

## The Innate Immune System in Acute and Chronic Wounds

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**Significance:** This review article provides an overview of the critical roles of the innate immune system to wound healing. It explores aspects of dysregulation of individual innate immune elements known to compromise wound repair and promote nonhealing wounds. Understanding the key mechanisms whereby wound healing fails will provide seed concepts for the development of new therapeutic approaches.

**Recent Advances:** Our understanding of the complex interactions of the innate immune system in wound healing has significantly improved, particularly in our understanding of the role of antimicrobials and peptides and the nature of the switch from inflammatory to reparative processes. This takes place against an emerging understanding of the relationship between human cells and commensal bacteria in the skin.

**Critical Issues:** It is well established and accepted that early local inflammatory mediators in the wound bed function as an immunological vehicle to facilitate immune cell infiltration and microbial clearance upon injury to the skin barrier. Both impaired and excessive innate immune responses can promote nonhealing wounds. It appears that the switch from the inflammatory to the proliferative phase is tightly regulated and mediated, at least in part, by a change in macrophages. Defining the factors that initiate the switch in such macrophage phenotypes and functions is the subject of multiple investigations.

**Future Directions:** The review highlights processes that may be useful targets for further investigation, particularly the switch from M1 to M2 macrophages that appears to be critical as dysregulation of this switch occurs during defective wound healing.



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### SCOPE AND SIGNIFICANCE

THIS REVIEW DISCUSSES the basis for the development of chronic wounds that fail to complete the initial clearance of microbial contamination by the innate immune system and stall in a late stage of the inflammatory process. The early inflammatory stage of wound healing is the prov-

ince of the innate immune system. This article reviews the complex literature on factors of the innate immune system that affect cutaneous wound healing and potential cellular and molecular mechanisms involved and discusses possible reasons that the innate immune system fails to establish conditions for healing. We

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will also discuss the switch from inflammation to the reparative phase that ultimately facilitates wound closure. The review will include a discussion of the normal relationship between commensal bacteria that interact with the innate immune system and the cells of the skin. This relationship is extensively modified in chronic wounds.

## TRANSLATIONAL RELEVANCE

Understanding the cellular and molecular mechanisms that lead to impaired wound healing may provide insight into novel aspects to the research on the nature of chronic wounds. Identification of cellular and molecular factors that impair wound healing, such as overt proinflammatory cytokines, dysregulation of macrophages, and imbalance of immunoregulatory molecules such as adenosine and

nitric oxide (NO), may provide novel therapeutic targets to aid to the wound healing response.

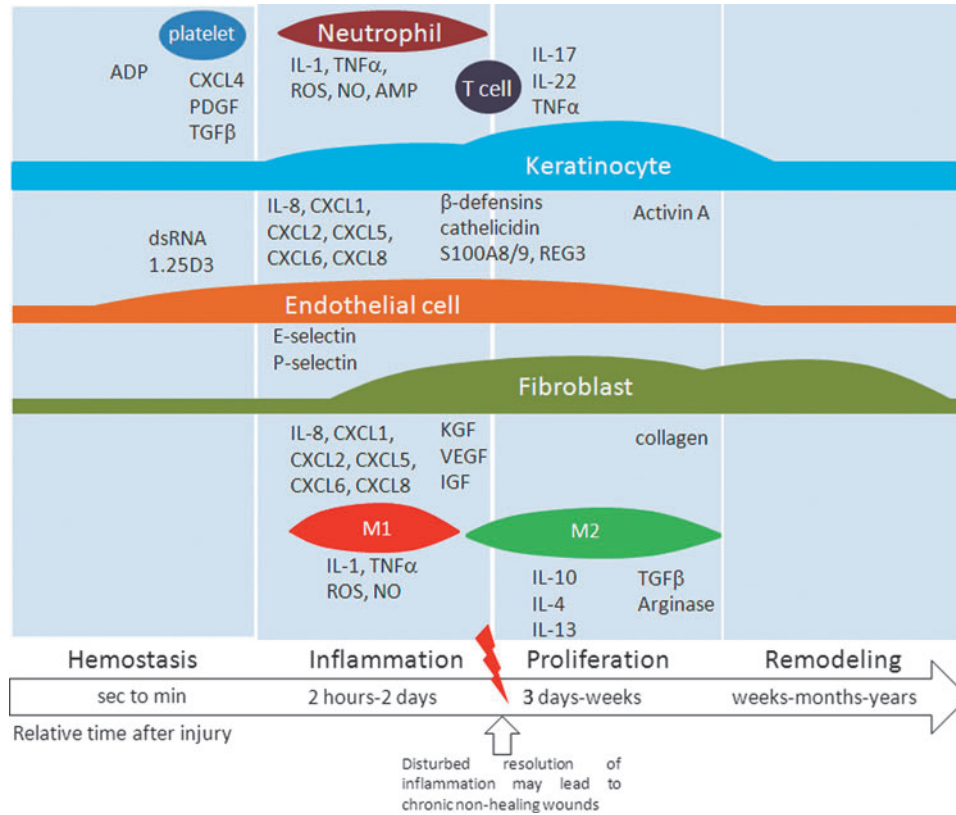
## CLINICAL RELEVANCE

Understanding innate immunity components that are critical in the early phases of wound healing will inform the design of wound repair therapies and the selection of new targets with the goal of improving wound healing and resolving impaired wounds.

## DISCUSSION

### Background

In normal skin, stable homeostasis and barrier function are established by interaction of resident keratinocytes, dendritic cells (DCs), T cells, mast cells, fibroblasts, and macrophages with each other



**Figure 1.** The successive processes of wound healing. Platelet aggregation and release of ADP, PDGF, tumor growth factor  $\beta$  (TGF $\beta$ ), and CXCL4 activate the local cells (fibroblasts and keratinocytes) and the immune cascade to initiate inflammation. Keratinocytes, as the major cell type of the epidermis, release proinflammatory cytokines and antimicrobial peptides and proteins (AMPs). Together, this immune response is aimed at decontaminating the wound bed by recruiting neutrophils and macrophages into the wound bed. Neutrophils as well as macrophages are professional microbe-killing cells eliciting reactive oxygen species (ROS), nitric oxide (NO), and AMPs. Meanwhile, endothelial cells in dermal venules express E and P-selectins, which guide the rolling and tethering of extravasating leukocytes to the wound bed. Skin-resident T cell on the one hand and infiltrating T cells on the other hand participate in the inflammatory stage through production of interleukin (IL)-17, IL-22, and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) further amplifying the host defense response. In addition, skin-resident T cells can also produce growth factors, which stimulate keratinocyte proliferation. Activation of macrophages, neutrophils, and keratinocytes leads to sterilization of the wound and subsequently to resolution of inflammation. At this time, proliferation of keratinocytes and fibroblasts stimulates wound closure. This step is directed through the fine regulation of inflammatory to reparative processes and a switch in the cytokine milieu of the wound. Macrophages, which are active in the inflammatory phase, termed M1 macrophages, undergo a phenotypic switch to an anti-inflammatory and reparative M2 phenotype. This switch is tightly regulated by cytokines and other molecules further depicted in Fig. 4. M2-like macrophages activate fibroblasts to produce growth factors that stimulate keratinocyte proliferation and migration.

and with the resident microbiome. When the skin barrier is breached, acute wound healing follows a well-coordinated series of processes,<sup>1</sup> illustrated in Fig. 1, comprising inflammation, proliferation, and remodeling phases. Each stage is activated by a series of cytokines and other mediators, which are, in part, released as the stage before approaches completion. In chronic wounds, this orderly progression appears to be halted somewhere in the late inflammatory stage before the initiation of wound closure. The arrested stage does not adequately pass on the signals for wound closure, but instead sets up a quasistable equilibrium with resident bacteria. Consequently, the wound does not heal, but forms an ulcer that can last for years. This inflammatory stage of wound healing is the province of the innate immune system. In the development of chronic wounds, at least two mechanisms of arrest may be considered. In the first, a process never reaches the level of completion at which signals, for example, growth factor production, initiating the next stage, for example, keratinocyte proliferation, are generated. In the second, a process, such as inflammation, follows a defined trajectory to completion and moves on to the next stage. If that first step does not complete its function (*e.g.*, clearance of contamination) and the next step is inappropriate (*e.g.*, wound closure), the wound healing response may stall. Examples of both these mechanisms may be found in the development of chronic wounds as discussed below.

The innate immune system comprises a diverse range of defense systems that act to provide primary protection against the harmful agents. The innate immune system includes macrophages, neutrophils, interferons (IFNs), interleukins (ILs), antimicrobial peptides and proteins (AMPs), mast cells, eosinophils, and acute-phase proteins, as well as the physical barrier established by the stratum corneum. The innate immune system is capable of very rapid activation and deployment in response to pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) through the Toll-like receptor (TLR) systems and other innate immune receptors to recognize viruses, bacteria, endogenous or exogenous danger signals, or foreign particles. The innate immune system interacts and instructs the adaptive immune system to elicit optimal immune responses, as recently discussed.<sup>2</sup>

### Host-microbiome interactions in healthy and wounded skin

It has recently become evident that barrier function and control of responses to microorgan-

isms in normal skin are complex and involve the establishment of a synergistic ecology between the host and colonizing microbiome.<sup>3</sup> Human skin is colonized by a diverse skin microbiome. Many of these skin microorganisms are harmless. Symbiotic microorganisms occupy a wide range of skin niches and protect against invasion by more pathogenic or harmful organisms. Commensal organisms such as strains of *Staphylococcus epidermidis* found in the skin secrete compounds that are themselves antibacterial, such as phenol-soluble modulins,<sup>4,5</sup> and also interact with the host to induce AMP expression<sup>6</sup> or reduce inflammation.<sup>6</sup> This interactive host-microbiome system was thought to be restricted to the stratum corneum, but it has become evident that the system extends further into the living layers of the skin as commensal organisms or their products have been detected in the subcutis in healthy skin.<sup>7</sup> The composition of the skin microflora varies in different regions of the body and, to lesser degrees, in different individuals and from time to time.<sup>8</sup> Following injury, this stable and mutually beneficial host-microbiome system is disturbed. Bacterial products stimulate the innate immune system and thus play a role in the inception of inflammation, and commensal skin bacteria invade and become pathogenic upon breach of the skin barrier. If the disturbance is minor, inflammation is minimal as in sterile suction blister wounds.<sup>9</sup> However many bacteria, including both commensal bacteria, such as *S. epidermidis*, and opportunistic microbes are capable of forming biofilms.<sup>10</sup> If this occurs, these microbes change their metabolism<sup>11</sup> and a new ecology is set up with a modified microbiome.<sup>12,13</sup> The species composition of the bacterial communities in chronic wounds may differ from normal skin, but presumably also show species similarities and may persist for many years. However, molecular studies have been unable to identify a unique organism that colonizes wounds of the same general type, for example, in diabetic foot ulcers or venous leg ulcers.<sup>14</sup> This is in contrast to burn wounds, in which a causative microbiological agent is usually readily identifiable. Burn wounds often become infected with *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, or enterococci.<sup>15</sup> It has been suggested that chronic wounds may benefit if treated by modifying the nonhealing wound microbiome using probiotics.<sup>16</sup>

### Inflame or not to inflame: the role of normal and abnormal inflammation

Following initial hemostasis, inflammation is a critical part of the normal wound healing process.

The importance of inflammation is believed to lie in the clearance of contaminating microorganisms. Inflammation may involve cells, such as macrophages, neutrophils, proinflammatory cytokines, vasoactive amines, reactive oxygen species (ROS), NO, extracellular adenosine triphosphate (eATP), double-stranded ribonucleic acid (dsRNA), hyaluronic acid, uric acid, eicosanoids, AMPs, and other mediators, which in concert create, trigger, or amplify skin inflammatory responses. Early events in the innate immune response to skin injury include the recruitment of neutrophils and the induction of antimicrobial peptides, particularly, in humans, the cathelicidin LL-37.<sup>17</sup> Neutrophils are recruited by a series of asparagine-leucine-arginine (ELR) motif-containing CXC chemokines that are secreted by keratinocytes and fibroblasts and subsequently by neutrophils and macrophages. Cathelicidins are produced by neutrophils, keratinocytes, and other cells. The rapidity of this response and the resulting microbicidal activity are essential to kill planktonic bacteria and to prevent the establishment of biofilms and other stable microbial communities.<sup>18</sup> In the absence of effective microbial decontamination, prolonged inflammation may enter a chronic state prone to failure to heal. Bacterial colonization does not necessarily cause inflammation,<sup>6,19,20</sup> but high endotoxin levels and other microbial products can drive inflammatory responses. Prolonged inflammation leads to increased levels of proteases that degrade extracellular matrix (ECM) and growth factors.<sup>21,22</sup> This hampers the wound entering the proliferative phase to facilitate wound closure. Interestingly, suction blister wounds, where the blister roof is still intact, do not show substantial inflammation,<sup>9</sup> whereas excisional wounds, ultraviolet (UV)-induced skin damage, superficial tape stripping or chemical skin irritants, and contact sensitizers cause inflammatory infiltrates,<sup>17,23–26</sup> suggesting that the route of trauma and the degree and nature of disruption may play an important role.

#### **Antimicrobial host defense proteins in injured skin**

Healthy mammalian skin comprises cells and molecules that are poised to provide a first line of defense against microbial pathogens and to increase immunity in response to tissue injury or microbial threats. AMPs provide a soluble barrier that forms a strong impediment to microbial invasion. AMPs, such as hBD1 and S100A7, S100A8, and S100A9 proteins, are present under homeostatic conditions, whereas other AMPs, such as cathelicidin, hBD2, hBD3, and regenerating islet-

derived protein 3A (REG3A), require induction through environmental or endogenous triggers. Previous studies on AMPs revealed several mechanisms of how AMPs protect the host from infections and how they act as multifunctional effector molecules to assist wound healing (Figs. 2 and 3). In addition to their antimicrobial functions, AMPs also regulate keratinocyte growth and differentiation, cytokine production, and influence adaptive immunity.<sup>25,27,28</sup> Abnormal or dysfunctional AMP production has been associated with susceptibility to microbial infections and several other cutaneous diseases, including nonhealing wounds.<sup>29</sup> In fact, recent mouse studies further revealed that murine  $\beta$ -defensin 3 (the ortholog of hBD2) and Reg3 $\gamma$  (ortholog of REG3A), but not S100A8, have wound healing-promoting properties as they could rescue defective wound healing in IL-17A<sup>-/-</sup> mice, which show defective AMP induction.<sup>6,30</sup>

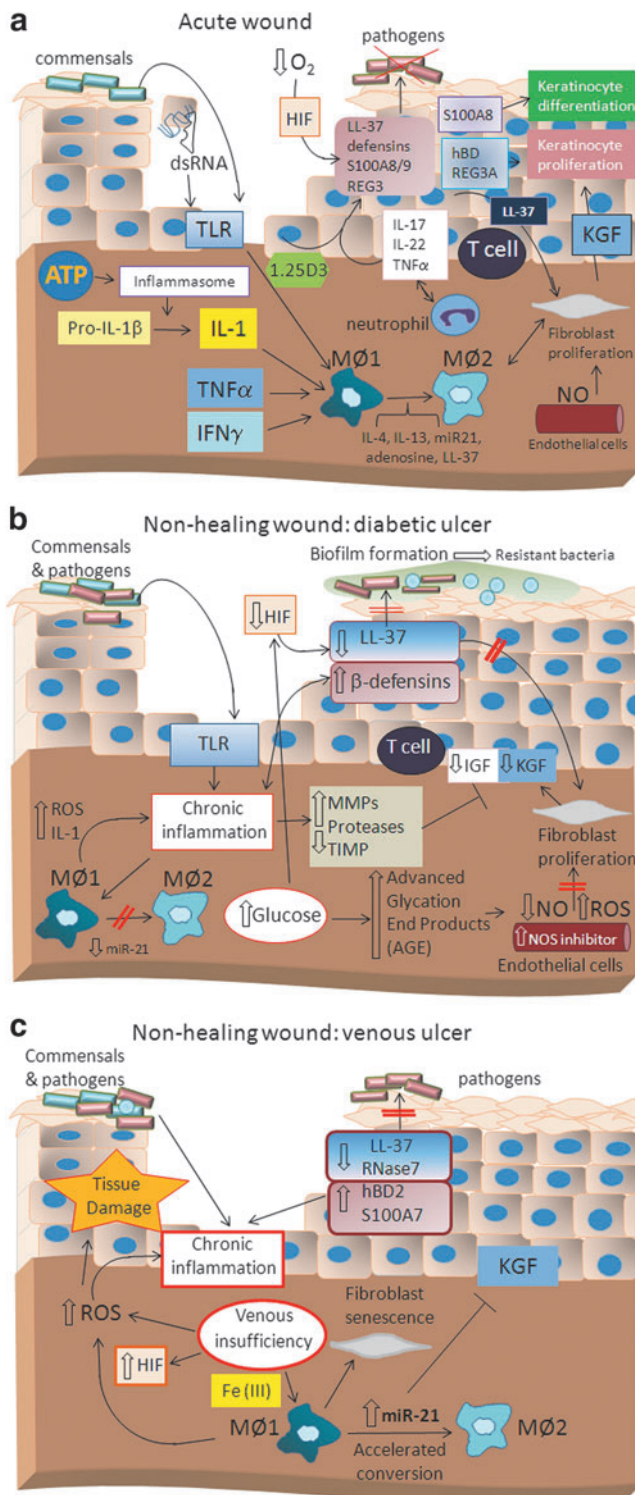
Upon skin injury, the protective skin barrier is breached and the skin becomes more vulnerable to invasion of microbes. Recognition of microbial products and endogenous danger molecules that generate an AMP response are critical components to mount host protection. The innate immune system senses invading microbes, their products, and host-generated danger signals, such as degraded hyaluronic acid and eATP, through classical pathogen recognition receptors (PRRs) or other innate immune receptors. These include TLRs, purinergic receptors, the receptor for advanced glycation endproducts (RAGE), and others. In addition to their well-known role as effectors of innate immunity by killing microbes, AMPs have additional roles as proteinase inhibitors, chemokines, neuropeptides, and in vascularization, which may contribute to their broad function in wound repair.<sup>27</sup>

Many AMPs are small cationic peptides binding to and interacting with the negatively charged membranes of microbes and are thereby able to kill the microbe through membrane disruption, whereas other AMPs inhibit bacterial metabolism or growth.<sup>31,32</sup> In the case of infection, inflammation, or injury, many AMPs in the skin are increased due to upregulated synthesis by keratinocytes and deposition from degranulation of recruited neutrophils and other immune cells. AMP expression is highly dependent on the cytokine milieu present in the skin and proinflammatory cytokines, such as IL-1, IL-6, IL-8, and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), are typically strong inducers of AMPs, whereas IL-4, IL-13, and IL-10 have been shown to dampen AMP responses.<sup>33–35</sup> AMPs can amplify the proinflammatory cytokine response and can thus, in some circumstances, assume

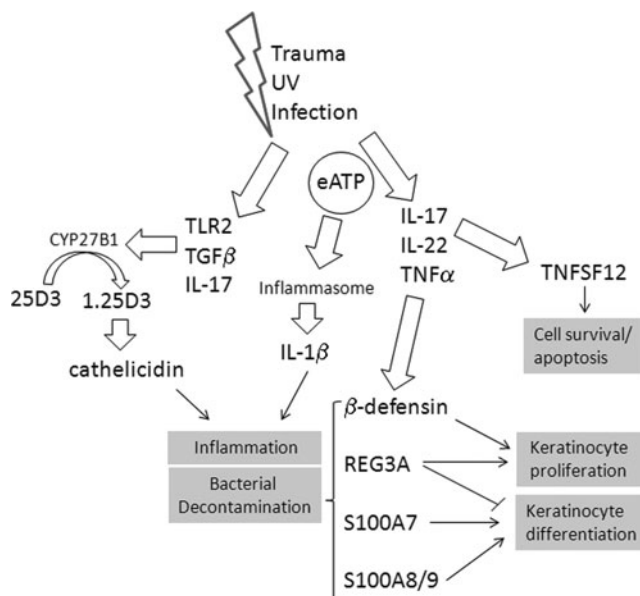
pathogenic roles as in psoriasis, rosacea, and other skin diseases.<sup>36–38</sup> Human  $\beta$ -defensins are a well-characterized group of AMPs that are abundantly expressed by multiple cell types such as keratinocytes and peripheral blood mononuclear cells (PBMCs). In the skin, hBD1 is constitutively expressed, whereas hBD2 and hBD3 are highly upregulated upon wounding. In inflamed skin, in-

cluding psoriasis and skin injury, hBD3, which like hBD2 was originally purified from psoriatic skin, is inducible by skin injury.<sup>38</sup> IL-1, TNF $\alpha$ , IFN $\gamma$ , IL-17, and IL-22 are strong inducers of hBD2 and hBD3 expression.<sup>25,36,39</sup> Furthermore, hBD2 expression is induced by high calcium concentrations and 1,25 vitamin D<sub>3</sub>.<sup>36</sup>

Similar to defensins, cathelicidin is derived from a precursor molecule called human cathelicidin antimicrobial protein (hCAP18) that can release an antimicrobial peptide after cleavage. In man, one cathelicidin gene, named human cathelicidin antimicrobial peptide (CAMP), has been identified that encodes the 18 kD pre-pro-protein, hCAP18, which includes the C-terminal human antimicrobial peptide, LL-37. Cathelicidin LL-37 is the best studied cathelicidin peptide in man, whereas in mice, *Camp* refers to the gene encoding cathelicidin-related antimicrobial peptide (CRAMP). Although expressed in only very low levels in epidermal keratinocytes under resting conditions, it is found in high levels in neutrophils, monocytes, natural killer (NK) cells, and T cells.<sup>40,41</sup> Wound



**Figure 2.** Model of (a) normal wound healing in acute wounds and nonhealing (b) diabetic and (c) venous ulcers. Acute wound healing is characterized by activation of the innate immune system aimed at the successful killing of pathogenic microbes through deployment of antimicrobial effector molecules called AMPs, such as cathelicidin LL-37,  $\beta$ -defensins, and S100 proteins, as well as regenerating islet-derived protein 3 (REG3). Activation of Toll-like receptor (TLR) and other pathogen or danger recognition receptors leads to inflammasome activation and IL-1 production as well as an antimicrobial peptide response. Furthermore, AMP responses are enhanced by low O<sub>2</sub> levels in the wound, known to activate HIF1 $\alpha$  and through IL-17 and IL-22 produced by resident T cells at the site of injury. IL-1, TLR activation, and interferon (IFN) $\gamma$ , as well as TNF $\alpha$  drive M1 macrophage differentiation. Following successful decontamination of the wound, the inflammatory phase resolves and the reparative stage, which is characterized by keratinocyte and fibroblast proliferation, begins. The switch from M1 macrophages to M2-like macrophages as well as distinct AMP function is an important regulator of the reparative phase. Wound reepithelialization requires regulated keratinocyte proliferation, migration, and differentiation, which is guided, at least in part, through growth factor production and AMPs. However, under pathogenic conditions, such as diabetic ulcers (b), the normal wound healing response is disturbed. Diabetes is associated with a chronic inflammatory state, which leads ultimately to an imbalance and dysregulation of skin immune function. Dysfunctional AMP, ROS, and protease production result in overt perpetuation of inflammation, tissue destruction, and ineffective decontamination of pathogenic bacteria. High glucose levels are associated with high levels of AGE products ultimately enhancing vascular NO synthase inhibitor, thereby inhibiting NO production. In contrast, venous insufficiency in venous ulcers (c) leads to iron overloading of macrophages, which engulf extravasated erythrocytes. Iron overloading in macrophages has been shown to give rise to M1 macrophages. However, high abundance of miRNA-21 has been associated with accelerated conversion to M2-like macrophages, leading to the paradox that despite increases in inflammatory M1 macrophages, large numbers of M2-like macrophages are generated prematurely. In addition, miRNA-21 inhibits growth factor signaling by yet undefined mechanisms and delays wound closure.



**Figure 3.** Role of innate immunity in skin wounds. Upon skin injury, the innate immune system becomes rapidly activated. TLR activation-induced CYP27B1 expression converts inactive 24D3 to 1,25D3, which is a strong inducer of cathelicidin antimicrobial peptide. TLR, IL-1, IL-17, and IL-22 signaling induces multiple antimicrobial molecules, such as  $\beta$ -defensins, REG3A, S100A7, S100A8, and S100A9. IL-17 and IL-22 are derived from skin-resident T cells and their activation is enhanced through purinergic signals, such as through extracellular adenosine triphosphate (eATP). Cathelicidin acts not only as a potent antimicrobial agent to kill gram-positive bacteria but it also serves immunomodulatory functions and can perpetuate inflammation.  $\beta$ -defensins also have a relatively broad spectrum of antibacterial activity toward gram-positive bacteria and enhance inflammation by recruiting immune cells to the wound site through their chemoattractive properties. S100A7 and S100A8/A9 have antimicrobial activity toward gram-positive and gram-negative bacteria. Together, the antimicrobial activity of various host defense peptides and proteins facilitates wound decontamination, a prerequisite for normal wound healing.  $\beta$ -Defensins and REG3 proteins, but not S100A7 or S100A8/A9, have been shown to stimulate keratinocyte proliferation, which is important for wound reepithelialization and repair. In addition, keratinocyte apoptosis and differentiation are critical to epithelial remodeling and reestablishing the skin barrier. These processes are regulated, at least in part, by S100 proteins and through TNFSF12, recently shown to be induced by IL-17 signaling.

fluids and skin wounds show elevated levels of cathelicidin expression in keratinocytes and other immune cells, reaching a maximum at 48 h.<sup>29</sup> hCAP18 is detected in the inflammatory infiltrate and in the epithelium migrating over the wound bed. Using a noninflammatory *ex vivo* wound healing model comprising organ-cultured human skin, it has been shown that hCAP18 is strongly expressed in healing skin epithelium and that treatment with affinity-purified antibodies raised against LL-37 inhibits reepithelialization in a concentration-dependent manner.<sup>29</sup>

Induction of cathelicidin in human keratinocytes and monocytes in skin wounds or upon infection is enhanced by 1,25 vitamin D<sub>3</sub>.<sup>42,43</sup> Interestingly, induction of cathelicidin by 1,25 vitamin D<sub>3</sub> in murine

cells is absent probably due to the absence of a vitamin D-responsive element in the murine cathelicidin promoter.<sup>43</sup> In chronic wounds, cathelicidin is one element of host defense molecules that shows marked changes. Cathelicidin is low in venous leg ulcers and absent at the margin of other nonhealing wounds.<sup>29</sup> In contrast, expression of other AMPs, such as S100A7, ribonuclease 7 (RNase7), hBD2, and hBD3, is not reduced in chronic wounds, but remains unchanged or is upregulated.<sup>44</sup>

S100 proteins comprise a large family of low-molecular-weight proteins that are characterized by the presence of calcium-binding EF-hands. The majority of S100 genes are located within the epidermal differentiation complex on chromosome 1.q21, and 13 S100 proteins are expressed in normal and/or diseased epidermis. Many S100 proteins are overexpressed in skin cancer, metastasis, psoriasis, arthritis, wound healing, and inflammation.<sup>45</sup> A relevant role in host defense function has been shown for S100A7, S100A8 (calgranulin A), S100A9 (calgranulin B), S100A12, and S100A15.<sup>45</sup> S100A7 and S100A8 are strongly regulated by IL-17 and IL-22 in keratinocytes.<sup>25,39</sup>

### Inflammatory cytokines and their receptors in the normal and dysfunctional wound healing response

Keratinocytes, endothelial cells, fibroblasts, DCs, neutrophils, monocytes, macrophages, and innate lymphocytes such as NK cells,  $\gamma\delta$  T cells, as well as skin-resident T cells play major roles in the immune response of wound healing. They produce large amounts of cytokines to initiate and regulate inflammation. In the early stages of normal wound healing, innate immune cells produce proinflammatory cytokines that exacerbate host defense functions by inducing antimicrobial molecules, attracting leukocytes, and creating an environment to protect from microbial infection. In the late stages of the inflammatory phase of wound healing, macrophages are found to switch from a proinflammatory to an alternatively activated phenotype, which has been assumed to be requisite for the switch from the inflammatory to the proliferative stages in wound healing as discussed below in more detail.<sup>46</sup> Although much work has been done, the defining mechanisms for chronic nonhealing wounds are not exactly known. What we do know is that nonhealing wounds are characterized by a dysregulation of the late inflammatory stage as nonhealing wounds rarely enter the proliferative phase of wound healing. Alterations in macrophage phenotypes and associated cytokine patterns play a critical role in the pathogenesis of nonhealing wounds.

Interleukin-8. IL-8 (CXCL8), formerly known as neutrophil-activating peptide-1, is one of a group of chemokines, including CXCL1, 2, 3, 5, 6, and 8, that are neutrophil chemokines through a high-affinity receptor, CXCR2, and neutrophil activators through a low-affinity receptor, CXCR1. CXCR2<sup>-/-</sup> mice display impaired neutrophil recruitment and delayed wound healing.<sup>47,48</sup> These chemokines are characterized by the sequence ELR at their N-terminus. Removal of this sequence by matrix metalloproteinase 9 (MMP9) proteolysis causes, in the case of CXCL8, a 10-fold increase in its activity.<sup>49</sup> This contrasts with the effect of proteolysis on later stages of wound healing, where MMP9 may cause breakdown of protein factors needed to orchestrate wound closure. MMP9 thus represents a double-edged element important in the early stage of wound healing, but destructive if it is not switched off at later stages.

By contrast with the CXCR2<sup>-/-</sup> mice, mice depleted of neutrophils using a rabbit antineutrophil serum show faster wound healing than controls.<sup>50</sup> Likewise, the chemokine receptor, CCR1, which is involved in neutrophil and macrophage recruitment and strongly upregulated after skin injury, is dispensable for wound healing. The CCR1<sup>-/-</sup> mouse, in which leukocyte recruitment is impaired, shows no change in wound healing in experimental acute wounds.<sup>51</sup> The disparity between the expected importance of a major innate immune system component and its dispensability for healing is thought to reflect the differing roles of neutrophils in the noninfected wounds made in the laboratory versus wounds that occur in the natural world that are more likely to be contaminated.

Interleukin-1. Currently, there are 11 members of the IL-1 family, of which IL-1 $\alpha$  and IL-1 $\beta$  are best characterized. Keratinocytes produce large amounts of IL-1 $\alpha$  and less IL-1 $\beta$ ,<sup>30,52</sup> whereas epidermal DCs, called Langerhans cells, as well as wound-infiltrating macrophages produce mostly IL-1 $\beta$ , but not IL-1 $\alpha$ . While IL-1 $\alpha$  is translated into a biologically active form, IL-1 $\beta$  is translated as a propeptide that requires processing by caspase-1 in the inflammasome. However, the secretion pathways and mechanisms of IL-1 $\alpha$  and IL-1 $\beta$  remain a matter of debate. The NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasome, comprises a multiprotein complex, which is required for activation of inflammatory caspases such as caspase-1. This complex comprises the adaptor protein (ASC) and procaspase-1, both of which are expressed under homeostatic conditions in keratinocytes, and NALP3 whose expression is induced upon wounding. IL-1 $\alpha$

and IL-1 $\beta$  both bind to the IL-1 type I receptor to elicit an active immune response, whereas binding to IL-1R type II blocks IL-1 activity because it acts as a decoy receptor. IL-1 expression is rapidly induced upon skin injury and is a key player in the wound response by amplifying inflammatory mediators. IL-1 $\beta$  is a key inducer of the antimicrobial response in the skin and is also involved in shaping adaptive T cell immune responses. IL-1 $\alpha$  levels are low in wound fluids from acute wounds, but are elevated in fluids from chronic wounds. As IL-1 $\alpha$  has been shown to stimulate collagenase production, IL-1 $\alpha$  overproduction may be mechanistically linked to impaired wound healing through cleavage of collagen.<sup>53</sup> Furthermore, skin injury, induced either by UV radiation, skin irritants, or trauma, has been shown to liberate ATP into the extracellular space, which acts as a trigger for NALP3 inflammasome activation and subsequent IL-1 $\beta$  secretion.<sup>30,52,54</sup> IL-1 $\beta$  together with IL-23 and IL-6 is important for Th17 differentiation, and IL-17 regulates antimicrobial barrier function as discussed below.

An elegant study by Lee *et al.* demonstrated that IL-1 $\alpha$  is negatively regulated by caspase-8.<sup>52</sup> Caspase-8 becomes downregulated upon wounding in the granular epidermal layer, and IL-1 $\alpha$  subsequently mediates keratinocyte proliferation in a paracrine manner by first stimulating fibroblasts of the underlying dermis to secrete cytokines, which then induce keratinocyte division. The functional relevance of IL-1 in the wound healing response is somewhat controversial as IL-1R<sup>-/-</sup> mice do not show differences in the rate of closure of excisional wounds. Skin wounds in IL-1R<sup>-/-</sup> animals have decreased inflammation, decreased collagen levels, and improved restoration of normal skin architecture compared with skin wounds in wild-type mice.<sup>55</sup> In contrast, mice deficient in the endogenous antagonist of IL-1R (IL-1ra) exhibit impaired wound healing compared with wild-type mice, as evidenced by attenuated collagen deposition and delayed neovascularization.<sup>56</sup> Conversely, neutrophil recruitment is exaggerated and accompanied by augmented expression of IL-1s, TNF $\alpha$ , and CXC chemokines, macrophage inflammatory protein 2-alpha (MIP-2), and KC together with a failure to induce tumor growth factor  $\beta_1$  (TGF $\beta_1$ ) and angiogenesis.<sup>57</sup> This IL-1ra<sup>-/-</sup> phenotype is strongly reminiscent of an arrest in the transition from proinflammatory to anti-inflammatory macrophage phenotypes seen in chronic wounds.

Tumor necrosis factor  $\alpha$ . TNF $\alpha$  is a pleiotropic cytokine produced by a variety of cell types, including keratinocytes, macrophages, mast cells,

and T cells. There are two types of receptors for TNF $\alpha$  signaling: one TNF receptor with a molecular mass of 55 kDa (TNF-Rp55) and one with a molecular mass of 75 kDa (TNF-Rp75). TNF-Rp55 is expressed ubiquitously in many cell types, whereas TNF-Rp75 expression is predominantly restricted to hematopoietic and endothelial cells. TNF-Rp55 mediates various activities of TNF $\alpha$ , including cytotoxicity, fibroblast proliferation, and induction of superoxide dismutase, whereas TNF-Rp75 mediates thymocyte and cytotoxic T cell proliferation. Not surprisingly, TNF $\alpha$  can act in a beneficial or deleterious mode in the wound healing response in a dose-dependent manner. Elevation of TNF $\alpha$  leads to decreased production of granulation tissue, whereas low doses of TNF $\alpha$  promote collagen disposition,<sup>58–61</sup> demonstrating the importance of accurate cytokine regulation during wound healing. TNF $\alpha$  also suppresses the TGF $\beta$  stimulation of ECM production.<sup>62</sup> High levels of TNF $\alpha$  are present in the wound fluid of chronic nonhealing wounds, leading to excessive inflammation ultimately resulting in impaired wound healing and tissue destruction.<sup>63</sup>

Granulation tissue formation is a prerequisite for complete wound closure and healing. There are contradictory reports on the effects of locally applied TNF $\alpha$  on granulation tissue formation in skin wound healing,<sup>59,64,65</sup> indicating there is no consensus on the effects of local TNF $\alpha$  application. Wound healing studies in TNF-Rp55<sup>-/-</sup> mice showed enhanced collagen accumulation with reduced leukocyte infiltration at wound sites.<sup>66</sup>

**Interleukin-6.** Various types of cells, including macrophages, T cells, fibroblasts, keratinocytes, and endothelial cells, produce IL-6, which exhibits various activities on a wide variety of cells, including lymphocytes, DCs, and keratinocytes. The IL-6 receptor is a protein complex consisting of an IL-6 receptor subunit (IL-6R) and IL-6 signal transducer glycoprotein 130 (gp130). Furthermore, *STAT3* is one of the key components for IL-6 receptor signaling whereby IL-6 can stimulate keratinocyte proliferation. IL-6 induces the production of C-reactive protein, which can induce an inflammatory M1 phenotype in macrophages. IL-6 enhances AMPs through direct signaling and through IL-17 as IL-6 mediates expansion of Th17 lymphocytes. The essential involvement of IL-6 in the skin wound healing process is evidenced by delayed wound healing in IL-6<sup>-/-</sup> mice.<sup>67,68</sup>

**IL-17A and IL-22.** IL-17A and IL-22 are two members of the cytokine family with substantial

roles in host defense and other immunoregulatory functions.<sup>25,36,39,69–72</sup> IL-17 is not only the master cytokine of Th17 cells but is also produced by CD8<sup>+</sup> T cells and innate lymphocytes, such as  $\gamma\delta$  T cells, NK cells, and NKT cells.<sup>73</sup> IL-22 is produced by Th22 cells and minor subsets of other T cells and NK cells. Subsets of Th22 cells are resident in human skin, where they act as sentinel cells and promote keratinocyte migration.<sup>72</sup> Skin-resident T cells produce increased levels of IL-17A early upon skin injury and UV-induced skin damage.<sup>25,30</sup> The requirement for IL-17A for the normal wound healing response has been demonstrated in a mouse model, where IL-17A<sup>-/-</sup> mice showed delayed closure of incisional wounds compared with wild-type controls.<sup>25</sup> IL-17A participates functionally in the healing response by augmenting the skin antimicrobial barrier and by regulating epidermal regeneration and differentiation.<sup>25,28</sup> IL-17A signaling is mediated through IL-17 receptor A (IL-17RA) and IL-17RC,<sup>74,75</sup> and keratinocytes upregulate IL-17RA upon skin injury.<sup>25</sup> *In vivo*, IL-17A produced by the enigmatic murine dendritic epidermal  $\gamma\delta$  T cell population (DETC) induces epidermal murine  $\beta$ -defensin 3 (hBD2 ortholog), S100A8, and Reg3 $\gamma$  (REG3A ortholog). The repair function of IL-17A in acute wounds is supported by several findings: recombinant IL-17A restores defective wound healing and AMP expression in IL-17A<sup>-/-</sup> skin<sup>25</sup>; skin-resident T cells, which are proficient in IL-17A production, are dysfunctional in nonhealing wounds<sup>76</sup>; and IL-17A is decreased at wound sites in severe burn wound patients who are highly susceptible to infections and sepsis.<sup>77</sup> Furthermore, patients with genetic mutations limiting the levels of IL-17A, such as Job's syndrome, suffer from recurring mucocutaneous infections and ulcers.<sup>78,79</sup> Whether repair of such wounds may be improved by therapeutic targeting of skin-resident T cells to produce IL-17A or recombinant IL-17 is not yet known.

IL-22 mediates its functions by binding to its heterodimeric receptor consisting of IL-10 receptor  $\beta$ 2 (IL-10R $\beta$ 2) and IL-22 receptor (IL-22R) and activating signaling pathways, which stimulate *STAT3* phosphorylation and nuclear translocation.<sup>80–84</sup> *In vitro* studies have suggested that IL-22 signals to epithelial keratinocytes of the skin to promote proliferation and migration and inducing AMPs.<sup>85,86</sup> An essential role for IL-22 in the wound healing response has been demonstrated in IL-22<sup>-/-</sup> mice. The delayed wound healing response in IL-22<sup>-/-</sup> mice has been attributed to defective fibronectin and collagen production by fibroblasts and impaired myofibroblast differentiation rather than to keratinocyte-mediated effects.<sup>87</sup>

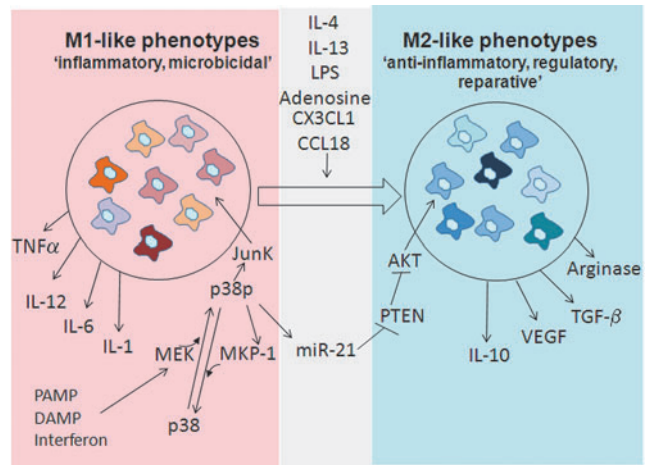


## microRNA

Changes in microRNA (miR) during wound healing have recently received considerable attention. These molecules suppress target mRNA expression through a complementary sequence usually in the 3'UTR and affect several genes involved in the innate immune response. Micro RNA (miR)-16, -20a, -21, -106a -130a, and -203 have been shown to be overexpressed in venous leg ulcer patients.<sup>88</sup> They contribute to delayed wound healing through inhibiting growth factor signaling and reepithelialization. miR-203 is also increased in psoriasis and its targets include p63, suppressor of cytokine signaling 3 (SOCS3), and IL-8,<sup>89</sup> whereas miR-21 and miR-130a target the leptin receptor.<sup>88</sup> These miRNAs appear to serve restoration of normal keratinocyte differentiation. Studies led by Pivarsci demonstrated that miR-146 acts as a regulatory element in keratinocyte innate immunity. miR-146 suppresses the production of inflammatory mediators under homeostatic conditions, serves as a negative feedback regulator following TLR2 activation, and represses neutrophil chemotaxis.<sup>90</sup> Its overexpression in human psoriatic skin has been suggested to reflect insufficient compensation of overt skin inflammation.<sup>91</sup> Furthermore, miR-146 targets sma and mothers against decapentaplegic-related protein 4 (SMAD4) and thus may inhibit the TGF $\beta$  induction of the M2 phenotype.<sup>92</sup> miR-21 appears to play a substantial role in the macrophage switch and is discussed further below.

## Switch from inflammation to repair

As inflammation achieves its goals and the wound is cleared of contamination, the acute wound healing process and inflammatory stage move to a reparative stage associated with keratinocyte proliferation, the development of granulation tissue, and keratinocyte migration. It is at this point that chronic wounds stall (Fig. 2a–c). One of the major events at this time is a change in the phenotype of macrophages from an inflammatory and microbicidal phenotype, grouped as M1 macrophages, secreting IL-1, TNF $\alpha$ , IL-6, IL-12, MMPs, and other cytokines, to an anti-inflammatory, regulatory, and reparative phenotype, classed and grouped as M2, producing arginase, TGF $\beta$ , CCL18, PGE2, and IL-10 (Fig. 4). Because of the complexity of macrophage biology, we refer to two excellent reviews.<sup>93,94</sup> Recent studies suggest multiple pathways by which macrophages are induced to develop into M2 and M2-like macrophages. One pathway occurs through an IL-4/IL-13-dependent signaling cascade,<sup>95</sup> a second is mediated through TLR and IL-1R signaling and adenosine-sensing



**Figure 4.** Macrophage plasticity in wound healing. Macrophages play multiple roles in wound healing. In the early stages of wound healing, macrophages release inflammatory cytokines that promote the inflammatory response by recruiting and activating additional leukocytes. Macrophages are also important for decontaminating the wound from microbes and clearing apoptotic cells, thereby paving the way for the resolution of inflammation and the beginning of the proliferative phase of wound healing. As the microenvironment changes within the wound bed, macrophages undergo a phenotypic switch to an anti-inflammatory, regulatory, and reparative state, which stimulates keratinocytes, fibroblasts, and endothelial cells to promote tissue regeneration. In this way, macrophages promote the transition to the proliferative phase of healing. Multiple pathways are critical in this macrophage switch from M1 to M2 and include IL-4, IL-13, TLR stimulation, adenosine signaling, CXCL1, and CCL18. Furthermore, miRNA-21 targets phosphatase with a tensin motif (PTEN) and subsequent reduction in PTEN extends the activation of protein kinase B (AKT), which turns off the inflammatory state and promotes the anti-inflammatory state.

receptor A(2A)R,<sup>56,95,96</sup> a third is mediated by CCL18,<sup>97</sup> and a fourth involves CX3CL1 (fractalkine).<sup>57</sup> While the M1 macrophages are involved in microbial decontamination and inflammation upon skin injury, M2 and M2-like macrophages induce anti-inflammatory, regulatory, and reparative functions aimed at promoting wound closure. In addition, a hybrid macrophage phenotype has been recently identified that shows some M2 as well as M1 features and is induced by CXCL4 (platelet factor 4) released by platelets at the inception of the injury response.<sup>98</sup> The *in vivo* relevance of CX3CL1 and its receptor (CX3CR1) for the wound healing response is supported by the finding that its expression is highly induced during the healing of acute excisional wound<sup>57</sup> and that CX3CR1<sup>-/-</sup> mice show delayed wound healing that could be restored by bone marrow transplants from wild-type mice, implying that the relevant site of the receptor is a bone marrow-derived cell, such as the macrophage. By comparison with wild-type, CX3CR1<sup>-/-</sup> mice showed reduced numbers of macrophages and particularly the products of anti-inflammatory macrophages such as TGF $\beta$  and VEGF at the wound site.

This was interpreted as indicating that fractalkine is involved in the recruitment of anti-inflammatory macrophages to the wound site.<sup>57</sup>

One of the intracellular signaling pathways controlling the phenotypic switch involves p38 and miR-21.<sup>99,100</sup> miR-21 affects several cellular actors in wound healing in multiple ways. Its promoter contains 3 activator protein 1 (AP-1) sites, a PU-1 site, and nuclear factor kappa B (NF $\kappa$ B) site. In macrophages, miR-21 plays a key role in the transition from an inflammatory to an anti-inflammatory phenotype. The p38/Jun stress pathway is activated by PAMPs and DAMPs, leading to the secretion of inflammatory cytokines, such as IL-1, TNF, and IL-6.<sup>101</sup> In a study of a muscle repair system, Perdiguerro *et al.* showed that the activity of p38 is controlled by mitogen-activated kinase phosphatase-1 (MKP-1), which it induces in a feedback loop.<sup>99,100</sup> It also induces miR-21, one of whose targets is phosphatase with a tensin motif (PTEN). The reduction in PTEN extends the activation of protein kinase B (AKT), which turns off the inflammatory state and promotes the anti-inflammatory state. It also leads, over some days, to a general reduction in cytokine secretion that is probably important for the ultimate resolution of the macrophage phase of wound healing.<sup>99,100</sup> Under normal conditions, this process appears to possess an intrinsic time course. If the healing process fails to pace this process, the phenotype of the macrophage may become inappropriate for the stage of healing, leading to failure. Fibroblasts and keratinocytes show a response to the elevation of miR-21 that differs from macrophages and stimulates migration.<sup>102</sup> This is of great importance for the inception of granulation tissue formation and wound closure. Thus, miR-21 appears to be involved in both the termination of the inflammatory phase of wound healing and the start of the proliferative repair phase.

miR-21 has been reported to be increased in the skin of db/db mice, but reduced in diabetic wound healing.<sup>103</sup> This may be the result of decreased expression in macrophages, fibroblasts, keratinocytes, or other cells.<sup>103</sup> In db/db mice, inhibition of p38 has been reported to improve wound healing, perhaps by reducing the inflammatory phase and delaying anti-inflammatory macrophage conversion to allow wound clearance to be completed, thus allowing healing.<sup>104</sup> This is supported by the prolonged secretion of chemokines in the diabetic mouse.<sup>105</sup> By contrast, in venous ulcers, miR-21 has been reported to be elevated.<sup>106</sup> It is also consistent with the observation that the addition of macrophages induced with IL-4 or IL-10 delays wound healing in db/db mice.<sup>107</sup> Thus, it is possible

that venous ulcers and diabetic ulcers each show a derangement of the macrophage phenotypic switch, but in different ways, in each case important to an inappropriate immune response. In venous ulcers, oxidative stress may activate p38, leading to a premature induction of the anti-inflammatory macrophage phenotypes. As this process follows an intracellular program through to a decline in macrophage activity, the process may be prematurely and inappropriately shut down. In diabetic ulcers, the inflammatory process may just never reach the completion, perhaps through inadequate nutritional supply.

An alternative mechanism for the failure of the macrophage phenotype switch is that it is impaired by extracellular conditions in the wound. Separate reports using two different mouse models of nonhealing wounds<sup>108,109</sup> have demonstrated that iron overloading and sustained inflammasome activation of macrophages results in defective transition from an inflammatory M1 to a prohealing M2 phenotype. Similar mechanisms have long been suggested for human patients.<sup>110</sup>

## CONCLUSION

The innate immune system comprises a network of interacting mechanisms that provide a rapid response to clear microbial contamination upon skin injury. In the development of chronic wounds, this process stalls in the late inflammatory stage. The major defect in nonhealing wounds occurs at the phenotypic switch from inflammatory macrophages to an anti-inflammatory phenotype. This step is controlled by IL-4/IL-13, TLR activation, adenosine, and fractalkine and is influenced by IL-1 and involves a signal transduction pathway whose function depends on a calibrated activity of the stress-associated MAPK, p38. Active p38 not only controls the secretion of inflammatory cytokines but also induces miR-21 that indirectly activates AKT and induces the anti-inflammatory macrophage phenotype. In fibroblasts and keratinocytes, it induces granulation tissue and migration. Interference with this mechanism leads to delayed wound healing and the system shows abnormalities in chronic wounds.

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## TAKE-HOME MESSAGES

- The innate immune system comprises a group of defense and contamination clearing functions capable of rapid deployment.
- First-line defense molecules include antimicrobial peptides, CXC chemokines secreted by fibroblasts and keratinocytes, IL-1, and TNF $\alpha$ .
- Recruited neutrophils and macrophages secrete cytokines that stimulate further recruitment of immune cells.
- The inflammatory phase is limited in its duration in normal wound healing and is succeeded by a prorepair phase. A central player in this switch is a phenotypic change in the macrophage.
- Failure of this switch is a candidate for the process that leads to chronic wounds. Factors involved in the control of this step through p38 include IL-4/IL-13, TLR and adenosine, and fractalkine.

the commercial development of a live fibroblast product for the treatment of chronic wounds.

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### Abbreviations and Acronyms

A(2A)R = adenosine type 2A receptor
AKT = protein kinase B
AMP = antimicrobial peptide and protein
AP-1 = activator protein 1
ASC = apoptosis-associated speck-like protein containing CARD
ATP = adenosine triphosphate
CAMP = cathelicidin antimicrobial peptide
CCL = group of chemokine ligands characterized by two adjacent cysteines
CRAMP = cathelicidin-related antimicrobial peptide
CXCL = class of chemokines characterized by the sequence cysteine, any amino acid, cysteine.
CXCR = CXC chemokine receptor
DAMP = danger-associated molecular pattern
db/db = mice lacking the leptin receptor
DC = dendritic cell
DETC = dendritic epidermal $\gamma\delta$ T cells
dsRNA = double-stranded ribonucleic acid
eATP = extracellular adenosine triphosphate
ECM = extracellular matrix
EF = calcium-binding motif
ELR = asparagine leucine arginine
hBD = human $\beta$ -defensin
IFN = interferon
IGF = insulin-like growth factor
IL = interleukin
KC = keratinocyte
KGF = keratinocyte growth factor
LPS = lipopolysaccharide
MAPK = mitogen activated protein kinase
MIP-2 = macrophage inflammatory protein 2-alpha
miR = micro RNA
MKP-1 = mitogen-activated kinase phosphatase-1
MMP = matrix metalloproteinase
NALP3 = NACHT, LRR and PYD domains-containing protein 3

**Abbreviations and Acronyms  
(Cont.)**

NF $\kappa$ B = nuclear factor kappa B  
NK = natural killer cell  
NKT = natural killer T cell  
NO = nitric oxide  
p38 = stress-related mitogen-activated kinase  
PAMP = pathogen-associated molecular pattern  
PBMC = peripheral blood mononuclear cells  
PGE2 = prostaglandin E2

PRR = pathogen recognition receptor  
PTEN = phosphatase with a tensin motif  
PU-1 = master transcription factor of the myeloid system  
RAGE = receptor for advanced glycation endproducts  
REG3A = regenerating islet-derived protein 3A  
RNase7 = ribonuclease 7  
ROS = reactive oxygen species  
SMAD4 = sma and mothers against decapentaplegic-related protein 4

STAT3 = signal transducer and activator of transcription 3  
SOCS3 = suppressor of cytokine signaling 3  
TGF $\beta$  = tumor growth factor  $\beta$   
Th = T helper cell  
TLR = Toll-like receptor  
TNF = tumor necrosis factor  
TNF-Rp55, TNF-Rp75 =  
TNF receptors  
UV = ultraviolet  
VEGF = vascular endothelial growth factor