 2 cytokine

combinations are provided by Th2 lymphocytes that are recruited to skin wound sites. In this view, disruption of inducible costimulator (ICOS)-ICOS ligand signaling that controls Th2 lymphocyte activation resulted in reduced IL-4 and IL-10 expression and severely delayed wound healing, which could be fully rescued by adoptive transfer of wild-type lymphocytes.⁵⁶ A dominant Th2 lymphocyte polarization was detected in peripheral blood of patients developing hypertrophic scars after thermal injuries,⁵⁷ but the role of Th2 cells in pro-fibrotic macrophage functions during normal wound healing needs to be elucidated.

Alternatively, a subset of CX₃CR1⁺ macrophages has been proposed to cause fibrosis and scar formation in healing wounds. CX₃CR1⁺ macrophages and their ligand fractalkine were found to progressively increase in the wound tissue and to peak in the granulation tissue phase. Consistently, disruption of CX₃CR1-fractalkine cross-talk impaired macrophage recruitment to wound sites, which was associated with reduced TGF- β_1 production, impaired myofibroblast differentiation and collagen deposition, and delayed wound healing.¹⁹ Whether these pro-fibrotic macrophages derive from newly recruited peripheral monocytes—for example, from CX₃CR1⁺ patrolling monocytes—or from pro- or anti-inflammatory wound macrophages by changes in their functional polarization, and how they contribute to fibrosis, needs to be addressed in further studies.

Fibrolytic macrophages. The view that macrophages are critical not only for the initiation, but also for the resolution of fibrosis, is convincingly sustained by the finding that late macrophage depletion impaired the clearance of the fibrotic scar induced by chemical liver injury.⁵⁸ Proposed mechanisms involved in scar remodeling include the direct degradation of fibrillar components by secreted metalloproteases, induction of myofibroblasts apoptosis, subsequent removal of apoptotic cells, and suppression of further inflammation via IL-10 release.⁵² Fibrolytic macrophages are functionally classified as “regulatory Mreg/M2c-like macrophages,” express the regulatory cytokine IL-10, metalloproteases, and Arginase-1, and can be induced *in vitro* upon stimulation with apoptotic cells, IL-10, or pentraxin-2.^{59,60} However, microarray profiling of macrophages associated *in vivo* with the resolution of liver fibrosis revealed a phenotype outside the M1/M2 classification, with simultaneous upregulation of metalloproteases (*Mmp9*, *Mmp12*), growth factors (*Igf1*), and phagocytosis-related genes.⁶¹

The finding that conditional depletion of macrophages during the remodeling phase (at day 9 after wounding) did not further affect skin wound healing¹² may question the relevance of fibrolytic macrophages for cutaneous wound-associated scarring. Nevertheless, skin fibrosis definitely responds to fibrolytic signals, as recently demonstrated by applications of TGF- β_3 , which induced fibroblast migration and ECM deposition patterns resembling fetal wound healing and prevented excessive myofibroblasts proliferation and scarring in humans and several experimental wound healing models.⁶² Whether any macrophage population contributes to these processes needs to be soon addressed by conditional depletion of macrophages at stages when the scar is already organized.

OUTLOOK

Several open questions and expectations emerge from the rapid advances in understanding macrophage functions during wound healing.

First, do the so-far-identified functional macrophage subsets represent discrete populations of macrophages that infiltrate and polarize *de novo* with changing conditions at the wound site? Or do they represent a continuous spectrum of phenotypes reflecting their dynamic adaptation to new environmental cues? This last view has been favored in the past years based on studies that describe macrophage phenotypes sampled at defined phases of the inflammatory response,^{12,18,21} but strong *in vivo* evidence for the switch from M1 toward different M2-like phenotypes during wound healing is scarce.²³ However, in experimental liver fibrosis, combined adoptive transfer and *in situ* labeling experiments revealed that Ly6C^{high} early pro-inflammatory monocytes give rise in the late phase of fibrosis resolution to Ly6C^{low} fibrolytic macrophages, demonstrating macrophage plasticity *in vivo*.⁶¹

On the other hand, monocytes are continuously recruited as long as chemotactic signals are released at wound sites. It is plausible that defined stimuli recruit monocytes with distinct phenotypic repertoires which will differentiate toward specific functional macrophage subsets. For example, fractalkine, the only known ligand for CX₃CR1, was shown to reach a peak expression in wounds by day 6 after wounding and to recruit *de novo* M2-like

TAKE-HOME MESSAGES

- Macrophages are invariably present and regulate all phases of adult wound healing.
- Pro-inflammatory macrophages promote host defence at the expense of tissue damage, anti-inflammatory macrophages dampen inflammation and protect from uncontrolled tissue damage, pro-fibrotic macrophages promote granulation tissue deposition and wound contraction with unavoidable scar formation, and a population of fibrolytic macrophages that contributes to tissue remodeling emerges.
- The polarization phenotypes of macrophages during physiological and pathologic wound healing do not fit the M1/M2 paradigm.
- Early inflammatory macrophage subpopulations express no other M2 marker except for VEGF initiate angiogenesis in normal wound healing. In CVUs, a subset of iron-overloaded inflammatory M1-like macrophages is responsible not only for tissue breakdown but also for excessive dermal fibrosis.
- Identification and detailed characterization of functional macrophage subsets *in vivo* is essential to provide effective therapeutic targets in impaired wound healing.

CX₃CR1⁺ macrophages with pro-angiogenic and pro-fibrotic functions.¹⁹ Furthermore, initial data generated in liver injury models suggest that one and the same precursor, the Ly6C^{high} inflammatory macrophage, may polarize either toward pro-fibrotic or to fibrolytic macrophages. Interestingly, induction of a phagocytic behavior through administration of liposomes resulted in increased fibrolytic macrophage numbers and improved fibrosis resolution.⁶¹ Since TGF- β_1 -producing pro-fibrotic macrophages differentiate as well upon phagocytosis of apoptotic cells, opsonization may be critical for the contrasting functional outcomes. While during the inflammatory phase the apoptotic neutrophils uptake depends on complement factors,⁴⁵ thrombospondin,⁴⁶ and integrin signaling, opsonization with pentraxin-2 and Extracellular-Signal Regulated Kinase signaling appears to induce regulatory/fibrolytic macrophages.⁴⁴

The picture becomes even more complicated with the recent identification of early Ly6C⁺ CCR2⁺ macrophage subsets, which, besides expressing classical M1 activation markers IL-6 and iNOS, also upregulate M2-pro-angiogenic VEGF and Tie-2, thus making the M1/M2-like classification unhelpful. These VEGF-producing macrophages very much resembling, at least phenotypically, the *in vitro* defined M2d-like subset,³⁸ were only detected in the early inflammatory wound tissue and non-redundantly initiated vascular sprouts formation in this phase.²⁰

We have recently identified a similar hybrid macrophage subpopulation that causally drives the severely impaired healing of CVUs. Here, hemo-

globin released from extravasated erythrocytes complexed by the serum protein haptoglobin is taken up by macrophages upon upregulation of the hemoglobin-haptoglobin receptor CD163, a typical M2 marker (Fig. 2).²¹ CD163 upregulation by low concentrations of hemoglobin/haptoglobin complexes is an efficient anti-infectious mechanism to restrict iron required for bacterial and viral growth to deactivate macrophage activation and limit oxidative stress in atherosclerotic plaques.⁶³ In CD163^{high} CVU macrophages, the continuous uptake of haemoglobin most probably yields high intracellular concentrations of heme-iron, which, *via* a so far unclear mechanism, induces an unrestrained pro-inflammatory macrophage activation with deleterious effects on wound healing.²¹ Macrophages can be targeted in CVUs by inhibition of TNF α , iron chelators, and oxygen radicals scavengers, which improve wound healing in several animal models (Fig. 2).⁶⁴ Besides CVUs, such approaches may provide substantial benefit for wound-healing disturbances that are associated with a series of iron overload conditions, as for instance occurring in hemochromatosis. This important finding highlights the necessity of identifying

and targeting environmental cues that are responsible for pathologic macrophage activation.

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