Toll-Like Receptor Function in Acute Wounds

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Significance: Inflammation is an integral part of immune response and supports optimal wound healing in adults. Inflammatory cells such as neutrophils, macrophages, dendritic cells, lymphocytes, and mast cells produce important cytokines, chemokines, and growth factors. These immune cells interact with keratinocytes, fibroblasts, and endothelial cells (ECs), as well as the extracellular matrix within a complicated network that promotes and regulates wound healing. Aberrant and persistent inflammation may result in delayed wound healing, scar formation, or chronic wounds. Targeting the molecules involved in the inflammatory response may have great potential therapeutic value.

Recent Advances and Critical Issues: Toll-like receptors (TLRs) are pattern recognition receptors that recognize pathogen-associated molecular patterns from microbes or danger-associated molecular patterns from damaged cells. The discovery of TLRs sheds new light on the mechanism by which the inflammatory or innate immune response is initiated in wound healing. Convincing evidence now shows that multiple types of cells, including infiltrating or resident inflammatory cells, keratinocytes, fibroblasts, and ECs, express specific types of TLRs. Experimental reduction of certain TLRs or treatment of wounds with TLR ligands has been shown to affect wound healing. A better understanding of the involvement of TLRs in the innate immune response during skin wound healing may suggest novel strategies to improve the quality of tissue repair.

Future Directions: Despite the indisputable role of TLRs in regulating the immune response in acute wound healing, the functions of TLRs that are relevant to human wound healing and chronic wounds are poorly understood.

Keywords: skin, wound, toll-like receptor, innate immunity, inflammation

SCOPE AND SIGNIFICANCE

TOLL-LIKE RECEPTORS (TLRs) are important molecules for activating and regulating the innate immune response. TLRs are expressed by both immune and nonimmune cells in the skin. This review provides (1) an overview of TLRs, (2) a description of the expression of TLRs in resident and infiltrating inflammatory cells in skin wound healing, (3) a discussion of recent findings regarding the function and signaling mechanisms

of TLRs in normal and diabetic acute wounds, and (4) examples of future directions, particularly in the targeting of TLR signaling.

TRANSLATIONAL OR CLINICAL RELEVANCE

Inflammation plays an essential role in optimal adult skin wound healing, and prolonged inflammation is detrimental to wound healing. Recent studies show that TLR activation is a key element in initiating

and mediating inflammation after injury. Targeting TLRs or their signaling pathways may provide novel therapeutic strategies for the treatment of chronic or poorly healing wounds.

AN OVERVIEW OF TLRs

At least 13 TLRs have been identified in mammals in the last two decades.^{1,2} Growing evidence shows that TLRs play a key role in host defense by regulating both innate and adaptive immune responses. TLRs are expressed on many cell types such as macrophages, neutrophils, dendritic cells (DCs), Langerhans cells (LCs), mast cells, lymphocytes, endothelial cells (ECs), keratinocytes, and fibroblasts.^{3,4} TLRs are important pattern recognition receptors (PRRs) recognizing pathogen-associated molecular patterns (PAMPs) or exogenous ligands. They bind to a number of diverse PAMPs on microbes, including those found in viruses, bacteria, fungi, and parasites. TLRs detect specific molecules on the surface or the inside of those organisms. In addition to detecting the conserved PAMPs, TLRs also recognize danger-associated molecular patterns (DAMPs), also known as alarmins or endogenous ligands. DAMPs are host biomolecules released from damaged cells or tissues after injury. DAMPs include nuclear or cytosolic components such as DNA, RNA, β -defensins, heat shock proteins (HSPs), S100 proteins, free fatty acids, and high-mobility group box 1 protein (HMGB1). DAMPs also derive from the breakdown products of extracellular matrix (ECM)

such as hyaluronan (HA) fragments, heparan sulfate, fibrinogen, and fibronectin extra domain A.^{1,2,5,6} Table 1 provides a summary of PAMPs, DAMPs, and their corresponding TLRs (adapted from^{7–15}).

All TLRs have an extracellular domain, a transmembrane domain, and a highly homologous cytoplasmic toll/interleukin (IL)-1 receptor domain (TIR). TLR1, 2, 4, 5, 6, 10, and 11 exist on the surface of the cell membrane, while TLR3, 7, 8, 9, 12, and 13 are present on the membranes of intracellular compartments such as endoplasmic reticulum, endosomes, lysosomes, and endolysosomes where they recognize microbial nucleic acids.^{1,2,5} There are two TLR signaling pathways. The MyD88-dependent pathway results in induction of NF- κ B-dependent transcription of inflammatory cytokines such as TNF- α , IL-1, IL-6, IL-8, IL-12, and MIP2, as well as production of specific costimulatory molecules and adhesion molecules. The TIR domain-containing adapter-inducing interferon (IFN)- β (TRIF)-dependent pathway (or the MyD88-independent signaling pathway) results in activation of NF- κ B and IFN regulatory factors, which in turn lead to the expression of inflammatory cytokines and type I IFN genes, respectively.^{1,2,5}

TLRs ON INNATE IMMUNE CELLS IN THE SKIN AND THEIR ROLES IN WOUND HEALING

Skin is the largest organ in the human body and serves as the first line of defense against insults

Table 1. Pathogen-associated molecular patterns, danger-associated molecular patterns, and their corresponding toll-like receptors^{7–15}

Endogenous Ligands (DAMPs)/Alarmins	TLRs	Exogenous Ligands (PAMPs) and Sources	TLRs
Biglycan, endoplasmic reticulum chaperones, free fatty acid, HA, human cardiac myosin, HMGB1, HSP60, HSP70, HSPGp96, and uric acid	TLR2	Triacylated lipoproteins (bacteria)	TLR1&2
		GPI anchor of mucin-like glycoproteins (<i>Trypanosoma cruzi</i>), Lipoteichoic acid (Mycobacteria), lipoproteins glycolipids and LPS (<i>Spirochetes</i>), lipoteichoic acid (bacteria), LPS, and fimbriae (<i>Porphyromonas gingivalis</i>), Outer membrane protein A (<i>Klebsiella</i>), Peptidoglycan (Gram-positive bacteria), Porin (<i>Neisseria meningitidis</i>), Zymosan (yeast)	TLR2
Biglycan, CD138, α -crystallin A chain, β -defensin 2, endoplasmic reticulum chaperones, free fatty acid, fibronectin, heparan sulfate, HA, HMGB1, HSP22, HSP60, HSP70, HSP72, HSPGp96, uric acid, OxPAPC, resistin, S100 proteins, surfactant protein A, and tenascin-C	TLR4	dsRNA (virus), synthetic poly (I:C)	TLR3
		Envelope proteins (Murine retroviruses), Flavolipin (<i>Flavobacteria</i>), F protein (RS virus), HSP60 (<i>Chlamydia</i>), LPS, and lipoteichoic acid (Gram-negative bacteria), Taxol (plant)	TLR4
		Flagellin (bacteria with flagella)	TLR5
		Profilin (<i>Toxoplasma gondii</i>)	TLR2&6
		Diacetylated lipoprotein (<i>Mycoplasma</i>), Heat-labile soluble factor (Group B streptococcus), Phenol-soluble modulin (<i>Staphylococcus</i>), Zymosan (yeast)	TLR7
Chromatin-IgG complexes and HMGB1	TLR9	Small single-stranded RNA (RNA virus)	TLR8
		Small single-stranded RNA (RNA virus)	TLR9
		Unmethylated CpG DNA (bacteria, viruses, insects)	TLR9
		Profilin (<i>Toxoplasma gondii</i>)	TLR11
		Profilin (<i>Toxoplasma gondii</i>)	TLR12
		RNA (bacteria and virus)	TLR13

DAMPs, danger-associated molecular patterns; HA, hyaluronan; HMGB1, high-mobility group box 1 protein; HSP, heat shock protein; LPSs, lipopolysaccharides; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor.

from the environment involving temperature changes, allergens, or poisons, and from trauma, such as lacerations or surgical wounds. The skin has always been primarily considered a passive barrier between the host and environment. Recent discoveries dispel this idea, though, as they show that a variety of cells in the skin play critical roles in innate and acquired immune responses in cutaneous immunopathology.³

Human skin comprises two major parts, the epidermis and dermis. The epidermis serves as a physical barrier and early warning system. The cells in the epidermis relevant to the skin immune response are keratinocytes, LCs, $\gamma\delta$ -T cells, and melanocytes. The dermis mainly comprises fibroblasts, resident immune cells, and ECM that is produced primarily by fibroblasts. Resident immune cells in noninflamed dermis include dermal DCs, macrophages, and mast cells. An inflamed dermis may contain neutrophils, macrophages, eosinophils, and lymphocytes, including T, natural killer T (NKT), and B cells.^{3,16,17}

Skin wound healing is a complicated pathophysiological process that consists of four overlapping phases: a hemostasis phase, characterized by fibrin clot formation and platelet activation/aggregation; an inflammatory phase, characterized by neutrophil and macrophage infiltration; a proliferative phase, dominated by reepithelialization, collagen synthesis, and angiogenesis; and a remodeling phase, involving the resolution of inflammation, pruning of excessive blood vessels, and collagen maturation.^{18,19} In this section, we will

discuss TLR expression in skin resident and non-resident cells and their roles in wound healing (summarized in Table 2).

Neutrophils

As part of the inflammatory phase, a massive infiltration of neutrophils is observed in the sub-epidermal region of wound edges soon after injury. Neutrophils protect the host from infection by combating invading microorganisms and clearing cellular debris.²⁰ On the other hand, neutrophils produce many bioactive substances such as reactive oxygen species, serine proteases, and matrix metalloproteinases (MMPs). Unbalanced production of these enzymes can have negative impact on the repair process by inducing bystander tissue damage.²¹ Therefore, neutrophils may both support and impair wound healing.^{8,20–22} Neutrophils express TLR1, 2, 4, 5, 6, 7, 8, 9, and 10. The agonists of TLRs found on neutrophils induce cytokine release and superoxide production by these cells.²³ The roles of TLRs on neutrophils seem likely to play an important role in skin wound healing, yet few studies have examined the specific role of these receptors following skin injury.

Macrophages

As neutrophil content diminishes, macrophages become the predominant cell type in the wound. Macrophages have two major roles in the healing process: engulfment of necrotic or apoptotic neutrophils, providing an important clearance mechanism, and production of cytokines, chemokines, and growth factors, which stimulate inflammatory

Table 2. TLRs found on specific cells and presence in wounds

Cells	TLRs	References	TLRs Shown to be Associated with the Cell Type in Healing Wounds	References
Adipocyte	TLR1, 2, 4, 7, 8	74,75		
B cells	TLR1–10	31	TLR4	55
Dendritic cell	TLR1, 2, 3, 7, 9, 13	3,10	TLR7, 9	35
Endothelial cell	TLR2, 4	77–79		
Fibroblast	TLR1–10	70		
Keratinocyte	TLR1–6, 9	58–67	TLR4	58
Langerhans cell	TLR1–10	3		
Macrophage	TLR1–10, 13	3,10,30,31		
Mast cell	TLR1–7, 9	3		
Melanocyte	TLR1–4, 6, 7, 9	84,85		
Neutrophil	TLR1, 2, 4–10	23		
Pericytes	TLR4	81		
Schwann cells	TLR1–7	86		
T cell				
CD4 ⁺	TLR2–4, 5, 7–9	40		
CD8 ⁺	TLR2, 3, 9	40		
$\gamma\delta$ T cells	TLR2 4, 9	46–48	TLR2, 4, 9	48
Treg	TLR4, 5, 7, 8	49		
NKT	TLR2–5, 7, 9	40		

NKT, natural killer T.

reaction, help recruit more inflammatory cells, and promote the proliferative phase of repair, including angiogenesis and tissue regrowth.^{19,24,25} Multiple studies now demonstrate that macrophages play an indispensable role in wound healing.^{26,27} However, macrophages are known to exhibit heterogeneous phenotypes within wounds. Macrophages in early wounds produce more proinflammatory cytokines (TNF- α and IL-6) and less TGF- β , whereas the opposite is observed in the later stage of wound healing.²⁸ Thus, wound macrophages share the characteristics of both classically and alternatively activated macrophages (M1 and M2, respectively).^{28,29} Macrophages express all TLRs,³ but predominantly express TLR1, 2, 4, 5, 8, and 13.^{10,30,31} Ligand stimulation of TLRs on macrophages induces the production of proinflammatory cytokines and the antimicrobial peptide, cathelicidin, and leads to increased phagocytosis.³ Similar to neutrophils, macrophage TLR function in wound healing has not been well studied.

Dendritic cells

DCs are derived from circulating monocytes and serve primarily as phagocytes and antigen-presenting cells. There are two major types of DCs termed classical (cDCs) and plasmacytoid, and both types are present in the skin.^{32,33} A few subsets of DCs have been described to exist in the mouse skin, including XCR1+cDC, LC, DN cDC, CD11b+cDC, and monocyte-derived DC based on their unique cell surface marker expression. Subsets of CD141+cDC, LC, CD1c+cDC, and monocyte-derived DC are present in human skin.³⁴ DCs express TLR1, 2, 3, 7, 9, and 13; activation of these receptors causes the production of cytokines and chemokines.^{3,10} Limited information is available regarding the roles of DCs in wound healing. However, plasmacytoid DCs have been shown to quickly infiltrate into wounds and to express IFNs- α & β through TLR7- and TLR9-dependent recognition of self-nucleic acids in acute murine and human skin wound models. Therefore, DCs seem to be important innate immune cells for the induction of early inflammatory responses.³⁵ More studies are warranted to elucidate the roles of DCs and TLRs on DCs in wound healing.

Langerhans cells

LCs are a subset of DCs and reside in the epidermis. LCs express TLR1–10 and respond to the stimulation of TLR2, 3, 4, 7, and 8 by producing inflammatory cytokines and chemokines.³ Given the location of LCs in the skin, LCs should be important players in wound healing, yet there is little available information about the role of these cells or their TLR receptors in wound healing.

Mast cells

Mast cells have been shown to express TLR1–7 and 9. Stimulation by the appropriate TLR ligand causes the production of a variety of inflammatory mediators.³ Upon activation by injury, mast cells degranulate and release cytokines, chemokines, growth factors, and enzymes, which may add fuel to the inflammatory response.³⁶ Blockade of mast cell activation results in reduced inflammatory cytokine production and smaller scars with better organized collagen fibers.^{37–39} However, the relationship between TLR activation of mast cells and the role of mast cells in skin wound healing has not been investigated.

T cells

CD4⁺ and CD8⁺ cells express functional TLR2–4, 5, and 7–9 and TLR2, 5, 8, and 9, respectively.⁴⁰ Substantial numbers of CD4⁺ and CD8⁺ T cells migrate to the wound bed in the later stages of healing.^{41,42} The roles of T cells in wound healing, however, are not clear as there are multiple conflicting results. In the complete absence of CD4⁺ or CD8⁺ T cells, wound closure is not affected.^{41,43} However, in the absence of CD8⁺ cells, wound breaking strength and collagen synthesis are shown to be markedly increased in one study,⁴³ but not changed in another.⁴¹ Despite the fact that wound closure is not affected in CD4 or CD8-deficient mice, there is upregulated expression of IL-1 β , IL-6, IL-17, IFN- γ , and CXCL-1 and downregulated expression of IL-4 in CD4-deficient mice. In contrast, wounds in CD8-deficient mice show downregulated expression of IL-1 β , IL-6, TNF- α , CXCL-1, and CCL-2 and upregulated expression of IL-4 compared with wild-type mice.⁴¹ Whether TLR engagement influences CD4⁺ or CD8⁺ T cell function in skin wound healing is unknown.

One special subset of T cells that is known to play a role in wound healing is $\gamma\delta$ T cells. These cells, which bear $\gamma\delta$ T cell receptor (TCR), are abundant in surface epithelia. $\gamma\delta$ T cells have important roles in skin wound healing as they can both produce and/or respond to IGF-1 and KGFs after TCR stimulation.^{44,45} $\gamma\delta$ T cells also express TLR2 and TLR4^{46,47} and respond to mitochondrial DAMP stimulation. In a burn wound model, expression of TLR2, 4, and 9 in $\gamma\delta$ T cells was seen to be significantly increased.⁴⁸ Therefore, activation of $\gamma\delta$ T cells may contribute to the subsequent inflammatory response in the healing processes. Similarly, another unique subset of T lymphocytes named regulatory T cells (Tregs, CD4⁺CD25⁺Foxp3⁺) express TLR4, 5, 7, and 8.⁴⁹ Tregs are presented in the skin wound tissue and facilitate skin

wound repair.⁵⁰ Therefore, it is possible that TLR activation on Tregs may also be involved in regulation of inflammatory response during wound healing. An additional subset of T cells, NKT, express TLR2–5, 7, and 9.⁴⁰ NKT cells infiltrate early skin wounds. In NKT cell-deficient mice, wound closure is accelerated and a transient enhancement of the infiltration of neutrophil and macrophage is seen.^{16,51} However, another study using a different NKT cell-deficient mouse line demonstrated delayed wound healing.¹⁷ It is not known if TLRs play any roles in activation of NKT cells during the response to injury.

B cells

Recent studies show that B cells express TLR1–10³¹ and activation of TLRs on B cells is required for CD4⁺ cell-mediated antibody production.⁵² B cells are present in both mouse and human skin wounds.^{53,54} Skin wounds in B cell-deficient mice have been reported to have delayed wound closure, decreased granulation tissue formation, decreased expression of FGF1, FGF2, IL-6, IL-10, PDGF, and TGF- β , reduced infiltration of neutrophils and macrophages, but not mast cells and CD3⁺T cells.⁵⁵ The TLR4 endogenous ligand, HA, stimulates B cells to produce IL-6, IL-10, and TGF- β through TLR4. Topical application of HA improves wound healing in wild-type mice, but not in B cell-deficient mice, suggesting that B cells regulate wound healing through HA-TLR4 signaling.⁵⁵ However, given the fact that few B cells are present in the wound, the significance of their roles through activation of TLR4 needs further study.

Keratinocytes

Keratinocytes are the main constituent of the epidermis and play a critical role in wound healing. Reepithelialization of wounds begins within a few hours after injury and continues into the proliferation phase. Studies show that keratinocytes are a major contributor to epidermal cytokine production. Many of the currently identified cytokines in wounds, such as IL-1 α & β , IL-6, 8, 10, 12, 20, 24, and TNF- α , can be produced by keratinocytes.^{56–58} Cytokine production by keratinocytes influences keratinocyte proliferation, migration, and differentiation processes, has multiple effects for the recruitment of inflammatory cells, and may have systemic effects on the immune system.^{56–58} Skin keratinocytes express TLR1–6 and 9.^{58–67} These TLRs are involved in keratinocyte activation, proliferation, and migration,^{58–67} indicating that TLR signaling in keratinocytes may play an important role in wound healing. However, there are no animal or human

studies specifically investigating the roles of TLRs on keratinocytes in skin wound healing.

Fibroblasts

Fibroblasts are the major cellular components in the dermis. In the proliferation phase, skin-resident fibroblasts or fibroblasts that have differentiated from blood-borne fibrocytes produce ECM molecules to provide structural support to the repairing tissue.⁶⁸ Emerging evidence shows that fibroblasts can also participate in regulation of inflammation. Fibroblasts can be induced to produce a variety of cytokines and growth factors such as VEGF, PDGF, FGF2, EGF, TGF- β , MMPs, and tissue inhibitors of MMPs. These molecules can affect functions of fibroblasts themselves as well as ECs, keratinocytes, macrophages, neutrophils, and mast cells.^{68,69} Human skin fibroblasts express TLR1–10.⁷⁰ Treatment of fibroblasts with ligands to TLR2, 3, 4, 5, and 9 with IFN- γ results in the production of CXCL9, 10, and 11, chemokines that are important for the recruitment of T cells and NK cells.^{71,72} Given the large number of fibroblasts in the wound bed area, TLR activation in these cells may play an important role in wound healing, especially in the remodeling phase.

Adipocytes

A large number of adipocytes exist in subcutaneous tissue, especially in type II diabetic and obese individuals. In diabetes, adipocytes may contribute to the observed chronic low-grade inflammation that is found in diabetic skin.⁷³ Recent studies show that normal human adipose tissue or adipocytes express TLR1, 2, 4, 7, and 8.^{74,75} TLR4 expression is increased in diabetic adipocytes.⁷⁴ Stimulation of adipocytes with lipopolysaccharides (LPSs), free fatty acids, or a TLR2 agonist (synthetic lipoprotein Pam3CSK4) results in increased IL-6, TNF- α , and NF- κ B expression,^{74–76} indicating that activation of TLR2 and TLR4 in adipocytes, especially in type II diabetic and obese individuals, may be involved in subcutaneous inflammatory response during wound healing.

Endothelial cells

Human dermal microvascular ECs express high levels of TLR4 and low levels of TLR2. These ECs respond to LPSs through the TLR4-NF- κ B signaling pathway, but do not respond to TLR2 ligands such as lipoproteins.⁷⁷ Furthermore, LPS, TNF- α , or IFN- γ treatments induce TLR2 expression in both human dermal microvascular and human umbilical vein ECs. LPSs and IFN- γ have a synergistic effect on the induction of TLR2 and also upregulate TLR4 expression.⁷⁸ Several other

inflammatory molecules can regulate TLR2 and TLR4 expression in ECs. The TLR4 agonist HA can induce TLR-dependent EC expression of the neutrophil chemoattractants, IL-8 and CXCL-1.⁷⁹ Thus, endogenous ligands generated from degraded ECMs may prompt ECs to recognize injury in the early stage of wound repair.

Pericytes

Pericytes, cells that wrap around mature vessels, are a major and critical component of mature blood vessel structure. Multiple studies show that appropriate pericyte coverage and function can enhance angiogenesis, wound closure, epidermal regeneration, and granulation tissue formation and may stimulate myofibroblast differentiation during the wound healing process.⁸⁰ Pericytes in the lungs express functional TLR4.⁸¹ However, there are no reports investigating the role of TLRs expressed by pericytes in skin wound healing.

Melanocytes

Melanocytes are melanin-producing cells located in the epidermis and have been shown to migrate into wounds.^{82,83} Human melanocytes constitutively express TLR1–4, 6, 7, and 9. Stimulation of melanocytes with TLR ligands such as peptidoglycan (TLR2 ligand), polyriboinosinic-polyribocytidylic acid [poly (I:C)] (TLR3 ligand), LPSs (TLR4 ligand), imiquimod (TLR7 ligand), or CpG 2006 (TLR9 ligand) results in expression of IL-8, IL-6, CCL2, CCL3, and CCL5. Activation of TLRs in melanocytes also results in upregulation of phosphorylated I κ B α and the nucleus translocation of NF- κ Bp65.^{84,85} However, the role of TLR stimulation and melanocyte function in wound healing has not been reported.

Peripheral nerve cells

The peripheral nervous system (PNS) is widely distributed in skin, so anatomical and functional damage of the PNS nearly always accompanies skin wounds. TLRs are expressed in several parts of the PNS. Schwann cells in the PNS express various functional TLRs, including TLRs1–7 especially with high levels of TLR3, 4, and 7.⁸⁶ Schwann cells function as the glia of the PNS. These cells play important roles in myelination and in the regeneration of neurons. Schwann cells also have immunologic functions as they may serve as antigen-presenting cells and can produce cytokines, chemokines, and adhesion molecules.⁸⁷ Sensory and motor neurons in PNS also express TLRs.⁸⁶ However, the function of TLRs expressed in skin PNS is yet to be explored in the context of wound repair.

One intersection of the PNS and wound healing that might involve TLRs is through specific neuropeptides and neurotrophic growth factors. The neuropeptide, substance P (SP), is produced by neurons as well as ECs, macrophages, granulocytes, lymphocytes, and DCs. SP is a pain transmitter and an important regulator of immune function, and SP can promote angiogenesis by mobilization of endothelial progenitor cells.⁸⁸ After injury, injured nerve endings release SP, and a positive role for SP in wound healing has been confirmed in multiple studies.^{89,90–92} SP may act through TLRs as SP is known to upregulate TLR2 in mast cells,⁹³ but whether the beneficial effects of SP on wound healing are mediated through TLRs is not yet known. Another possible bridge between the PNS and TLRs in wound healing is nerve growth factor (NGF). NGF is produced by fibroblasts, keratinocytes, mast cells, and immune cells and NGF production is positively regulated by TLR2 activation.⁹⁴

TLR FUNCTION IN ACUTE SKIN WOUNDS

As is evident from the descriptions above, the functions of TLRs in the innate immune system have been extensively studied in last two decades. Despite the widespread presence of TLRs on cells commonly found in skin wounds, examination of their roles in skin wound healing was largely neglected until a few years ago. Recent studies by our laboratory and others now suggest that the expression of TLRs, including TLR1–9 and 13, but not TLR12, is significantly elevated in mouse skin wounds at the early stage of healing (Fig. 1).^{58,95} Functional studies in mice, which are summarized below, have demonstrated a role for TLR2, 3, 4, and 9 in skin healing.

Skin wound healing is significantly delayed in TLR2, TLR4, or TLR2 and 4 double-deficient mice with decreased infiltration of neutrophils and macrophages.⁹⁶ The numbers of macrophages expressing TGF- β and keratinocytes expressing CCL5 in the wounds of these mice were also downregulated compared with wild-type mice. Topical administration of TGF- β and CCL5 markedly improves wound healing in TLR2, TLR4, and double-deficient mice.⁹⁶

TLR3 is markedly increased in mouse skin wounds and colocalizes with keratinocytes, fibroblasts, neutrophils, macrophages, and ECs.^{97,98} TLR3-deficient mice have delayed skin wound closure, reduced granulation formation and neovascularization, and impaired recruitment of neutrophils and macrophages.⁹⁸ Treatment with

Figure 1. mRNA expression of TLRs in 1-mm excisional mouse skin wounds over the time course of healing (For detailed methods, see Chen *et al.*⁹⁵). Wounds tissues were harvested at 6 h, 12 h, 1, 3, 5, 7, and 10 days after wounding. Total RNA was extracted, labeled, and hybridized to Affymetrix Mouse Genome 430 v 2.0 chip according to Affymetrix-recommended protocols. Signal intensities were normalized and background was corrected. A one-way ANOVA was used to identify significantly differentially expressed gene sets across all time points after wounding. (A) TLR1–6; (B) TLR7–13. NS, normal skin; TLR, Toll-like receptor.

TLR3 agonist poly (I:C) significantly promotes healing in either mouse or human skin wounds.⁹⁷ In the mouse model, poly (I:C) also increases granulation formation, angiogenesis, and recruitment of neutrophils and macrophages.⁹⁷ In addition, poly (I:C) can promote hair follicle regeneration after injury through activating TLR3 and its downstream effectors of IL-6 and STAT3, as well as hair follicle stem cell markers.⁹⁹ These data suggest that TLR3 pathway activation triggers genes involved in all aspects of wound healing from inflammation and proliferation to regeneration.

TLR4 is primarily expressed in keratinocytes at the wound edge from 6 h to day 3 after injury.⁵⁸ Wound closure in TLR4-deficient mice is significantly impaired from day 1 to 5 after injury compared with wild-type mice.⁵⁸ Temporal increases in macrophages, neutrophils, and lymphocytes, as well as a decrease of the expression of inflammatory cytokines (IL-1 β and IL-6), were observed in the wounds of TLR4-deficient mice. Cytokine production by injured normal human epidermal keratinocytes was shown to be stimulated through the TLR4-p38 and JNK MAPK signaling pathway.⁵⁸ Thus, TLR4 has been shown to be an important regulator of wound inflammation.

TLR9 deficiency significantly delays skin wound repair.¹⁰⁰ Topical application of TLR9 ligand, CpG oligodeoxynucleotides (ODNs), promotes macrophage infiltration and improves wound closure in wild-type mice, but not in TLR9-deficient mice.¹⁰⁰ CpG ODN accelerates wound healing and is associated with the increased production of VEGF and neovascularization induced by CpG ODN.¹⁰⁰

Wounds in mice deficient for MyD88, a molecule in the TLR signaling pathway, heal more slowly

than wounds in wild-type mice. This delayed healing is accompanied by less contraction, decreased formation of granulation tissue, and decreased blood vessel density. These results suggest that signaling through the MyD88-dependent pathway plays an important role in regulation of wound healing.¹⁰¹ Since MyD88 is the major adapter molecule in the TLR signaling pathway, the results indirectly confirm that TLR signaling is important for wound healing.

TLRs IN DIABETIC WOUND HEALING

Several studies have explored the role of TLRs in the impaired healing seen in diabetic mice. Unlike chronic wounds such as human diabetic foot ulcers, skin wounds in diabetic mice do heal, although more slowly and with a more severe inflammatory response than normal mice. Both the TLR2 and TLR4 receptors have been implicated in the healing deficit in diabetic mice. TLR2 signaling pathway proteins (MyD88, pIRAK, and TRIF) and the downstream inflammatory cytokines (IL-1 β and TNF- α) are observed to be significantly higher in the wounds of streptozotocin (STZ)-induced diabetic mice than in nondiabetic mice. Wounds in diabetic TLR2-deficient mice close significantly faster than those in diabetic wild-type mice, suggesting that the absence of TLR2-mediated inflammation alleviates wound closure deficit in diabetic mice.¹⁰² TLR4 expression is also significantly higher in wounds of STZ-induced diabetic mice than in nondiabetic mice. Similar to the TLR2-deficient mice, wound healing is improved in diabetic TLR4-deficient mice with less proinflammatory cytokine expression than wounds of

diabetic wild-type mice.¹⁰³ Some evidence for a role of TLRs in diabetic healing has been derived from human samples as well. Tissues from type 2 diabetic foot ulcers have higher levels of TLR1, 2, 4, 6, MyD88, IRAK-1, NF- κ B, IL-1 β , and TNF- α expression than wounds from healthy subjects.¹⁰⁴ Therefore, contrary to wound healing in normal mice or healthy human subjects, TLR activation seems to negatively impact diabetic repair and contributes to the prolonged inflammation in diabetic skin wound.

A summary of the function of TLRs involved in acute wound healing is provided in Table 3.

MICRORNA-MEDIATED REGULATION OF TLR SIGNALING

MicroRNAs are a recently discovered large group of noncoding, short (22 nucleotides long) single-stranded RNAs, and thousands of miRNAs have already been identified in human and animals. Acting at the post-transcriptional level, these small

RNAs play vital roles in regulating gene expression, including those genes involved in TLR signaling pathways. Two recent comprehensive reviews well summarize miRNA regulation of TLR signaling.^{105,106} In brief, miR-105, miR-19a/b, miR-143, and miR-146a target TLR2; miR-223 and miR-26a target TLR3; let-7i, let-7e, miR-146a, miR-146b, miR-223, and miR-511 target TLR4; and miR-146a also targets TLR5. MicroRNAs also regulate signaling pathway molecules that are downstream of TLRs. For example, miR-146a&b modulate IRAK-1&2, TRAF6, and MyD88. MiR-155 regulates MyD88, IKK β and IKK ϵ , TAB2, and RIPK1, to name a few. In addition, microRNAs mediate regulation of TLR-induced transcription factors such as NF- κ B and STAT. For example, miR-9 and miR-210 regulate NF- κ B, and miR-17-5p, miR-20a, and miR-223 regulate STAT3. While the microRNA regulation of TLRs seems likely to be important in wound healing, the specific ways that microRNAs regulate TLRs and their associated molecules in skin wound healing have not been studied.

Table 3. Summary of TLRs in acute skin wounds

TLRs	Wound Model	Findings	References
Acute skin wounds			
TLR2&4	Mouse, 6-mm punch wound	Wound closure is delayed in TLR2, 4, and 2&4 double-deficient mice with decreased infiltration of neutrophils and macrophages and downregulated CCL5 and TGF- β . Topical administration of TGF- β and CCL5 markedly improves wound healing in these mice.	Suga <i>et al.</i> ⁹⁶
TLR3	Mouse, 4-mm punch wound	Wound closure is delayed in TLR3-deficient mice with impaired recruitment of neutrophils/macrophages and decreased expression of CXCL2, CCL2, and CCL3.	Lin <i>et al.</i> ⁹⁸
TLR3	Mouse, 4-mm punch wound Human, laser surgery of nevus	Topical application of TLR3 ligand, poly (I:C), improves wound closure in both mice and humans and increases granulation formation, angiogenesis, and recruitment of neutrophils and macrophages in mouse model. TLR3 colocalizes with keratinocytes, fibroblasts, neutrophils, macrophages, and endothelial cells in a mouse model.	Lin, <i>et al.</i> ⁹⁷
TLR3	Mouse, 1 cm ² excisional full-thickness	Poly (I:C) promotes hair follicle regeneration through activation of TLR3 and its downstream effectors of IL-6 and STAT3, resulting in increased hair follicle stem cell markers.	Nelson <i>et al.</i> ⁹⁹
TLR4	Mouse, 3-mm punch wound <i>In vitro</i> scratch wound	Wound closure is delayed in TLR4-deficient mice. Temporal increases in macrophages, neutrophils, and lymphocytes, as well as decreased inflammatory cytokines, are also observed. EGF expression by wound edge keratinocytes is decreased. Inflammatory cytokine production by injured normal human epidermal keratinocytes is stimulated through the TLR4-p38 and JNK MAPK signaling pathway.	Chen <i>et al.</i> ⁵⁸
TLR9	Mouse, 6-mm punch wound	Wound closure is delayed in TLR9-deficient mice. Topical application of TLR9 ligand promotes macrophage infiltration and improves wound closure in wild-type mice, but not in TLR9-deficient mice. Accelerated wound healing is associated with increased production of VEGF and neovascularization.	Sato <i>et al.</i> ¹⁰⁰
Acute diabetic wounds			
TLR2	STZ-induced diabetic mouse, 3- and 8-mm punch wounds	Wounds in diabetic TLR2-deficient mice close faster with lower levels of MyD88 signaling, NF- κ B activation, inflammatory cytokine production, and oxidative stress than in wild-type diabetic mice.	Dasu <i>et al.</i> ¹⁰²
TLR4	STZ-induced diabetic mouse 6-mm punch wound	Wounds in diabetic TLR4-deficient mice close faster with less IL-6, TNF- α , and NF- κ B expression than in wild-type diabetic mice.	Dasu and Jialal ¹⁰³

STZ, streptozotocin.

NEGATIVE REGULATION OF TLR SIGNALING

As the proliferation stage begins in wounds, the intensity of inflammation gradually reduces and then disappears in the remodeling phase. However, if inflammation persists, it is detrimental to reepithelialization, ECM synthesis, and angiogenesis, which may lead to chronic wounds. We speculate that wound inflammation largely results from TLR activation. Given the fact that injury or trauma causes damage to tissue and cells and that most wounds experience at least some level of microbial contamination, TLR activation would seem likely to play a critical role in eliciting the significant increase of inflammatory mediators that occur in wounds. Therefore, negative regulation of TLR signaling is probably quite important in quenching the inflamed wound and in maintaining a balanced immunological reaction in the skin. The mechanisms of negative TLR signaling have been well discussed.^{2,107,108} Briefly, these mechanisms include the following: (1). Inhibition of ligand binding. Soluble forms of TLRs as decoys compete with the cell membrane-bound TLRs for ligands. (2). Downregulation of expression or breakdown of TLRs and their signaling molecules. MicroRNAs such as miRs-21, 146, and 155 inhibit the expression and function of molecules in the TLR signaling pathway as described in the previous section. Suppressor of cytokine signaling proteins stimulate degradation of TIRAP/MyD88 adaptor-like or TRAF proteins. In addition, Triad3A can bind to cytoplasmic domain of TLR4 and TLR9 and induce ubiquitination and degradation of the targets. (3). Dissociation of adaptor complexes. Some variant forms of TLRs and TIR domain-containing adaptor proteins can act as antagonists to prevent binding among intact forms of adaptor molecules, thus stopping downstream signaling pathways. (4). Epigenetic modulation. The structure and function of TLRs and/or their downstream signaling molecules are altered through chromatin remodeling and histone modification. (5). Evasion of pathogens. Pathogens are capable of developing strategies to escape TLR recognition through the production of proteinases or by using the host ubiquitin system to degrade TLR signaling proteins. The mechanism of negative regulation of TLR signaling in wound healing is yet to be explored.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Prior studies show that skin wound healing is significantly delayed in the absence of TLR2, 3, 4,

and 9; these delays often involve decreased inflammatory cell infiltration and cytokine expression, as well as impaired reepithelialization, granulation formation, and angiogenesis (Table 3). Excessive TLR function also seems to play a role in the impaired healing of diabetes. Therefore, it is clear that TLRs are important factors in the skin immune response that regulates multiple aspects of wound healing. Given that multiple TLRs are expressed by an assortment of skin cell types, each of which could react to a variety of ligands (DAMPs and/or PAMPs) known to be in the wound environment, there is a need for more investigation of the specific roles of TLRs and their ligands in wound healing regulation. The majority of the studies summarized to date employed globally deficient mice. A further understanding of the roles of TLRs in specific cell types in wounds will require the use of conditional TLR knockout mice or other local suppression. Almost no information is available regarding the negative regulation of TLR signaling in wound healing, thus this area also warrants exploration. In addition, since most studies have relied upon mouse models, the implications of the findings need to be verified in human skin wound samples. The expression and function of TLRs in chronic wounds such as diabetic foot ulcers, pressure wounds, and venous and arterial ulcers—wounds that affect millions of patients without good treatment options—need more study. An improved knowledge of the function of TLRs in the regulation of wound healing may suggest new therapeutic targets that can be used to modify and improve the healing process. Such discoveries will assist clinicians as they tackle the dilemmas faced in the daily clinical practice of wound care.

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TAKE-HOME MESSAGES

- TLRs are PRRs that recognize PAMPs from microorganisms or DAMPs from damaged cells.
- Activation of TLR signaling pathways can induce important wound mediators such as transcription factors, cytokines, chemokines, and growth factors in nearly all cell types involved in wound healing.
- In mice, the absence of TLR2, 3, 4, or 9 leads to dysregulated cytokine production, delayed wound closure, reduced granulation formation, and reduced angiogenesis. Topical application of TLR3 or 9 ligand can improve wound healing.
- In the condition of diabetes, TLR2 and 4 appear to stimulate excessive inflammation. Wounds in diabetic TLR2-deficient or diabetic TLR4-deficient mice heal faster than those of control diabetic mice.
- More information is needed about the function of the entire TLR family in wound healing, including the role of TLRs in specific cell types, the regulation of TLR signaling, and roles of TLRs in nonhealing human wounds.

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Abbreviations and Acronyms

CCL	= C-C motif ligand
cDCs	= classical dendritic cells
CXCL	= C-X-C motif ligand
DAMPs	= danger-associated molecular patterns
DC	= dendritic cell
EC	= endothelial cell
ECM	= extracellular matrix
HA	= hyaluronan
HMGB1	= high-mobility group box 1 protein
HSP	= heat shock protein
IFN	= interferon
IL	= interleukin
JNK	= Jun N-terminal kinase
LC	= Langerhans cell
LPSs	= lipopolysaccharides
MAPK	= mitogen-activated protein kinase
MMP	= matrix metalloproteinase
NGF	= nerve growth factor
NKT	= natural killer T
ODN	= oligodeoxynucleotide
PAMP	= pathogen-associated molecular pattern
PNS	= peripheral nervous system
Poly (I:C)	= polyriboinosinic–polyribocytidylic acid
PRR	= pattern recognition receptor
SP	= substance P
STZ	= streptozotocin
TCR	= T cell receptor
TIR	= cytoplasmic toll/interleukin (IL)-1 receptor domain
TLR	= Toll-like receptor
TRIF	= TIR domain-containing adapter-inducing interferon- β