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Toll-Like Receptor Signaling in Burn Wound Healing and Scarring

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Significance: Damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) emanate from burn-injured tissue and enter systemic circulation. Locally and systemically, they activate pattern-recognition receptors, including toll-like receptors (TLRs), to stimulate cytokine secretion, which in the severest burns typically results in extreme systemic cytokine levels, a dysfunctioning immune system, infection, impaired healing, and excessive scarring. This system-wide disruption of homeostasis can advance to life-threatening, multiorgan dysfunction syndrome. Knowledge of DAMP- and PAMP-TLR signaling may lead to treatments that ameliorate local and systemic inflammation and reduce scarring and other burn injury sequela.
Recent Advances: Many PAMPs and DAMPs, the TLRs they activate, and their downstream signaling molecules have been shown to contribute to local and systemic inflammation and tissue damage following burn injury.
Critical Issues: Whether TLR-pathway-targeting treatments applied at different times postburn injury might improve scarring remains an open question. The evaluation of this question requires the use of appropriate preclinical and clinical burn models carried out until after mature scar has formed.
Future Directions: After TLR-pathway-targeting treatments are evaluated in porcine burn wound models and their safety is demonstrated, they can be tested in proof-of-concept clinical burn wound models.

Keywords: burns, experimental models, inflammation, scar, tissue death

SCOPE AND SIGNIFICANCE

Toll-like receptor (TLR) signaling is involved in damaged tissue sensing and wound repair, but also contributes to burn wound progression and systemic inflammation. TLR signaling is activated by pathogen-associated molecular patterns (PAMPs) of bacteria present in injured tissue and damage-associated molecular patterns (DAMPs) of injured tissue to produce cytokines. Early after burn injury, DAMPs as well as cytokines are elevated in circulation and contribute to systemic inflammation and secondary tissue damage that increase susceptibility to infection, impair healing, and worsen scarring. The potential of TLR signaling as a therapeutic target for improving burn outcomes is the subject of this review.

TRANSLATIONAL RELEVANCE

In animal burn models, suppressing TLR signaling has reduced inflammation in wounds and systemically. During the inflammation phase of healing, suppressing excessive inflammation by a variety of experimental means can mitigate tissue damage and improve healing. Lessening inflammation

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during the proliferation and remodeling phases can also potentially benefit scar outcomes. However, after the inflammation phase, reducing inflammation has impaired healing in some animal models. Thus, treatments to improve scar outcome must be properly timed and titrated and the risks of stalled healing and infection must be managed.

CLINICAL RELEVANCE

Hypertrophic scars develop typically after the prolonged inflammation of slow-healing burn wounds.[1] In clinical studies, the size of burn injury correlated with the level of circulating DAMPs (e.g., decorin and cell-free nucleic acids) and cytokines. In addition, early (2 weeks postburn) serum levels of decorin, a TLR2 and TLR4 ligand, were a factor (along with early interleukin [IL]-1β and late transforming growth factor [TGF]-β) that was suggested to predict hypertrophic scar.[2] Thus, reducing DAMP- and PAMP-TLR signaling can potentially improve deep burn outcomes by mitigating injury progression, systemic inflammation, and the prolonged inflammation and healing associated with hypertrophic scarring.

BACKGROUND

In the 1960s before early eschar excision and grafting was the standard of care for deep burns and before TLRs were discovered, burned skin extracts injected into the abdomen of mice produced an 80% mortality rate, whereas nonburned skin extracts had no effect. A thermally denatured lipid–protein complex was isolated from the burn extracts that when injected into mice mimicked many effects of burns on the immune system, such as increased susceptibility to *Pseudomonas* infection, suppressed immune responses to sheep erythrocytes and bacterial endotoxin, and inhibited IL-2-dependent cell growth in culture.[3]

Since these early studies of burned-tissue signaling, numerous DAMPs and PAMPs have been found to activate TLRs, and the crystal structures of some of these PAMP-TLR complexes have been solved.[4] DAMPs and PAMPs are among the numerous signaling molecules that activate the innate immune system, protecting damaged tissue from infection, and participating in the repair of burn-injured skin. Nonetheless, TLR signaling pathways also contribute to tissue-damaging inflammation.

DISCUSSION

The need for anti-scar treatments

Deep partial-thickness burn wounds destroy blood vessels and skin appendages (Fig. 1). These wounds typically heal in 3–8 weeks with severe scars that can be raised, red, hard, with abnormal sensations, contraction, severe functional impairment, psychological morbidity, and costly long-term healthcare.[5] Unlike full-thickness burns that require eschar excision and grafting, deep partial-thickness burn wounds retain some dermal elements that provide regenerative capacity but contribute to hypertrophic scarring. Hypertrophic scars develop in more than half of deep partial-thickness burn wounds, typically after prolonged inflammation, and once formed, treatments are only minimally effective.[6] Therefore, treatments are needed that promote regenerative healing before these scars form. TLR signaling pathways are among the candidate molecular targets for such treatments. Hitting these targets can limit inflammation and fibrosis, but risks infection and inhibited healing.

Outlines of TLR signaling

TLR signaling stimulated by DAMPs and PAMPs produces a cytokine-rich milieu for clearing necrotic debris and infection and setting the stage for angiogenesis and granulation tissue formation. TLR signaling activates the expression of many molecules, including cytokines and adhesion molecules that promote leukocyte recruitment and activation.[7] There are 13 TLRs in mice and 10 in man. Of the 10 human TLRs, 6 of them are located on the cell surface (TLR1, 2, 4, 5, 6, and 10) where they bind a diversity of molecule types, whereas TLR3, 7, 8, and 9 are in endosomes and sense nucleic acids. Ligand binding to TLRs activates the transcription factors CREB, AP-1, NFκB, IRF3, and IRF7 (Fig. 2).

The DAMPs that activate TLRs and other pattern-recognition receptors can be categorized into: (1) proteins secreted through a nonclassical secretion mechanism involving secretory lysosomes—for example, high mobility group box (HMGB)1 and galectin-3; (2) molecules released by necrotic cells—for example, S100 proteins, HMGB1, IL-1α, galectin-3, HSP60, HSP70, HSP72, histones, and nucleic acids; and (3) extracellular matrix molecules—for example, hyaluronan, heparin sulfate, fibronectin, and degraded matrix constituents. Numerous DAMPs have been identified. Theoretically, every molecule that normally resides inside cells and is extruded or is part of the extracellular matrix and is disrupted by tissue damage may potentially function as a DAMP; and hydrophobic surfaces in general have been proposed to act as DAMPs.[8] Interestingly, the unique proteins detected in human plasma after trauma mostly reside inside cells.[9]

PAMPs in burn wounds come from pathogens and skin microbiota that enter the dermis through

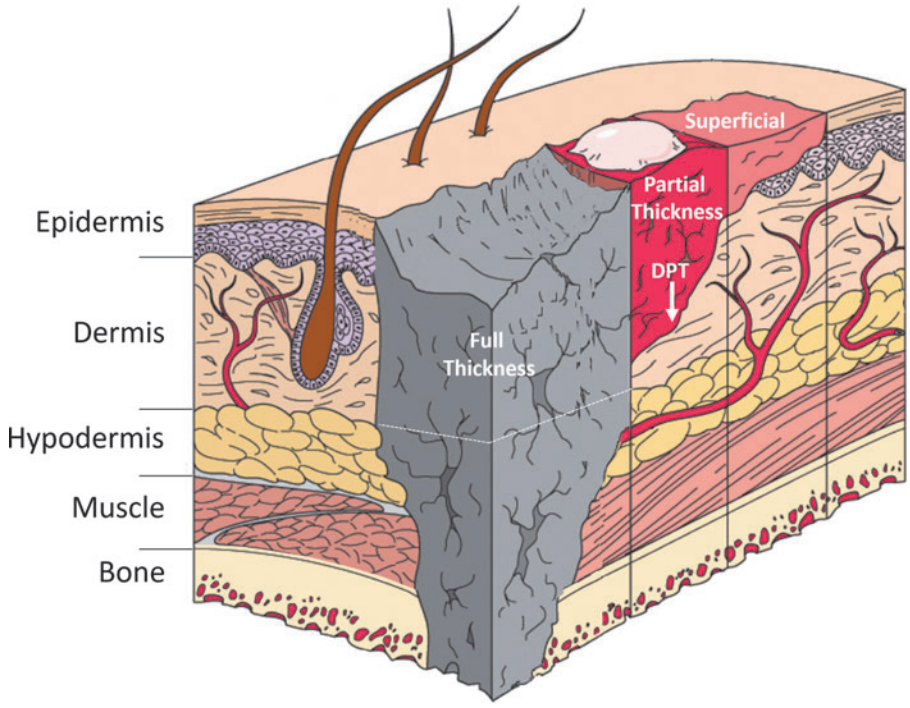

Figure 1. Depths of cutaneous burn injuries. Shown are superficial, partial-thickness, deep partial-thickness (DPT), and full-thickness (to hypodermis, shown by *dotted line*, or beyond) cutaneous burn injuries.

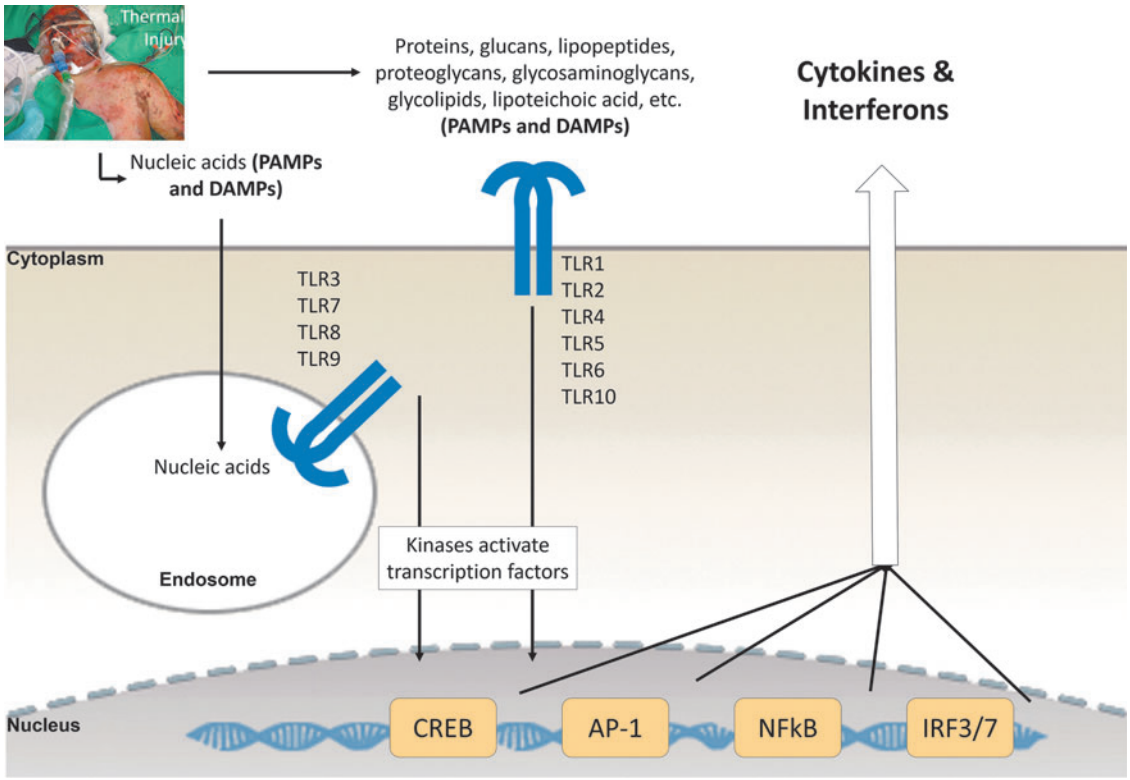

Figure 2. Outlines of DAMP- and PAMP-TLR signaling resulting in the production of cytokines and interferons. For the details of these outlines, see O'Neill *et al.*[4] (image modified from[4]). DAMPs and PAMPs activate similar receptors and converge on similar signaling pathways. The transcription factor–activating kinases are prominent drug targets. Image of thermally injured patient was provided by Rodney K. Chan. DAMP, damage-associated molecular pattern; PAMP, pathogen-associated molecular pattern; TLR, toll-like receptor.

the breached epidermal barrier. In normal skin, bacterial counts have been quantified using quantitative real-time polymerase chain reaction (16S rRNA gene) to be 1,000,000; 50,000; and 10,000 per square centimeter for the lower epidermal layers (punch biopsies), the intermediate layers (scrapes), and the epidermal surface (swabs), respectively,[10] and the bacteria were of diverse species.[11] Therefore, once the epidermis is disrupted by burn, these bacterial PAMPs likely activate TLR signaling, possibly most strongly in the microenvironment surrounding hair follicles where bacteria are concentrated.[12] With time as the epidermal barrier remains disrupted, ambient external pathogens have opportunity to infiltrate the wound.

TLRs are expressed by circulating leukocytes and a number of cells in the skin, including keratinocytes, Langerhans cells, T and B cells, mast cells, endothelial cells, myofibroblasts, and primary fibroblasts that can release cytokines.[13]

Burn injury progression

Over the first 2 days after a partial-thickness burn injury, the damaged tissue can expand. This "burn wound progression" is thought to result from progressive ischemia due to thrombosed vessels, increased capillary permeability, hypoperfusion, and oxidative damage, which are exacerbated by locally released signaling molecules from extracellular matrix and ruptured necrotic cells (i.e., DAMPs and PAMPs) that activate surviving proximal cells to produce inflammatory mediators.[14] In addition, burn progression likely involves ischemia/reperfusion (I/R) injury because burned-tissue blood flow on the day of injury fluctuated repetitively (Laser Doppler Imaging) in parallel to changes in base deficit.[15] In other types of tissue damage involving I/R injury, the importance of TLR signaling, including HMGB1-TLR signaling, has been demonstrated in a large number of *in vivo* studies (see Ref.[16] Tables 1 and 2). In peri-burn tissue during injury progression, necrotic cells released HMGB1 from chromatin into the cytoplasm and extracellular space.[17] Extracellular HMGB1 under the highly oxidizing conditions of burns is expected to be in the C23-C45 disulfide form, which activates TLR4 to induce cytokines.[18] In addition, oxidized lipoproteins can stimulate TLR2 and TLR4, resulting in inflammation,[19] suggesting that oxidized macromolecules in burn tissue may activate this inflammatory pathway.

Although HMGB1-TLR signaling has not been studied in burn-injured tissue *per se*, in an I/R hepatic injury model (mouse), HMGB1-neutralizing antibody decreased injury and, conversely, administration of recombinant HMGB1 worsened it, but only in TLR4-competent mice, suggesting that HMGB1-TLR4 signaling can mediate tissue damage under I/R redox conditions, as occurred during wound progression.[20] Furthermore, when HMGB1 was knocked out specifically in hepatic epithelial cells, there was a profound reduction of infiltrating neutrophils and inflammatory-gene expression,[21] suggesting that HMGB1 may be generally required for recruiting neutrophils to necrotic tissue where they may amplify tissue injury.

Burn eschar contributes to inflammatory signaling and scarring

The burn eschar likely contributes to inflammatory signaling and scarring at least partially by acting as a reservoir of DAMPs and PAMPs. Patients with >40% total body surface area (TBSA) burns whose eschars were excised early showed reductions in circulating cytokines, hypermetabolism, and mortality.[22] In addition, in a mouse study that compared early (day 1) and late (day 8) excision of 8%-TBSA full-thickness burns (without grafting), early excision of the eschar prevented the extreme inflammation and immune dysfunction occurring after late excision (analyzed on day 2 and 6 post eschar excision) that is associated with susceptibility to infection, organ dysfunction, and scarring.[23] In porcine models, early eschar excision accelerated reepithelialization and reduced scarring compared with no excision or delayed excision.[24,25] These results suggest that the longer the necrotic eschar remains *in situ*, the more the inflammation and scarring, which may result at least partially from continuously-released DAMPs and PAMPs within the eschar.

Water loss from breached and immature epidermis signals inflammation through S100A12-TLR4

Evaporative water loss from breached epidermis increases sodium concentration, which can increase inflammation and scarring.[26] An increase in sodium concentration of 10% mimics the increase in sodium concentration due to water loss from injured epidermis. Keratinocytes exposed to 10% higher sodium or cultured stratified keratinocytes exposed to reduced hydration secreted increased amounts of the TLR4-activating protein S100A12 (and expressed increased transcripts for COX-2, IL-1β, and IL-8). In addition, reduced hydration caused a sixfold increase in α-smooth muscle actin (SMA) transcripts of fibroblasts in keratinocyte–fibroblast cocultures, which was abolished by RNA interference (RNAi) knockdown of S100A12 in the

keratinocytes before coculture. Furthermore, recombinant S100A12 activated fibroblasts alone in culture, and this activation was diminished by specific antagonists of TLR4 or receptor for advanced glycation end product (RAGE), which additively inhibited the fibroblast activation. Moreover, intradermal delivery of recombinant S100A12 to rabbit-ear excision wounds resulted in hypertrophic scar.[26]

In burn wounds, water loss also likely upregulates the secretion of S100A12 that activates TLR4, which upregulates inflammatory mediators, activating fibroblasts to increase scarring. Water loss stays elevated long after reepithelialization of cutaneous wounds in general; for example, split-thickness skin-graft donor sites completed reepithelialization by postwound day 14, but water loss from the immature epidermis remained elevated for 200–400 days.[27] In addition, *S100A12* was highly expressed in hypertrophic scar,[28] which occurs commonly after deep partial-thickness burns. And, *S100A12* expression was elevated from postburn day 0–17 in the margins of partial-thickness burn wounds in a clinical study[29] (Fig. 3).

These studies suggest that pharmacological targeting of pathways downstream of water loss could potentially improve scarring, and they also support the use of occlusive water-impermeable barriers (e.g., silicone sheets) applied after reepithelialization to reduce scarring (as discussed[26]). In addition, topical treatments that reduce signaling resulting from water loss before reepithelialization might possibly benefit scar outcome, consistent with the benefits of moist wound healing.

PAMP-TLR signaling in burn wounds

Although infections impair healing, low-level bacterial colonization of wounds can sometimes help healing, depending on the level of colonization and the wound type.[13] The normal inflammation at the edges of full-thickness incision wounds was reduced in $Tlr3^{-/-}$ mice, suggesting that double-stranded RNA (dsRNA) from damaged cells normally activates TLR3 signaling. However, *Staphylococcus epidermidis* lipoteichoic acid (LTA) applied to the wounds inhibited the increase in IL-6 and tumor necrosis factor (TNF)-α at the wound edge of wild-type but not $Tlr3^{-/-}$ mice.[30] *In vitro*, in primary human keratinocytes, *S. epidermidis* LTA activated TLR2 signaling that inhibited dsRNA-TLR3 signaling. Mechanistically, LTA-TLR2 signaling induced TRAF1, which inhibits TRIF, an adaptor protein required for TLR3 signaling. Thus, *S. epidermidis* appears to balance the normal inflammatory dsRNA-TLR3 signaling during healing, and this mechanism might possibly function in the microenvironment of the wound edge where local inflammation may be downregulated when keratinocytes touch *S. epidermidis*.

Although LTA-TLR2 signaling inhibited dsRNA-TLR3 inflammatory signaling of keratinocytes *in vitro* and inhibited cytokine production at the

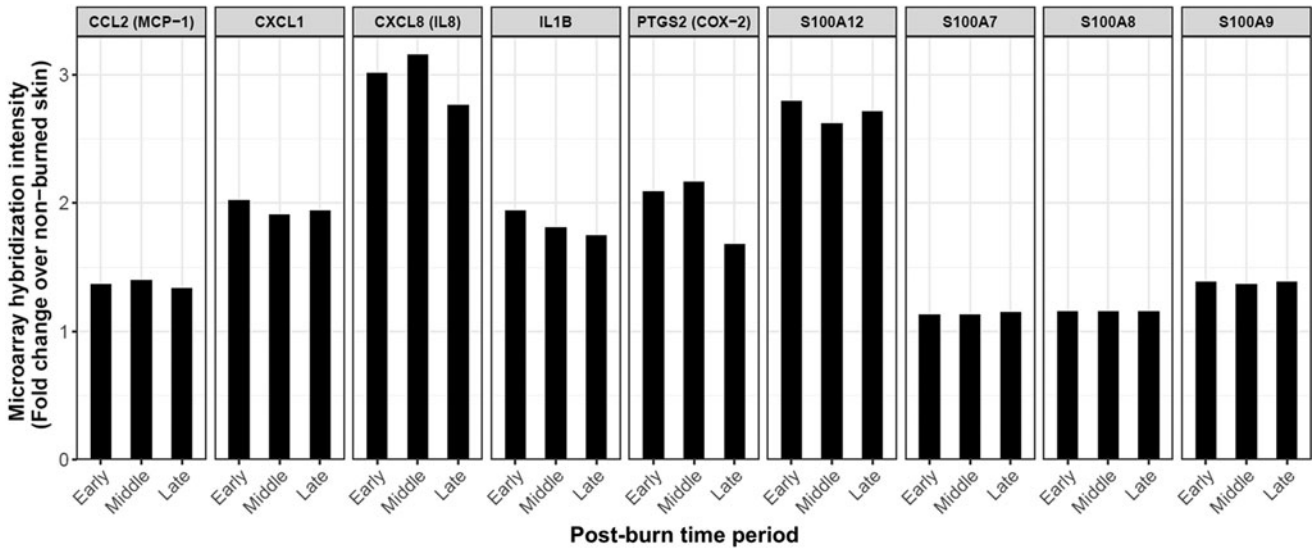

Figure 3. Elevated gene expression in the margins of clinical partial-thickness burn wounds. These data were obtained from a clinical study by Greco and Nanney and colleagues that used the Affymetrix U133 plus 2.0 GeneChip™[29] to evaluate global gene expression in the wound edge of burns at postburn time periods: Early (0–3 days), Middle (4–7 days), and Late (7–17 days). The significantly elevated genes graphed were extracted from Gene Expression Omnibus accession record GSE8056 (using R[69]). The genes shown were upregulated in cultured keratinocytes in response to high sodium, dependent on the activity of the sodium channel Na_x (scn7a).[28]

incision wound edge, LTA-TLR2 signaling conversely stimulated cytokine production by other cell types *in vitro*: macrophages and dendritic and endothelial cells.[30] Thus, the activation of TLR2 signaling by skin flora might benefit or impair wound healing depending on the wound locale.

Deep partial-thickness burn wounds, compared to incision wounds, have a large surface area where LTA-TLR2 signaling might be inflammatory. At the wound perimeter and at appendage stubs,[31] such as hair follicles where bacteria are concentrated,[12] LTA-TLR2 signaling might be predominantly anti-inflammatory—hypothetically, to protect sites of regenerative growth.

Such speculative beneficial effects of skin microbiota, however, might be eliminated by anti-microbial standard-of-care treatments, such as silver sulfadiazine cream, possibly explaining at least partially why this treatment has both retarded healing in several studies and increased hypertrophic scarring (rabbit ear excisional wound model).[32]

The importance of PAMP-TLR signaling in noninfected wounds has also been suggested by studies of germ-free mice. Never exposed to bacterial products, these mice expressed much less TRAF1 in their skin,[30] as expected since they lack Staphylococcal LTA to stimulate TLR2 and increase TRAF1 (possibly to balance dsRNA-TLR3 inflammatory signaling). However, despite lacking this anti-inflammatory mechanism, the wounds of germ-free mice had fewer neutrophils, more mast cells, more macrophages expressing healing genes, increased angiogenesis, accelerated healing, and reduced scar—which were reversed when germ-free mice were "conventionalized" by receiving the conventional-mouse microbiota.[33] Thus, the improved wound healing of germ-free mice might result from their complete absence of PAMP signaling in wounds and/or to an alternatively configured immune system resulting from the lifetime absence of microbiota.

Conversely, bacterial colonization benefited wound healing in some studies (as described in[13]), suggesting that heightened inflammation may aid in clearing necrotic debris and increasing blood flow. But these studies used rodents that are million-fold more resistant to endotoxin or bacterial loads compared to humans.[9] In human partial-thickness burn wounds that already have high inflammation, bacterial colonization may not be beneficial.

Large burns disrupt homeostasis systemically

As with other traumatic injury, large burn injury, ~20% TBSA and greater, can hyperactivate inflammatory cascades that can result in systemic cytokine storm with a paralyzed immune system that can progress to multiorgan dysfunction syndrome (MODS) in the most severe cases.[34] The inflammatory cascade is initiated in the wound as signals, including DAMPs, ignite local inflammation. The inflammation, as well as damaged and clogged blood vessels, cause local edema, increased hydrodynamic pressure, I/R injury, and redox imbalance, which contribute to local spreading of tissue damage (burn injury progression) and an immune response that can spillover into the circulation to result in system-wide capillary leak, edema, and the release of large amounts of oxygen and nitrogen radical species. Stress hormones surge, up to more than 10-fold baseline, and can persist together with hypermetabolism and catabolism for up to and beyond 2 years after severe burn injury.[35] Circulating cytokine levels are altered before the metabolic abnormalities, and larger burns produce greater and more persistent perturbations in circulating inflammatory mediators, immune functions, and stress hormones, as well as more severe catabolism and hypermetabolism to compensate for the evaporative water and heat loss due to the denuded skin.[22] The systemic effects of large burns include increased susceptibility to infection, organ damage and dysfunction, disrupted healing, hypertrophic scarring, and at the extreme end, MODS and mortality (Figs. 4 and 5).

In a large study of >20% TBSA burns, white blood cell (WBC) gene expression was drastically changed (~80% of genes), referred to as a "genomic storm," which showed simultaneous upregulation of innate immunity and compensatory anti-inflammatory responses, together with down-regulation of adaptive immunity.[9] Among the upregulated innate immunity genes were 8 of the 10 human TLRs (Fig. 6), with the two down-regulated TLRs being the endosome-localized TLR3 and TLR7 that recognize dsRNA and single-stranded RNA, respectively.[9]

DAMPs/cytokines at early postburn times

Extensive WBC gene expression changes following severe burns[9] likely result at least, in part, from DAMPs elevated in circulation. In a mouse model of ~30% TBSA full-thickness scald injury, mitochondrial DNA (mtDNA) was elevated three-fold in plasma at 3 h postburn, and it remained significantly elevated up to 4 days before returning to baseline by day 10.[36] In mice with 25% TBSA full-thickness burn, DAMPs (cytochrome C, HMGB1, fibronectin, and hyaluronan) were elevated in plasma as early as 3 h postburn at the same time that TNF-α, IL-6, and IL-10 were elevated, but these cytokines stayed elevated longer,

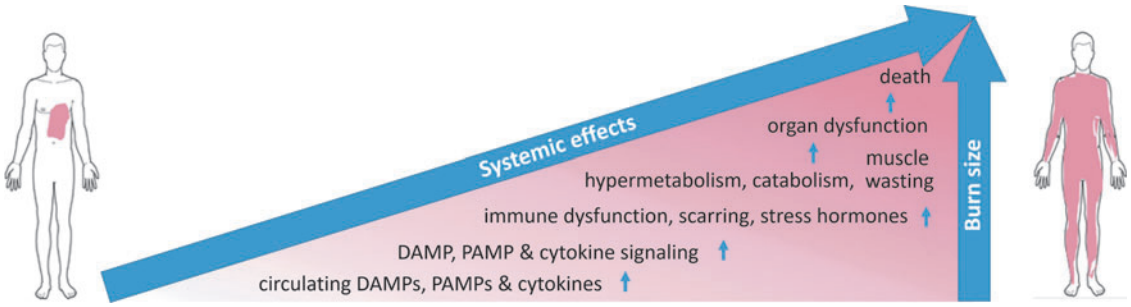

Figure 4. Correlation between burn size and systemic effects. The relationship is not necessarily linear.

until 24 h postburn.[37] However, by day 3, all the DAMPs and cytokines dropped to baseline, except hyaluronan which remained elevated out to the final time point on day 7.

In a prospective clinical study, cell-free *nuclear* DNA (nDNA) was elevated ~10-fold (2,685 genome-equivalents/mL) in the plasma of flame/flash burn patients and ~2-fold in the plasma of scald burn patients several hours after injury; and in the scald burn cohort, the circulating nDNA quantity correlated with the burn TBSA and the number of operations needed.[38] In addition, using a direct rapid fluorometric technique, cell-free DNA at patient admission was elevated 5-fold and correlated with the degree of burn and TBSA.[39] Both nDNA and mtDNA can activate TLRs, and condensed complexes of these self-DNAs with proteins such as histones, HMGB1, and LL-37 can protect them from nucleases and may be crucial for their delivery to endosomes and TLR9 (discussed in[40]).[41]

In addition, another DAMP, decorin, was elevated in patient serum for 2 weeks after burn injury, correlated with TBSA, and predicted hypertrophic scar better than burn size.[2] Decorin is a small leucine-rich proteoglycan that can activate TLR4 (and downstream p38, extracellular signal–regulated kinase [ERK], and NF-κB pathways) and lead to increased secretion of TNF-α, pro-IL-1β, and leukocyte chemoattractants.[2]

DAMPs from mitochondria, in addition to mtDNA, appear to contribute to systemic and local wound inflammation since they activate neutrophils. *In vitro*, these DAMPs (supernatants of mitochondrial sonicates), but not purified mtDNA,

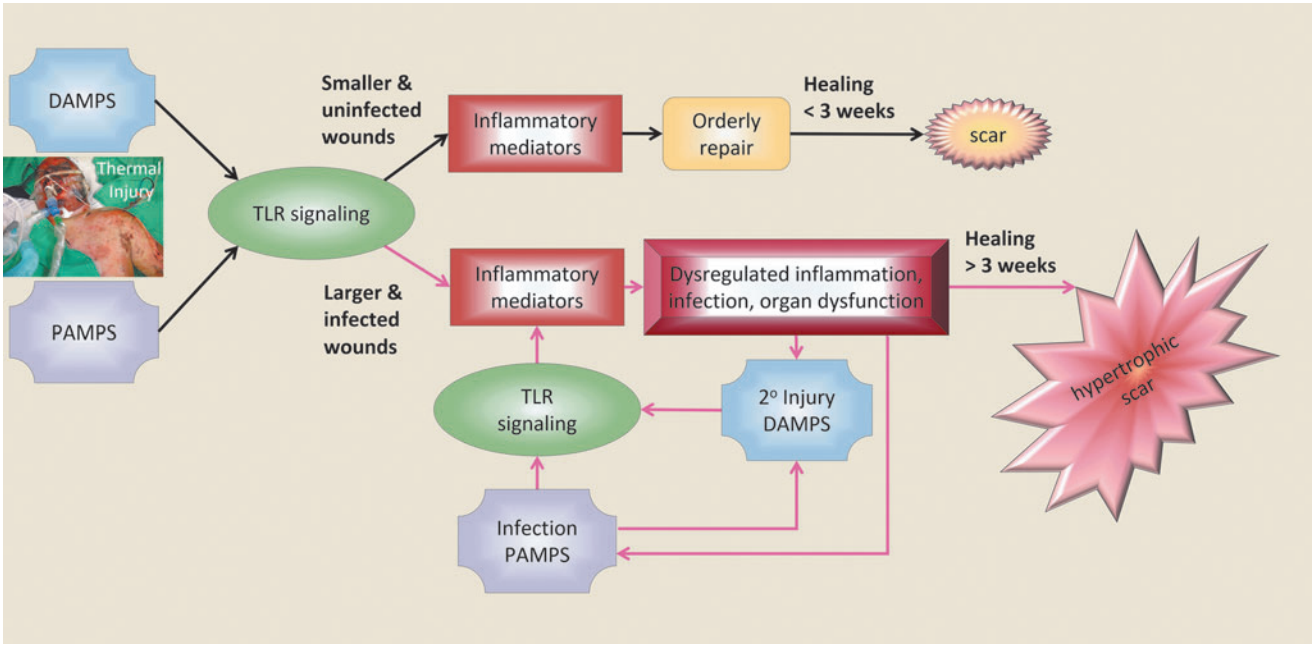

Figure 5. Disruption of systemic homeostasis following burn injury. Larger, deeper, and infected wounds produce uncontrolled inflammation with dysregulated systemic inflammation, susceptibility to infection, organ damage and dysfunction, impaired healing, and high risk for hypertrophic scar (*red arrows*).

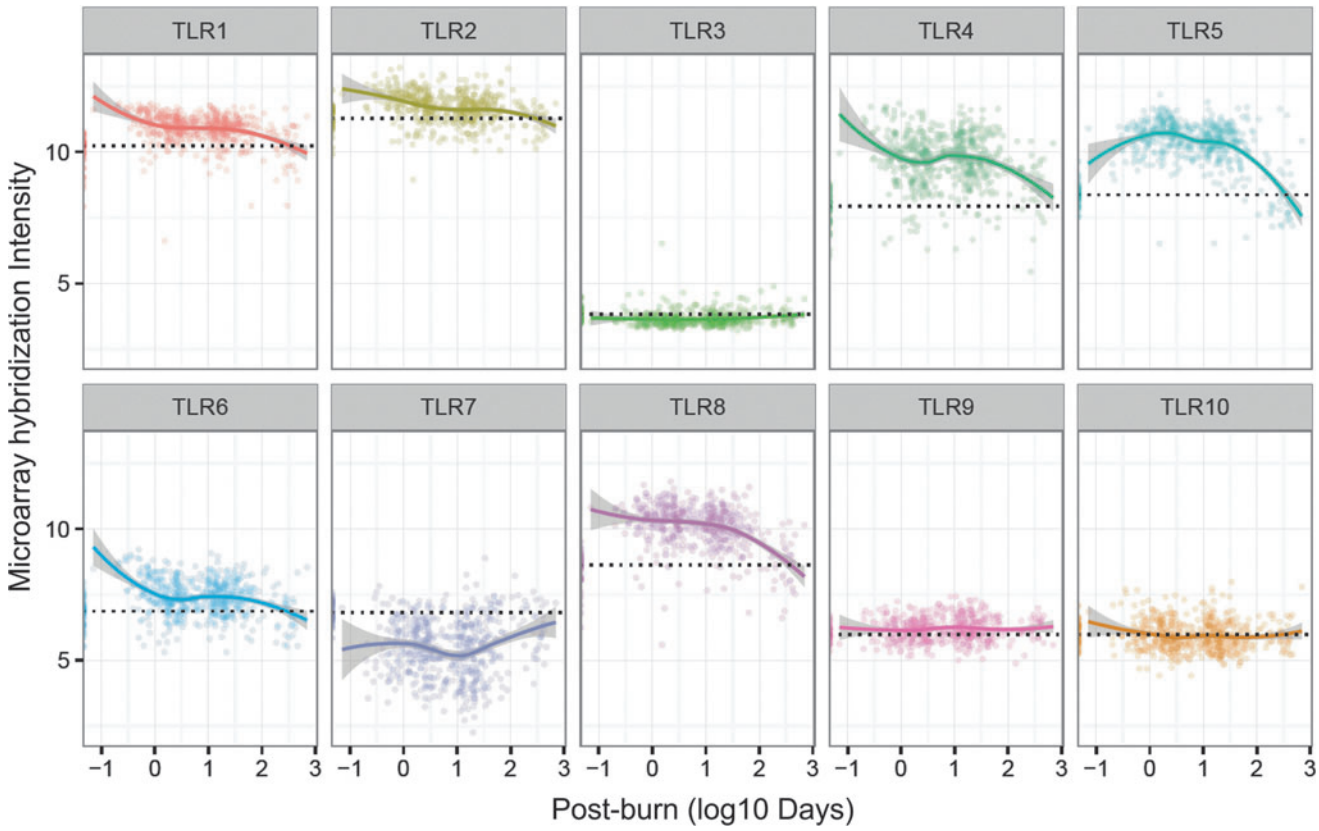

Figure 6. TLR gene expression in WBCs of severely burned patients ($n=244$) over postburn time and healthy subjects ($n=35$). Blood was sampled from patients with severe burns (>20% total body surface area; admitted within 96 h of injury; The Inflammation and the Host Response to Injury Large-Scale Collaborative Research Program).[9] Gene expression was analyzed using the Affymetrix U133 plus 2.0 GeneChip. These data were extracted from Gene Expression Omnibus GSE37069. The healthy subject expression values are plotted along the y axis with their mean indicated by a *dotted line*. The burn-patient TLR expression values were fitted using locally estimated scatterplot smoothing regression, with the 95% confidence interval shown as *gray shading*. Interestingly, TLRs that heterodimerize (TLR1/TLR2 and TLR4/TLR5) were expressed similarly in WBCs over postburn time. In addition, TLR9 and TLR10 were expressed similarly after burn injury, and both are known to be expressed predominantly in human B cells and to be upregulated with similar kinetics after B cell activation, including activation by CpG DNA.[70] WBC, white blood cell.

activated neutrophils, as well as their p38 and ERK1/2 MAPK signaling,[42] and inhibition of these TLR-downstream kinases by small molecules blocked the neutrophil activation.[43] In addition, mitochondrial DAMPs administered intravenously to rats, in an amount equivalent to 5% of their liver, caused marked injury to lung tissue.[42]

Overall, the results suggest that DAMPs released from traumatized tissue activate circulating leukocytes, as well as various cell types in periburn tissue, to produce excessive amounts of inflammatory mediators that contribute to organ damage. In addition, the paralyzed leukocyte function in the state of genomic storm[9] undoubtedly contributes to infection susceptibility and perturbed healing.[44]

Altered immune functions postburn

Elevated circulating DAMPs and cytokines in burns have been associated with altered functions of immune cells, consistent with the heightened innate immunity and suppressed adaptive immunity shown by the leukocyte genomic storm data. For example, leukocyte responses to TLR agonists were altered in mice at 3–7 days postburn (25% TBSA full thickness): *ex vivo* leukocytes stimulated with the DAMPs zymosan (TLR2 agonist) or lipopolysaccharide (LPS) (TLR4 agonist) were primed to produce more cytokines.[45] In addition, dendritic cells were dysfunctional following burn injury (25% TBSA, mouse model): in the early days postburn, *ex vivo* treatment with the TLR9 ligand, unmethylated CpG oligodeoxynucleotide, caused splenic conventional dendritic cells to produce a cytokine profile that was anti-inflammatory and could not activate $CD4^+$ T cells to produce Th1 and Th17 cytokines, while plasmacytoid dendritic cells showed impaired ability to secrete pro-inflammatory cytokines and activate T cell proliferation, and both of these defects were associated with low levels of transcripts of TLR9 and several key molecules of the

TLR signaling pathway.[46] Furthermore, from 1 to 7 days following burn, spleen cells were primed to produce greater amounts of pro-inflammatory cytokines after *ex vivo* exposure to TLR2 and TLR4 ligands; and burn-injured mice challenged with LPS expressed higher levels of inflammatory cytokines in the lung, liver, spleen, and plasma, primarily due to dendritic cells and macrophages, as judged by intracellular cytokine staining.[47] Moreover, increased TLR2 or TLR4 signaling in Kupffer cells has been suggested to be a source of elevated circulating cytokines in burned mice. Kupffer cells isolated from burned rats (30% full thickness) at 24 h postburn and exposed to HMGB1 *ex vivo* produced more TNF-α and IL-1β proteins than Kupffer cells isolated from sham-burned animals.[48] In addition, in these cells, burn injury enhanced HMGB1-induced activation of p38 MAPK, JNK, and NF-κB, and preincubation of the cells with antibody to TLR2 or TLR4 reduced this activation, as well as cytokine production.

These studies suggest that burn injury, within a day, causes priming of the responsiveness of immune cells—in circulation and residing in lymphoid tissues and organs, including lung and liver—to produce greater quantities of inflammatory mediators in response to activation of TLRs by DAMPs, resulting in altered immune system functions.

Organ dysfunction in severe burns

Larger and deeper burns are at greater risk for MODS and death, and patients with MODS have elevated inflammatory markers.[49] A role for TLR4 in postburn systemic inflammation and organ damage has been indicated. First, in a mouse burn model (25%-TBSA full thickness), adhesion of leukocytes to mesenteric venules (i.e., distal to the burn wound) at 30 min postburn was lower in *Tlr4*$^{-/-}$ mice (C3H.HeJ) as was microvascular leakage at 1–3 h postburn.[50] Second, endothelial cell monolayer cultures exposed to burn plasma became permeable, which was attenuated by small interfering RNA (siRNA) to TLR4. Overall, the data suggest that in systemic inflammation after trauma, including burns, TLR-4 plays a role, which in addition does not involve LPS, as LPS-resistant *Cd14*$^{-/-}$ mice showed the wild-type level of inflammation in response to trauma.[50] Furthermore, at 48 h postburn in a rat model of 30% TBSA (no fluid resuscitation), secondary tissue damage was indicated by elevated TNF-α and IL-1β in serum, as well as elevated HMGB1 mRNA and protein in lung, liver, and kidney.[51]

Altered healing following severe burn injury

In a mouse model of regenerative wound healing in which punch holes in the ear regenerate tissue (MRL/MpJ), a dorsal full-thickness 15% TBSA burn caused an increase in inflammatory mediators in serum, lung, and the earhole wound remote from the burn. In addition, the burn injury caused the earhole wounds to fail to undergo regenerative healing; instead, the wounds were infiltrated with inflammatory cells, ulcerated, and necrotic.[52]

TLR signaling in hypertrophic scar fibroblasts

Consistent with prolonged inflammation being a known factor contributing to hypertrophic scarring, cultured fibroblasts from hypertrophic scars of burn-injured patients, versus normal skin fibroblasts from the same patients, showed upregulated TLRs and greater expression of cytokines in response to TLR activation.[53] The hypertrophic scar fibroblasts had upregulated mRNA for all 10 TLRs and MyD88 (a TLR adaptor), as well as upregulated inflammatory-mediator proteins (prostaglandin E2, IL-6, IL-8, and monocyte chemoattractant protein [MCP]-1). In response to LPS stimulation, the hypertrophic scar fibroblasts produced more MyD88, IL-6, IL-8, and MCP-1 mRNA and protein, but siRNA knockdown of MyD88 decreased these. Thus, hypertrophic scar contains fibroblasts primed for inflammatory reactions, producing greater amounts of cytokines that attract leukocytes which produce pro-fibrotic growth factors such as TGF-β and MCP-1, which stimulate fibroblasts to produce excessive extracellular matrix.[53] TLRs of cells of healing wounds with roles in organ fibrosis have been recently reviewed.[54]

Therapeutic targeting of TLR signaling

Reviewed elsewhere are strategies for reducing burn wound progression,[55] dampening cutaneous wound inflammation for improving healing,[56] and treating diseases (metabolic) driven by DAMPs[57]; in this study, we review therapeutic strategies that can potentially ameliorate burn wound progression, systemic inflammation, and hypertrophic scarring through targeting of TLR pathways. Targets of TLR signaling pathways span from the DAMPs and PAMPs that trigger TLRs and extend to downstream kinases that activate the transcription factors driving the expression of inflammatory mediators (Fig. 2).

Within hours after burn injury, topical application of p38 MAPK inhibitors can reduce inflammation, possibly reducing burn wound progression, organ damage, and scarring. For example, in a rat model of *partial*-thickness burn (30% TBSA), a topical p38 MAPK inhibitor (SB202190) applied immediately after the burn injury reduced dermal inflammatory cytokines, neutrophil infiltration, microvascular damage, and hair follicle cell

apoptosis at 6–24 h postburn.[58] In addition, in a *full*-thickness burn (30% TBSA) model in mouse, topical p38 MAPK inhibitors applied 4 h postburn reduced cytokines in the dermis and circulating leukocytes.[59] Thus, p38 inhibitor treatment could penetrate the full-thickness burn eschar and be effective within a 4 h window, which suggests the feasibility of using early topical treatments to suppress inflammation that can damage tissue locally and systemically. However, these studies did not evaluate end points past 24 h.

Pointing to the potential efficacy of p38 MAPK inhibitors to reduce scarring, MAPK inhibition reduced fibroblast contraction *in vitro* (fibroblast-populated collagen-lattice contraction assay) and reduced wound contraction when applied topically to excision wounds, immediately and daily for 10 days, in a rat model.[60] Similar results were obtained in red Duroc pig (as described[61]). In addition, in a preliminary study in Duroc pig, topical p38 inhibitor attenuated inflammation and improved healing of deep partial-thickness excision and burn wounds; importantly, infection of the wounds, which received neither antibiotic nor dressing, when followed out to 20 weeks, was absent.[62]

Further suggesting a role of p38 MAPK in hypertrophic scarring, a prospective-cohort genome-wide association study of postburn hypertrophic scarring, which tested MAPK-pathway gene single nucleotide polymorphisms for association with the four Vancouver Scar Scale variables in a joint regression model, found a rare missense variant (1.5% minor allele frequency) in the gene for PTPN5, an inhibitor of MAPK, which was associated with decreased severity of postburn hypertrophic scar ($p = 1.3 \times 10^{-6}$), although this result awaits confirmation in an independent clinical cohort.[61]

Topical MAPK inhibition also protected against severe burn-induced organ damage in a rat model of 30% TBSA partial-thickness burn.[63] Topical application of p38 MAPK inhibitor to the burn, immediately and at 8 and 16 h, ameliorated cardiac function deficits at 24 h. This protective effect did not appear to be due to systemic absorption of the inhibitor because p38 MAPK activation in cardiac tissue was not changed. In addition, *in vitro*, burn-injured skin homogenates or serum added to cardiomyocytes impaired their contractility, but not when the homogenates or serum came from rats whose burns were treated with the topical p38 MAPK inhibitor. Thus, these results suggest that local treatment of burns can reduce systemic mediators of organ dysfunction.

Therapeutic targeting of the signaling involved in water loss–induced inflammation involving TLR4 has shown effectiveness in proof-of-concept studies in a hypertrophic scar model (rabbit ear excision wounds). The water loss–induced increase in sodium concentration is sensed and signaled through two sodium channels, and blocking these channels—Na_x by RNAi and ENaC by amiloride—after re-epithelialization, reduced scar formation, as did blockade of more downstream molecules, including TLR4 (using TAK-242), RAGE, p38α, COX-2, and IL-1.[64] These findings are likely relevant to burn wounds that have high water loss for long times after reepithelialization.

HMGB1 is a candidate therapeutic target for mitigating burn wound sequela. HMGB1 was released from necrotic cells of peri-burn tissue during burn injury progression, likely in the form with C23–C45 disulfide that activates TLR4. HMGB1 can be inhibited by glycyrrhizin—a triterpene glycoconjugate derived from licorice root (Glycyrrhiza glabra) that has been used in Japan for decades to treat patients with hepatitis B and C (up to 140 mg/day).[65] Glycyrrhizin directly binds HMGB1 ($K_d \sim 150\ \mu M$), partially explaining its anti-inflammatory properties.[65] During diverse types of acute inflammation, glycyrrhizin treatment reduced the activation of downstream molecules (NF-κB, STAT3, and the MAPKs, JNK, p38, and ERK).[66]

The potent, well-tolerated synthetic TLR4 antagonist Eritoran is a small molecule that has mitigated diverse types of I/R injury[67] and therefore may mitigate I/R injury in burn wounds. In addition, antibody to HMGB1 reduced hepatic I/R injury, and knocking out HMGB1 specifically in hepatic epithelial cells profoundly reduced numbers of infiltrating neutrophils and inflammatory gene expression.[21] Thus, blocking HMGB1 or its TLR4 receptor may benefit burn wound outcomes.

Suggesting that inhibiting inflammation early after burn can be beneficial, in a clinical study, a monoclonal antibody against ICAM-1[68] administered intravenously at 6, 12, and 24 h postburn inhibited immune cell extravasation, which was associated with a threefold increase in the number of patients that healed within 21 days. However, scar at 6 months evaluated by laser Doppler and Vancouver scale was not different, possibly because only 40% of the 110 patients could be evaluated.

Collectively, these studies suggest that targeting TLR-signaling pathways to reduce inflammation has potential to improve burn wound outcomes.

SUMMARY

Deep partial-thickness burn wounds that heal over long times with prolonged inflammation

typically result in hypertrophic scars. To prevent these scars from forming, treatments are needed that can be applied to wounds early in the repair process to promote regenerative healing. Treatments that target TLR signaling pathways can limit inflammation and fibrosis and can potentially act at multiple stages of wound repair to protect against scarring. In addition, blocking TLR signaling pathways can potentially mitigate systemic inflammation/MODS.

TAKE-HOME MESSAGES

- The extent of the systemic sequela of burn injuries, including circulating cytokines, immunosuppression, impaired wound healing, hypertrophic scarring, susceptibility to infection, hypermetabolism, catabolism, muscle wasting, and mortality, is directly related to burn size.
- Deep partial-thickness burn wounds that heal over long times with prolonged inflammation typically result in hypertrophic scars.
- Molecules from burn-injured tissues (DAMPs) are released and interact locally and systemically with TLRs of cells to signal danger. Molecules from microbes, called PAMPs, also activate TLRs. Cells having these receptors overactivated produce excessive amounts of inflammatory mediators that are damaging to organs, stimulate fibrosis, and inhibit healing, which contribute to hypertrophic scarring.
- Inhibitors of TLR signaling pathways are available that can be tested in animal models for efficacy in inhibiting the adverse effects of TLR activation.
- For evaluating treatments to mitigate scarring after burn injury, animal models are used (rodent, rabbit, and pig), and each has strengths and weaknesses; therefore, testing of treatments should be started in clinical studies as soon as their safety is established.

TLR signaling appears to be involved in burn wound progression since from peri-burn tissue, HMGB1 was released from chromatin into the cytoplasm and extracellular space[17] on the day of injury where it might activate TLR4 to promote tissue-damaging inflammation. Second, I/R injury occurs during burn wound progression, and TLR signaling is known to be involved in I/R injury of other organs. In addition, oxidative damage is high in burn tissue, and oxidized lipoproteins can stimulate TLR signaling. In addition to HMGB1, other TLR ligands, such as nucleic acids, are released from burn-injured tissue, contributing to inflammatory signaling and burn wound progression.

DAMPs are present in circulation within a few hours after burn injury and correlate with TBSA (e.g., cell-free nuclear and mtDNA, decorin, etc.). At the same time, cytokines in circulation are elevated and leukocyte gene expression changes are pervasive (i.e., genomic storm), indicating simultaneous upregulation of innate immunity and compensatory anti-inflammatory responses, as well as down-regulation of adaptive immunity. Consistent with the upregulated innate immunity genes (including 8 of 10 TLRs), immune cells in circulation and residing in lymphoid tissues and organs, including lung and liver, are primed to produce greater quantities of inflammatory mediators in response to TLR activation by DAMPs. In addition, adaptive immune functions are paralyzed. Overall, the results suggest that DAMPs released from traumatized tissue activate various peri-burn skin cells, circulating leukocytes, and cells of distant organs to produce excessive amounts of inflammatory mediators that inhibit healing, activate fibroblasts, increase susceptibility to infection, and contribute to organ dysfunction and risk for mortality.

Immediately after epithelial barrier rupture, water loss increases. Evaporating water leaves a higher sodium concentration, which can increase keratinocyte secretion of S100A12, which can activate fibroblasts (increased α-SMA) through TLR4 and RAGE. In addition, intradermally injected S100A12 increased hypertrophic scar (rabbit ear wound model). Inhibiting molecules in this pathway (sodium channels, TLR4, RAGE, p38 MAPK, COX-2, and IL-1) starting after reepithelialization resulted in reduced hypertrophic scarring in the rabbit ear model of excision wounds; although not a burn wound, these findings are likely relevant to burn wounds in which, like other traumatic skin wounds, water loss remains high for months after reepithelialization.

Therapeutic targeting of HMGB1 or TLR4 has mitigated inflammation and injury in several animal models of organ injury (not burn). In addition, topical p38 MAPK inhibitor applied immediately to burn wounds in a mouse model reduced dermal and circulating cytokines, infiltrating neutrophils, and early tissue damage; and when applied up to 4 h postinjury in a rat model, reduced dermal and circulating cytokines. Although these studies did not evaluate long-term outcomes, other preliminary studies suggest that topical p38 MAPK inhibition can inhibit wound contraction in mice and may have had beneficial effects on healing in a pig model. In addition, topical p38 MAPK inhibition protected against severe burn-induced cardiac function deficits at 24 hrs.

To promote regenerative healing, reduce hypertrophic scarring, and mitigate systemic sequela of burn injury, many drugs that target TLR signaling pathway molecules are available for testing. Some of these have demonstrated proof-of-concept efficacy in mitigating short-term end points, mostly in small-

animal models. The development of treatments for improving final wound outcome requires evaluating wounds several months after injury, optimally in pigs and then in proof-of-concept clinical models.

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Abbreviations and Acronyms

AP-1 = activator protein 1
COX = cyclooxygenase
CREB = cAMP response element binding
DAMP = damage-associated molecular pattern
DPT = deep partial-thickness
dsRNA = double-stranded RNA
ENaC = epithelial sodium channel
ERK = extracellular signal–regulated kinase
HMGB = high mobility group box
HSP = heat shock protein
I/R = ischemia/reperfusion
ICAM-1 = intercellular adhesion molecule
IL = interleukin
IRF = interferon regulatory transcription factor
JNK = c-Jun N-Terminal Kinase
LPS = lipopolysaccharide
LTA = lipoteichoic acid
MAPK = mitogen-activated protein kinase
MCP = monocyte chemoattractant protein
MODS = multiorgan dysfunction syndrome
mtDNA = mitochondrial DNA
MyD88 = myeloid differentiation primary response gene 88
Na_x = sodium voltage-gated channel type 7
nDNA = nuclear DNA
NFκB = nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP = pathogen-associated molecular pattern
PTPN5 = protein tyrosine phosphatase, non-receptor type 5
RAGE = receptor for advanced glycation end product
RNAi = RNA interference
S100A12 = S100 calcium binding protein A12
siRNA = small interfering RNA
SIRS = systemic inflammatory response syndrome
SMA = smooth muscle actin
TBSA = total body surface area
TLR = toll-like receptor
TNF = tumor necrosis factor
TRAF = TNF receptor-associated factor
TRIF = TIR domain–containing adapter inducing interferon-β
WBC = white blood cell