Integration of Canonical and Noncanonical Pathways in TLR4 Signaling: Complex Regulation of the Wound Repair Program

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Significance: Chronic inflammation and maladaptive repair contribute to the development of fibrosis that negatively impacts quality of life and organ function. The toll-like receptor (TLR) system is a critical node in the tissue response to both exogenous (pathogen-associated) and endogenous (damage-associated) molecular pattern factors (PAMPs and DAMPs, respectively). The development of novel TLR ligand-, pathway-, and/or target gene-specific therapeutics may have clinical utility in the management of the exuberant inflammatory/fibrotic tissue response to injury without compromising the host defense to pathogens.

Recent Advances: DAMP ligands, released upon wounding, and microbial-derived PAMPs interact with several TLRs, and their various coreceptor partners, engaging downstream pathways that include Src family kinases, the epidermal growth factor receptor, integrins and the tumor suppressor phosphatase and tensin homolog (PTEN). Toll-like receptor 4 (TLR4) activation enhances cellular responses to the potent profibrotic cytokine transforming growth factor- β 1 (TGF- β 1) by attenuating the expression of receptors that inhibit TGF- β 1 signaling.

Critical Issues: Common as well as unique pathways may be activated by PAMP and DAMP ligands that bind to the repertoire of TLRs on various cell types. Dissecting mechanisms underlying ligand-dependent engagement of this complex, highly interactive, network will provide for adaptation of new and focused therapies directed to the regulation of pathologically significant profibrotic genes. Inherent in this diversity are therapeutic opportunities to modulate the pathophysiologic consequences of persistent TLR signaling. The recently identified involvement of receptor and nonreceptor kinase pathways in TLR signaling may present novel opportunities for pharmacologic intervention.

Future Directions: Clarifying the identity and function of DAMP-activated TLR complexes or ligand-binding partners, as well as their engaged downstream effectors and target genes, are key factors in the eventual design of pathway-specific treatment modalities. Such approaches may be tailored to address the spectrum of TLR-initiated pathologies (including localized and persistent inflammation, maladaptive repair/fibrosis) and, perhaps, even titrated to achieve patient-unique beneficial clinical outcomes.

Keywords: TLR4, wound repair, signaling, fibrosis, inflammation, TGF- β 1

SCOPE AND SIGNIFICANCE

ACTIVATION OF A fibro-inflammatory genomic program accompanies the host response to pathogens and tissue injury mediated by pathogen-associated mo-

lecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), respectively, via the toll-like receptor (TLR) system. Persistent TLR signaling often results in nonresolving

inflammation, tissue fibrosis, intravascular coagulation, multiorgan failure, and poor patient outcomes. This review discusses some of the more recent findings that highlight the growing complexity of TLR signaling.

TRANSLATIONAL AND CLINICAL RELEVANCE

In response to ligands released from pathogenic microorganisms or upon tissue injury, activation and maintenance of TLR signaling often result in systemic as well as localized inflammation, disseminated coagulopathy, exuberant wound repair, excessive scarring, and fibrosis. The time course and amplitude of the inflammatory phase are largely dictated by the duration of the stimulus, the postreceptor pathways engaged, and the target genes impacted. There is a growing recognition of the involvement of “noncanonical” toll-like receptor 4 (TLR4) signaling effectors (*e.g.*, phosphatase and tensin homolog [PTEN], integrins and the epidermal growth factor receptor [EGFR], and transforming growth factor- β 1 [TGF- β 1] networks) as important downstream participants. These provide new opportunities to manage the pathophysiologic consequences encountered upon aberrant activation of this important member of the TLR family.

DISCUSSION OF THE LITERATURE

Introduction

TLR4 is a member of a family of 10 human pattern recognition receptors initially implicated in the regulation of the innate immune system (Fig. 1). In addition to its prototypic PAMP ligand, bacterial lipopolysaccharide (LPS), TLR4 can also be activated by various endogenous components released following an injury.^{1–3} These DAMP molecules include the heat shock and high-mobility group box 1 proteins (HMGB1) as well as several extracellular matrix (ECM) elements, including hyaluronic acid, proteoglycans, the matricellular protein Tenascin C, and the alternatively spliced extra domain A (EDA) isoform of fibronectin. Activation of TLR4 by ECM-derived DAMPs induces the expression of fibroinflammatory genes that promote tissue remodeling and normal as well as maladaptive wound repair. When the response becomes exacerbated, DAMPs drive both chronic inflammation and pathological fibrosis.⁴ There is evidence to suggest possible cooperation between these two classes of TLR “ligands.” In an *in vivo* model of persistent cutaneous injury using a combination of PAMPs and DAMPs, excessive and sustained inflammation results in impaired healing and increased scarring.⁵ Dermal fibroblasts isolated from hypertrophic scars (HTS) express

transcripts for many TLRs, including TLR4, and their intracellular adaptors at levels considerably greater than that of normal fibroblasts.⁶ Hypertrophic scarring is a frequent outcome in severe burn injury, and the combination of bacterial colonization and thermal tissue damage, in the context of elevated TLR signaling, may lead to persistent inflammation and exuberant scar formation.⁶ Indeed, synthesis of proinflammatory cytokines in response to LPS was significantly increased in HTS fibroblasts compared with normal cells. Collectively, it has become apparent that (1) increased TLR2/4 surface levels and/or functional activation result in impaired cutaneous wound healing due, in large part, to prolonged inflammation, (2) TLRs have different roles in injury repair, and (3) outcomes depend on TLR expression, timing of activation, the specific cell type(s) involved, and the adaptor network engaged.⁷

Two of the best studied DAMPs are the ECM proteins EDA-fibronectin and Tenascin C. While not normally present at detectable levels in adult tissues, both are upregulated following injury and highly expressed in inflamed and fibrotic tissues as well as in the skin of systemic sclerosis patients. Animal studies, moreover, support a role for these DAMPs in disease progression.⁸ Elevated EDA-fibronectin levels are evident in keloids and psoriatic lesions,^{9–11} while EDA-fibronectin-deficient mice exhibit diminished inflammatory and fibrotic responses.^{12–15} These findings provide the basis for the hypothesis that products of tissue injury and sustained generation of DAMPs activate a TLR4-dependent feed-forward loop that promotes chronic inflammation and persistent fibrosis.^{12,16,17} TLR4 DAMP-type ligands, however, are structurally diverse and the mechanism(s), by which they initiate TLR4 signaling, are not well understood. In contrast, the molecular events underlying TLR4 activation by LPS are better defined, requiring the two ancillary molecules CD14 and MD-2. CD14 is a glycosyl phosphatidylinositol (GPI)-linked protein which captures LPS from solution and presents it to the TLR4/MD-2 complex. MD-2 is a TLR4 binding secreted protein which cooperates with CD14 to stimulate TLR4 dimerization and downstream signaling.¹⁸ Similar to LPS, DAMPs may not interact directly with TLR4, but may bind to accessory factors as part of a larger TLR4 complex.¹⁹

Involvement of the EGFR and integrins in TLR4 signaling

TLR4 activation and downstream signaling are also regulated by the cellular context in which TLR4 receptor complexes assemble as well as by the contributions of costimulated parallel pathways.

Figure 1. There are 10 members of the TLR family in humans. These are evolutionary-conserved homologs of the *Drosophila* Toll protein that play a key role in the innate immune response to pathogen-derived and endogenous danger signals (see Chen and DiPietro⁷⁶ for a complete description of TLR ligands). TLR10 is unique among the repertoire of TLRs as it appears to function to suppress inflammatory signaling and currently has no known ligand. TLRs 1, 2, 4, 5, 6, and likely 10 are found on the cell surface whereas TLRs 3, 7, 8, and 9 localize to intracellular compartments (e.g., endosomes and lysosomes). TLR, toll-like receptor.

Recent studies implicate both the EGFR and integrins in the TLR response to pathogens; it appears, moreover, that the EGFR is required for LPS to signal via TLR4.²⁰ Although the mechanism is unclear, the EGFR inhibitor erlotinib blocks LPS-induced expression of tumor necrosis factor α (TNF- α) and interleukin-6 (IL-6), while attenuating LPS-induced endotoxicity, indicating that the EGFR is essential for LPS-induced signaling *in vivo*.²⁰ Perhaps not unexpectedly, since LPS-induced TLR4 activation of NF- κ B is impaired by EGFR knock-down or erlotinib, EGFR kinase inhibitors (AG1478, erlotinib, gefitinib) have proven to be effective in treating septic shock in preclinical models. Gefitinib and erlotinib reduce pulmonary damage and promote increased survival in mouse models of sepsis,^{20,21} while erlotinib blunts the proinflammatory and proliferative responses following hepatic injury.²² AG1478 also reduced fibrosis and inflammation in obesity-related cardiac disease and hyperlipidemia-induced renal injury.^{23,24} Induction of proinflammatory and profibrotic outcomes upon TLR4 signaling, furthermore, depends on EGFR transactivation through complex pathways involving several kinase networks.^{22–25} Linkages, however, are unclear and interactions between TLR4 and EGFR may be functional as complexes between the two receptors have not been detected.^{20,21}

Integrins are also implicated in pathogen-induced TLR activation; such cooperation occurs in both immune and nonimmune cells where integrins enhance the innate immune response. Certain integrins may serve as TLR coreceptors, binding directly to ligands to initiate TLR signaling. In human monocytes and 293 cells, complexes between the $\alpha_v\beta_3$ integrin and TLR2 coordinate an inflammatory response.^{26,27} In addition to ligand recognition, functional interactions between integrins and TLRs are also evident at the level of intracellular trafficking. *Herpes simplex virus*/ $\alpha_v\beta_3$ integrin interaction, for example, activates TLR2 signaling by modulating subcellular localization.^{27–29} Similarly, binding of *Borrelia burgdorferi* to the $\alpha_3\beta_1$ integrin enhances TLR2-mediated responses by stimulating internalization and endosomal targeting of *B. burgdorferi*/TLR2 complexes.³⁰ The $\alpha_m\beta_2$ (CD11b/CD18) integrin regulates both LPS binding to TLR4 as well as downstream signaling by promoting TLR4 trafficking into the endosomal compartment.³¹

TLR responses are also modulated by integrin-dependent events activated in parallel; such effects on TLR pathways can be either positive or negative as well as bidirectional. Positive regulation occurs in dendritic cells, where $\alpha_9\beta_1$ integrin-stimulated ERK activation enhances TLR2- and TLR4-mediated cytokine production.³² In macrophages,

the $\alpha_v\beta_3$ integrin cooperates with TLR4 in a two-step mechanism resulting in TNF- α expression. In this model, LPS activation of TLR4 induces synthesis of the matricellular protein WISP-1, an $\alpha_v\beta_3$ ligand that further potentiates TNF- α expression via TLR4 signaling.³³ Integrins also negatively regulate TLR function, particularly in monocytes derived from $\beta 2^{-/-}$ mice which exhibit a hyperresponsiveness to TLR agonists due to increased activation of NF- κ B.³⁴

Bidirectional cross talk between integrins and TLRs is evident in macrophages where TLR-dependent activation of the $\alpha_m\beta_2$ integrin, in turn, inhibits further TLR signaling by stimulating the syk kinase-dependent phosphorylation, and subsequent degradation, of the TLR adaptor MyD88.³⁵ Similar cross talk also occurs in leukocytes where TLR2 agonists activate the $\beta 2$ integrin,³⁶ resulting in positive or negative regulation of the TLR2 and TLR4 pathways.³⁷ These data illustrate the complexity of TLR signaling in response to pathogens and provide a cellular and molecular context for ligand- and cell type-specific TLR responses. The requirements for TLR4 activation by the products of tissue damage (DAMPs) as well as their downstream targets, in contrast, are not well understood; the available data suggest that they are also ligand- and cell type-dependent. The association of TLR4 with distinct accessory proteins or coreceptors may dictate the assembly of ligand-specific TLR4 complexes, thereby tailoring the inflammatory response to achieve specific biological outcomes.^{38,39} Little is known, however, regarding the impact of integrins and EGFR on DAMP-initiated fibro-inflammation. Based on findings from studies on pathogen-initiated TLR signaling, it appears likely that adhesion receptors and receptor tyrosine kinases may play important roles in the regulation of the fibro-inflammatory responses to tissue damage.

Intersection of the TLR4 and TGF- β 1 networks

LPS activation of TLR4 signaling markedly enhances fibroblast responses to the profibrotic cytokine TGF- β 1.⁴⁰ The mechanism appears to involve downregulation of the TGF- β 1 inhibitory receptor BAMBI (through a TLR4/MyD88/NF- κ B pathway) that, in turn, may sensitize cells to the relatively abundant levels of TGF- β 1 at the wound site.^{41,42} A specific TGF- β 1 gene signature that includes the potent profibrotic serine protease inhibitor plasminogen activator inhibitor-1 (PAI-1; SERPINE1), connective tissue growth factor (CTGF, CCN2), EDA-fibronectin, collagen I, TGF- β 1, vimentin, p53, p21, miR-21, miR-29, and α -smooth muscle

actin (α -SMA)^{43–46} is consistently associated with fibrosis in human cells and mouse models. Several of these genes are upregulated in the cutaneous tissue of systemic sclerosis patients as well as in the LPS-treated mouse skin, via a TLR4 pathway and downstream MyD88 signaling, and attenuated by TGF- β 1 neutralizing antibodies.⁴⁷ The two most likely members of the TGF- β 1-induced repertoire with potential impact on TLR4 signaling are EDA-fibronectin and the SERPIN PAI-1. TGF- β 1 upregulates DAMP-type EDA-fibronectin expression in various cell types, including dermal fibroblasts; this fibronectin variant may function as an endogenous activator of the TLR4 pathway in much the same way as does exogenously-delivered EDA-fibronectin.^{40,48}

Recent findings also implicate PAI-1 in this pathway, perhaps as a matricellular DAMP.^{49,50} Macrophage activation in response to PAI-1 was dose-dependent and LPS-independent and partially blocked by a TLR4 neutralizing antibody. PAI-1-stimulated TNF- α and macrophage inflammatory protein-2 (MIP-2) expression, moreover, was reduced in TLR4 $^{-/-}$ compared with wild-type macrophages suggesting that PAI-1 is involved in the regulation of host inflammatory responses via TLR4.⁵¹ PAI-1 knockdown attenuated LPS-induced increases in TLR4, MD-2, MyD88, TNF- α , IL-1 β , and NF- κ B expression in macrophages, while vector-driven PAI-1 overexpression enhanced these responses.^{52,53} Although the mechanism is unclear, it appears that PAI-1 regulates the endotoxin-stimulated TLR4/MD-2 inflammatory pathway, at least in cells of the macrophage lineage. This is likely to have a significant impact on fibrogenic outcomes following tissue injury as exogenous PAI-1 treatment significantly increased TGF- β 1, collagen 1 α 1, collagen 1 α 2, and monocyte chemoattractant protein-1 (MCP-1) transcript abundance in cultured cells.⁵⁴ The TLR4/RAGE DAMP-type ligand HMGB1 also activates a subset of genes in the TGF- β 1 profibrotic signature that includes PAI-1, CTGF, and TGF- β 1,⁵⁵ suggesting that DAMPs and LPS may utilize signaling pathways that may be exploited in the design of interventional strategies. Collectively, it appears that TLR4 may function as a molecular “switch,” activated by endogenous DAMPs to initiate repair while upregulating the TGF- β 1 pathway promoting the persistent expression of profibrotic genes to create and maintain a progressive fibrotic microenvironment.^{40,56}

Ligand-dependent TLR4 activation leading to EGFR recruitment²⁰ and enhanced fibroblast responses to TGF- β 1 via BAMBI downregulation⁴⁰

has implications with regard to both pathways. Similar to LPS-initiated TLR4 signaling, control of TGF- β 1 target gene expression also involves Src kinases and EGFR transactivation.^{57–59} TGF- β 1-stimulated PAI-1 expression, moreover, was preceded by EGFR phosphorylation on Y845 (a Src kinase target residue) and required pp60^{c-src} activity.⁵⁹ EGFR inhibition or infection with adenoviruses encoding the EGFR^{Y845F} or kinase-dead EGFR^{K721A} mutants or dominant-negative pp60^{c-src} overexpression effectively decreased TGF- β 1-stimulated PAI-1 induction implicating EGFR activity and, specifically, the pp60^{c-src} EGFR^{Y845} phosphorylation site in the response. Consistent with these findings, TGF- β 1 failed to induce PAI-1 synthesis in triple Src kinase-deficient (SYF^{-/-}) fibroblasts, while reexpression of a wild-type pp60^{c-src} construct in SYF^{-/-} cells rescued the PAI-1 response to TGF- β 1.^{57–59} It appears, therefore, that Src family kinase EGFR activation is a critical event in both TLR4 and TGF- β 1 signaling to downstream effector pathways.

Role of PTEN in TLR4 signaling

Recent studies suggest a role for PTEN, the principle negative regulator of the phosphoinositide 3-kinase (PI3K) pathway and protein kinase B (Akt) activation, in the progression of tissue fibrosis. PTEN deficiency is characteristic of idiopathic pulmonary fibrosis, and genetic ablation of PTEN in the alveolar epithelium promotes lung fibrosis via an Akt pathway.^{60–62} Similarly, PTEN deficiency in dermal fibroblasts drives skin fibrosis in a mouse model.⁶³ PTEN silencing induces dedifferentiation and cell cycle arrest, both biomarkers of maladaptive repair, and cooperates with TGF- β 1 to further stimulate expression of the fibrotic signature genes CTGF, PAI-1, vimentin, α -SMA, and EDA-fibronectin.^{45,46} The mechanism of PTEN downregulation in the context of fibrosis is not known but is likely the result of increased TGF- β 1 levels in the injury microenvironment. TGF- β 1 reduces, moreover, the levels of the C-terminal SMAD2/3 protein phosphatase PPM1A, a terminator of TGF- β 1 signaling.⁶⁴ While the specific molecular events are unknown, persistent TGF- β 1 stimulation reduces PPM1A levels via the Rho/rho-associated, coiled-coil-containing protein kinase (ROCK) pathway enhancing and maintaining, thereby, SMAD3-dependent transcription of this profibrotic signature.^{43,46,57} Rho-ROCK signaling leads to inhibition of PTEN-PPM1A activity in TGF- β 1-stimulated cells resulting in a reduction of nuclear PPM1A, maintaining pSMAD2/3 levels required for profibrotic gene expression.⁵⁷ Similar

to their involvement in EGFR and TGF- β 1 signaling, Src kinases are key intermediates in the Rho/ROCK pathway leading to the expression of the profibrotic genes PAI-1 and CTGF.⁵⁷ These data implicate Src kinases as upstream regulators of the RhoA-ROCK-SMAD2/3 axis leading to control of PPM1A activity impacting downstream expression of specific profibrotic genes.

While LPS-activated TLR4 signaling enhances cellular responses to TGF- β 1 by downregulating BAMBI, PTEN levels are also decreased likely contributing to fibroblast activation in pulmonary fibrosis.⁴¹ PTEN deficiency has also been implicated in the profibrotic phenotype of scleroderma fibroblasts as well as in keloid pathophysiology and cutaneous fibrosis.^{63,65} The impact of DAMP ligands is less clear. PTEN expression is lost in several models of injury-induced fibrosis, and targeted PTEN depletion reduces PPM1A levels while promoting SMAD3 phosphorylation as well as SMAD3 nuclear translocation.^{43,46} PPM1A suppression further enhanced TGF- β 1-induced SMAD3 phosphorylation and fibrotic gene expression, while PPM1A overexpression inhibited both responses.^{45,46} Thus, these findings implicate PTEN as an upstream regulator of PPM1A function in dysfunctional tissue repair and establish PPM1A as a novel repressor of the SMAD3 fibrotic pathway. Stable silencing of PTEN, moreover, induced many of the same fibrotic genes as LPS or TGF- β 1 (*e.g.*, CTGF, PAI-1, vimentin, α -SMA, and fibronectin).^{45,46} Relatively, little is known about PPM1A regulation in general or in the context of tissue injury. PTEN, however, interacts with PPM1A in scleroderma⁶⁵ and PTEN silencing in macrophages increased LPS-induced expression of TNF- α , IL-1 β , IL-6, IL-8, and IL-10.⁶⁶ There are apparently contrary findings, however. LPS activation of TLR4 also stimulates miR-718 expression (in macrophages), which impacts PI3K/Akt signaling by targeting PTEN, promoting Akt phosphorylation leading to a decrease in the production of proinflammatory cytokines.⁶⁷ pAkt, in turn, downmodulates expression of TLR4 and several of its signaling effectors through let-7e exerting, thereby, multilevel negative regulation to the TLR4 pathway. Thus, depending on the actual magnitude and duration of the stimulus (*e.g.*, LPS and/or TGF- β 1), PTEN may function as a rheostat to influence the amplitude and kinetics of the inflammatory response. Increased TGF- β 1 sensitivity due to reductions in BAMBI levels, coupled with decreased PTEN expression, may facilitate the transition from the inflammatory to the tissue repair phases of wound healing and, perhaps if

Figure 2. TLR4 signaling in response to ligand binding, either directly to the TLR or through cooperative interactions with various coreceptors, mobilizes two major core parallel pathways, resulting in the activation of the transcription factor NF- κ B and the p38/JNK MAP kinases via the upstream intermediate MyD88. The collateral or noncanonical pathways that impact TLR4 signaling outcomes involve several Src family kinases, transactivation of the EGFR (and downstream signaling intermediates), integrins, and the caveolin-1/FAK/RhoA/Rock/PTEN axis, as well as the canonical SMAD-dependent TGF- β pathway. The complex interactions among these diverse, although inter-related, pathways positively and negatively regulate the outcome of TLR4 signaling (detailed in this review). The available data suggest that while Akt activation may attenuate the amplitude and duration of the inflammatory response following TLR4 signaling, TGF- β 1 regulation of RhoA/ROCK signaling controls PTEN activity that, in turn, affects SMAD2/3 transcription of the profibrotic genes (e.g., PAI-1, CTGF) through the SMAD phosphatase PPM1A. Akt, protein kinase B; CTGF, connective tissue growth factor; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; PAI-1, plasminogen activator inhibitor-1; PTEN, phosphatase and tensin homolog; ROCK, rho-associated, coiled-coil-containing protein kinase; SMAD, Sma+mothers against decapentaplegic; TGF- β , transforming growth factor- β ; TLR4, toll-like receptor 4.

persistent over the long term, promote exuberant fibrosis. Since it has recently been determined that PPM1A also regulates NK- κ B activation,⁶⁸ the available data collectively suggest significant interaction between the TLR4 and PTEN pathways.

Implications for cutaneous wound healing

Whether the repair of cutaneous injuries is enhanced or inhibited by TLR engagement depends on, among other factors, the repertoire and levels of TLRs expressed, the involved cell types and their location at the wound site, and the timing of TLR-ligand presentation.^{7,69} Additional confounders include the time course and amplitude of the in-

flammatory response, the presence of comorbidities (e.g., diabetes), and microbial colonization of the wound bed, each of which is a significant contributor to wound chronicity. Indeed, persistent activation of TLR2/4 and elevated expression of proinflammatory cytokines typified nonresolving venous ulcers, whereas healing lesions had much reduced TLR2/4 levels.⁷⁰ In the case of TLR4, both the nature of the ligand (DAMP vs. PAMP) and the cutaneous compartment stimulated (dermis vs. epidermis) appear important in the repair outcome. DAMP-type TLR4 ligands, moreover, appear critical in control of the inflammatory and subsequent fibrotic response in sterile cutaneous

wounds.⁷¹ Microarray analysis, moreover, confirmed that TLR4 expression is elevated in the wound edge epithelial cohort in a mouse model of skin injury.⁷² This is likely critical to tissue repair since excisional wound closure was delayed in TLR4 mutant mice compared with their wild-type counterparts, correlating with the attenuated production of IL-1 β and IL-6. Consistent with these *in vivo* results, TLR4 blockade with neutralizing antibodies attenuated *in vitro* keratinocyte migration following monolayer scratch injury and inhibited wound-induced p38/JNK activation as well as IL-1 β expression. These data indicate the requirement for the TLR4-p38/JNK pathway in the regulation of inflammation and wound repair as the presence of a mutated nonfunctional receptor or interference with TLR4 signaling, blunted both processes.⁷² Collectively, these findings underscore both locational and cell type controls on TLR4 signal transduction and, when coupled with the impact of the expression levels and activation status of specific TLRs, provide some explanation as to discrepancies in the literature regarding the effect of the TLR network on healing of chronic versus acute cutaneous wounds. Patients presenting with difficult-to-heal injuries, moreover, appear to have multilevel deficiencies in TLR pathways. Recent reports suggest that there is increased TLR4 expression, signaling as well as receptor activation in type-1 diabetes, and that streptozotocin-induced diabetic mice upregulate TLR2/4.⁷³ TLR4 downstream effectors were significantly reduced in TLR4^{-/-} animals receiving streptozotocin compared with similarly-treated wild-type controls as were levels of proinflammatory cytokines suggesting that TLR4 is a major contributor to the persistence of the diabetes-associated inflammatory response. Wound site infection further complicates repair events, particularly in diabetic patients. Biofilm-compromised cutaneous wounds in diabetic mice had significantly lower TLR2/4 levels compared with either bacteria-free diabetic or wild-type controls.⁷⁴ Diabetic wounds failed to upregulate IL-1 β or TNF- α expression following infection, whereas wild-type mice exhibited a >140-fold increase in both. By 10 days postinjury, diabetic wound sites had a much greater microbial burden (due to a reduced neutrophil burst) and a significant delay in reepithelialization. Wounds in TLR2^{-/-}, TLR4^{-/-}, and double-knockout TLR2^{-/-}/TLR4^{-/-} mice, moreover, exhibited attenuated healing, reduced

TAKE-HOME MESSAGES

- The TLR4 system regulates the cellular response to both exogenous pathogen-associated (PAMPs) and endogenous damage-associated (DAMPs) factors that can elicit both common and unique outcomes as a function of cell type.
- TLR4 activation by ECM-derived DAMPs induces expression of fibro-inflammatory genes that promote tissue remodeling, maladaptive wound repair, and tissue fibrosis.
- Pathways downstream of TLR4 involving Src family kinases, the EGFR and TGF- β , as well as the RhoA $\rightarrow$ PTEN/PPM1A network, highlight the growing complexity of TLR4 signaling.
- TLR responses are also modulated, both positively and negatively as well as bidirectionally, by integrin-dependent pathways, in which the integrins function as cofactors in TLR signaling.
- The development of novel TLR ligand-, pathway-, and/or target gene-specific therapeutics may have clinical promise in the management of the exuberant inflammatory/fibrotic tissue response to DAMPs released at the site of injury.

numbers of infiltrating macrophages and decreased TGF- β and CCL5 expression relative to wild-type animals.⁷⁵ Macrophage TGF- β induction by the DAMP ligand hyaluronan was also reduced in the absence of the two TLRs and, in particular, in response to TLR4 deficiency. Topical delivery of TGF- β and CCL5 improved healing in TLR-null mice. Macrophages and the cutaneous tissue of TLR2^{-/-} mice also had reduced TLR4 levels, suggesting that the observed effects of TLR2 loss may be partially due to TLR4 downregulation. These data indicate that TLR4, but perhaps not TLR2, impacts the process of cutaneous wound repair through mechanisms involving TGF- β and CCL5,⁷⁵ perhaps utilizing the pathways detailed in this review.

SUMMARY

The available data support a model whereby the duration and amplitude of TLR4 activation, and the collateral involved pathways, impacts both normal and maladaptive wound repair. Identification of the mechanisms underlying EGFR, RhoA $\rightarrow$ PTEN/PPM1A, and TGF- β 1 signaling and their involvement in PAMP- and DAMP-induced TLR4 stimulation may provide novel targets for treatment of fibro-inflammatory diseases (Fig. 2). The ongoing clarification of molecular events downstream of TLR4 that dictate the extent of the inflammatory response to microbial pathogens and the endogenous damage-associated factors released upon tissue injury clearly implicate these highly interactive-pathways in TLR4 signaling. Findings suggest, moreover, that different nonreceptor Src A/B-type family tyrosine kinases are

involved in activation of the EGFR (Lyn), RhoA/PTEN/PPM1A (Yes), and TGF- β 1 (pp60^{c-src}) pathways downstream of TLR4. The growing complexity of TLR4 signaling involving various coreceptors and the integration of both canonical and noncanonical pathways, with each potentially mobilizing a unique set of effectors, may provide new therapeutic targets to differentially manipulate the pathophysiologic consequences of TLR4 activation in response to PAMP versus DAMP ligands.

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Abbreviations and Acronyms

Akt = protein kinase B
 CD = cluster of differentiation
 CTGF = connective tissue growth factor
 DAMP = damage-associated molecular pattern

ECM = extracellular matrix
 EDA = extra domain A
 EGFR = epidermal growth factor receptor
 GPI = glycosyl phosphatidylinositol
 HMGB1 = high-mobility group box 1 protein
 HTS = hypertrophic scar
 IL = interleukin
 LPS = lipopolysaccharide
 MCP-1 = monocyte chemoattractant protein-1
 MD-2 = lymphocyte antigen 96
 MIP-2 = macrophage inflammatory protein-2
 PAI-1 = plasminogen activator inhibitor-1
 PAMP = pathogen-associated molecular pattern
 PI3K = phosphoinositide 3-kinase
 PTEN = phosphatase and tensin homolog
 RAGE = receptor for advanced glycosylation end products
 ROCK = rho-associated, coiled-coil-containing protein kinase
 SERPIN = serine protease inhibitor
 SMA = smooth muscle actin
 SMAD = Sma+mothers against decapentaplegic
 Src = sarcoma
 TGF- β 1 = transforming growth factor- β 1
 TLR = toll-like receptor
 TLR4 = toll-like receptor 4
 TNF- α = tumor necrosis factor α