SURGICAL INFECTIONS
Volume 11, Number 2, 2010

DOI: 10.1089/sur.2009.053

Toll-Like Receptor-4 Mediates Intestinal Barrier Breakdown after Thermal Injury

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Abstract

Objective: Toll-like receptor 4 (TLR-4) activation after sterile injury leads to organ dysfunction at distant sites. We have shown previously that intestinal barrier breakdown and alteration of tight junction proteins follows thermal injury; however, the role of TLR-4 in this process remains unclear. We hypothesized that increased intestinal permeability and barrier breakdown after burns is a TLR-4 dependent process; hence, knocking down the TLR-4 gene would have a protective effect on burn-induced intestinal dysfunction.
Methods: Male C57BL/6J (TLR-4 wild type [WT]) and C57BL/10ScN (TLR-4 knockout [KO]) mice were assigned randomly to either 30% total body surface area steam burn or sham injury. At 4 h, permeability to intraluminally administered fluorescein isothiocyanate (FITC)-dextran was assessed by measuring the fluorescence of the serum. Intestinal samples were analyzed for the presence of the tight junction protein occludin by immunoblotting and immunohistochemistry. Tumor necrosis factor (TNF)-α concentrations in the serum and intestines were measured by enzyme-linked immunosorbent assay at 2 h post-burn.
Results: Serum concentrations of FITC-dextran were decreased in TLR-4 KO mice compared with TLR-4 WT mice after burn injury (92.0 micrograms/mL and 264.5 micrograms/mL, respectively; $p < 0.05$). After injury, no difference in intestinal permeability was observed between the TLR-4 KO mice and the TLR-4 WT sham-treated mice. The TLR-4 KO mice had preservation of occludin concentrations after thermal injury in both immunoblot and immunohistochemistry assays, but concentrations were decreased in TLR-4 WT animals. The serum concentrations of TNF-α serum were higher in TLR-4 WT burned animals than in the sham-treated mice. The TLR-4 KO animals had unmeasurable concentrations of TNF-α. No differences in TNF-α were observed in the intestinal tissue at 2 h.
Conclusions: Mice with TLR-4 KO have less intestinal permeability to FITC-dextran than do TLR-4 WT mice after burn injury as a result of alterations in the tight junction protein occludin. These findings suggest that the greater intestinal permeability and barrier breakdown after burn injury is a TLR-4-dependent process. Toll-like receptor 4 may provide a useful target for the prevention and treatment of systemic inflammatory response syndrome and multisystem organ failure after injury.

Toll-like receptors (TLRs) are a family of transmembrane receptors located throughout the body. Toll-like receptor 4, in particular, plays a role in innate immunity and was described originally as the receptor for lipopolysaccharide (LPS) [1–3]. This receptor may have more than one responsibility: It may respond to endotoxin as well as to various endogenous damage-associated molecular patterns (DAMPs), which are believed to be released from damaged tissue into the systemic circulation after non-infection injuries [4–6]. Toll-like receptor 4 has been implicated in hepatocellular [7, 8], myocardial [9, 10], and respiratory [11, 12] dysfunction after sterile injury; however, there is a paucity of literature evaluating the role of TLR-4 in intestinal dysfunction after thermal injury.

The gastrointestinal system has long been believed to play a key role in mediating distant organ dysfunction after injury. The breakdown of the intestinal mucosal barrier, which keeps luminal contents from entering the bowel wall, has been

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implicated in the genesis of the systemic inflammatory response syndrome (SIRS), sepsis and multisystem organ failure (MSOF) after insults such as hemorrhagic shock and burns [13, 14]. When this barrier is compromised, luminal contents may stimulate a significant inflammatory response in the wall of the intestine, generating inflammatory mediators that further systemic inflammation and organ dysfunction.

The tight junction (TJ) is a complex of transmembrane and intracellular proteins that seal the intercellular space and prevent passage of substances. The relation between increased intestinal permeability and loss of intestinal barrier function after burn injury is well documented [14, 15]. Our laboratory has shown that burn-induced increases in intestinal permeability are associated with the activation of myosin light chain kinase (MLCK) resulting in a decrease in TJ proteins, specifically occludin and ZO-1 [16].

In this study, we investigated the role of TLR-4 in mediating intestinal barrier breakdown after injury. We postulated that increased intestinal epithelial permeability after burn injury is dependent on TLR-4 activation.

Materials and Methods

Thermal injury model

All procedures were done according to the University of California, San Diego (UCSD), Institutional Animal Care and Use Committee guidelines, and study protocols were approved by the UCSD Institutional Review Board. Male C57BL/6J (TLR-$4^{+/+}$: Wild type [WT]) and C57BL/10ScN (TLR-$4^{-/-}$: Knockout [KO]) mice (Jackson Laboratories, Bar Harbor, ME), ages 8 to 16 weeks, were used. The C57BL/10ScNJ mice contain a spontaneous deletion of the *Tlr4*^{lps} gene locus, which codes for the TLR-4 protein, resulting in both a loss of mRNA and protein and lack of responsiveness to LPS stimulation [17, 18]. These mice have been studied extensively and are used in a wide variety of inflammation and immunological research [19–24].

Animals were housed in the UCSD vivarium with 12-h light–dark cycles and given free access to food and water. They were randomly assigned to either thermal injury or sham treatment. A total of 24 animals were divided into four groups: 12 for the intestinal permeability model and 12 for tissue procurement, with each group being subdivided into treated and sham-treated animals. Isoflurane anesthesia was administered, and the hair was removed from the dorsum of the torso. Animals were then arranged in a template exposing 2×4 cm of dorsal skin and placed in a column of steam above water boiling at 100°C for 7 sec. This produces an approximately 30% total body surface area (TBSA) full-thickness burn, as described by our laboratory and others [25, 26]. Burned animals were then given subcutaneous injections of normal saline (4 mg/kg) and buprenorphine (0.05 mg/kg) in a non-burned area and returned to their cages for recovery. Sham-treated animals underwent anesthesia and were shaved and placed in the template without exposure to the steam.

Intestinal permeability model

Four hours after thermal injury, to precede maximum histologic abnormalities in the intestine [27], animals were again anesthetized, and a laparotomy incision was made. The distal ileum was identified, and a segment measuring 5 cm was isolated with silk suture. A 200-microliter volume (125 mg/mL) of 4-kDa fluorescein isothiocyanate (FITC)-dextran was injected into the lumen of the isolated distal ileal loop, and the laparotomy incision was closed with silk sutures. After 30 min, the animals were euthanized, and blood was collected via cardiac puncture. The serum was separated, and the fluorescence of FITC-dextran was measured at 520 nm.

Histology sections

Previous research in our laboratory indicated that histologic intestinal injury is maximal at 6 h after injury [27]; therefore, animals were sacrificed at this time, and intestinal sections were collected and preserved immediately in 10% formaldehyde solution. Sections were submitted for paraffin blocking and hematoxylin and eosin staining by UCSD Histology Core Services. Two 5-micrometer sections of ileum were selected randomly from two mice in each experimental group and evaluated by a pathologist blinded to the treatment given. Sections were scored according to the intestinal injury scoring system previously used by our laboratory and others [28, 29]: 0 = no damage; 1 = (mild) focal epithelial edema and necrosis; 2 = (moderate) diffuse swelling and necrosis of the villi; 3 = (severe) necrosis with evidence of neutrophil infiltration in the submucosa; 4 = (major) widespread necrosis with massive neutrophil infiltration and evidence of hemorrhage. The injury scores were averaged for each experimental condition, and images were taken at 200× magnification with light microscopy.

Western blot analysis

Tissues were frozen immediately with liquid nitrogen at the time of collection and stored at −80°C until use. Intestinal protein extraction was performed by Dounce homogenizing tissue with Tissue Protein Extraction Reagent (Pierce, Rockford, IL) and Halt Phosphatase and Protease Inhibitor (Pierce). Samples were then centrifuged, and the supernatant liquid was retained. Forty micrograms of protein was loaded in each sample well. Samples were separated using 6–18% Tris-glycine gel and transferred to nitrocellulose membranes. After blocking with 5% bovine serum albumin, samples were exposed overnight to rabbit anti-occludin antibody (Zymed Laboratories, Carlsbad, CA; 1:500). The samples were then treated with anti-rabbit IgG horseradish peroxidase-linked antibody (Cell Signaling, Danvers, MA; 1:2,000), followed by chemiluminescent detector solution (Pierce). Beta-actin loading control (Cell Signaling, Danvers, MA; 1:500) was measured for each gel. Band pixel density was calculated using Un-Scan-It software (Silk Scientific, Orem, UT). The relative band density was calculated by dividing the pixel density of the lane of interest by the pixel density of beta-actin. The average relative band density of the sham-treated animals was calculated. These data are reported as fold change over sham treatment ± standard deviation. The immunoblots were repeated three times, and the results are reported as means ± standard deviations.

Immunohistochemistry

At the time of tissue collection, intestinal sections were placed in OCT compound (Sakura Finetek, Torrence, CA) and

stored at −80°C. Two 10-micrometer sections from each animal were fixed with 4% paraformaldehyde, blocked with 5% bovine serum albumin, and exposed to rabbit anti-occludin antibody (1:100) overnight. This was followed by treatment with secondary antibody (goat anti-rabbit IgG; Alexa Flour-488; Invitrogen, Carlsbad, CA; 1:200). Images were viewed at 60× with confocal microscopy and analyzed using FlouView FV1000 software (Olympus, Center Valley, PA).

Tumor necrosis factor-α concentrations

Plasma and intestinal concentrations of tumor necrosis factor (TNF)-α were measured 2 h after burn injury using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). Intestinal TNF-α concentrations were measured using undiluted whole-cell protein extracts of the distal ileum. The TNF-α concentrations are reported as pg/mL ± standard error of the mean (SEM) (n ≥three per group).

Statistical analysis

In general, all results are presented as mean ± standard deviation. Gut Injury Scores were analyzed using the Kruskal-Wallis test, given the ordinal nature of the data. Other continuous data were analyzed using analysis of variance with Bonferronni corrections to evaluate differences between groups. A p value of < 0.05 was considered significant.

Results

Histologic abnormalities after thermal injury

At 6 h after burn injury, the histologic appearance of the distal ileum from TLR-4 WT mice was markedly abnormal, with an average Gut Injury Score of 2.75 ± 0.3 ($p < 0.01$; Fig. 1B) compared with the WT sham-treated animals (Gut Injury Score 0 ± 0; Fig. 1A). The ileum from the TLR-4 WT burned mice contained a significant number of shortened villi, alterations in epithelial nuclear polarity, and areas of focal necrosis of the mucosa. The histologic appearance of the TLR-4 KO ileum was nearly normal (Gut Injury Score 0 ± 0; Fig. 1D) and most closely resembled the WT sham-treatment ileum. The TLR-4 KO sham-treatment ileum did not show significant alterations in histologic appearance (Gut Injury Score 0.5 ± 0.6; Fig. 1C).

Intestinal epithelial permeability to FITC-dextran after thermal injury

Serum concentrations of FITC-dextran were elevated in TLR-4 WT mice at 4 h after thermal injury compared with WT sham-treated animals (264.5 ± 43.3 vs. 31.1 ± 21.0 micrograms/mL; $p < 0.01$; Fig. 2). The TLR-4 KO animals in both the sham-treated and thermal-injury group had low concentrations of serum FITC-dextran (sham-treated 72.2 ± 38.3 micrograms/mL; burn 92.0 ± 31.9 micrograms/mL). The serum FITC-dextran concentration in TLR-4 KO mice exposed

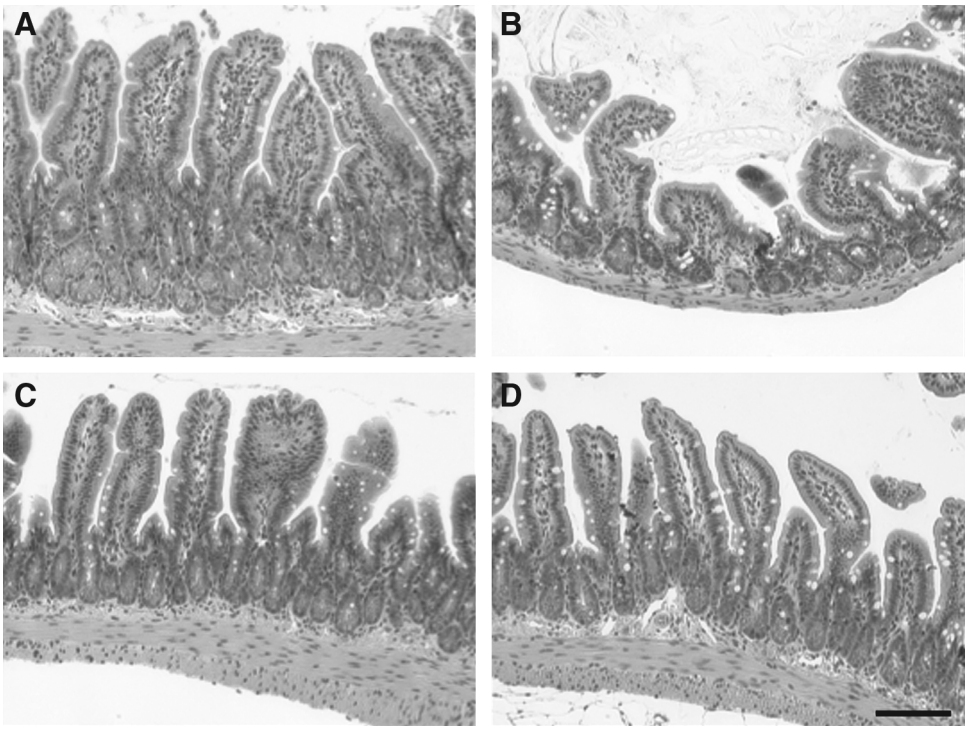


FIG. 1. Histologic abnormalities after thermal injury are ameliorated in TLR-4 knockout (KO) mice. (**A**) Normal histologic appearance of ileum from TLR-4 wild-type (WT) animal subjected to sham treatment. Note intact and uniform villi, mucosa with uniform polarity and no nuclear abnormalities, and absence of inflammatory cells. (**B**) Ileum from TLR-4 WT animal 6 h after 30% total body surface area steam burn. Ileal villi are markedly shortened and no longer of uniform length, and there are focal areas of loss of nuclear polarity and mucosal necrosis. (**C**) Ileum from sham-treated TLR-4 KO animal has normal histologic appearance. (**D**) Ileum from TLR-4 KO animal 6 h after 30% total body surface area burn injury has near-normal histologic appearance. Original magnification 200× (size bar = 0.1 mm).

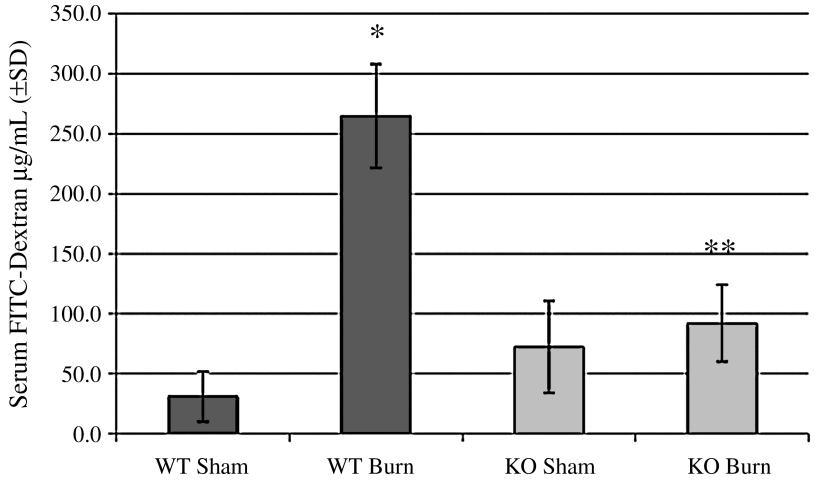


FIG. 2. Intestinal epithelial permeability to fluorescein isothiocyanate (FITC)-dextran after thermal injury is decreased in TLR-4 knockout (KO) mice. The FITC-dextran was administered intraluminally 4 h after a 30% total body surface area steam burn, and fluorescence of the serum was measured. Dark bars represent concentration of serum FITC-dextran in TLR-4 wild-type (WT) mice, and light bars represent TLR-4 KO animals (*$p < 0.01$ compared with WT sham-treated animals; **$p < 0.05$ compared with WT burned animals).

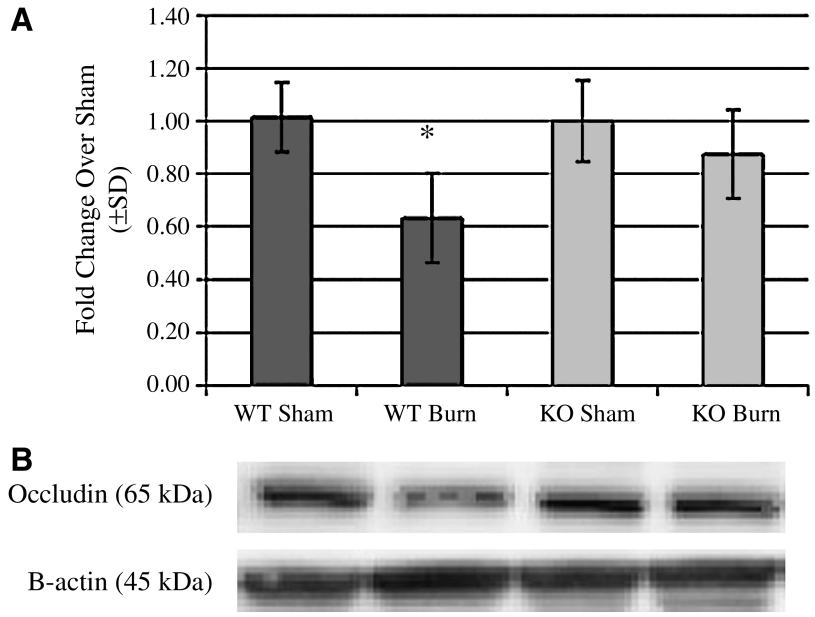
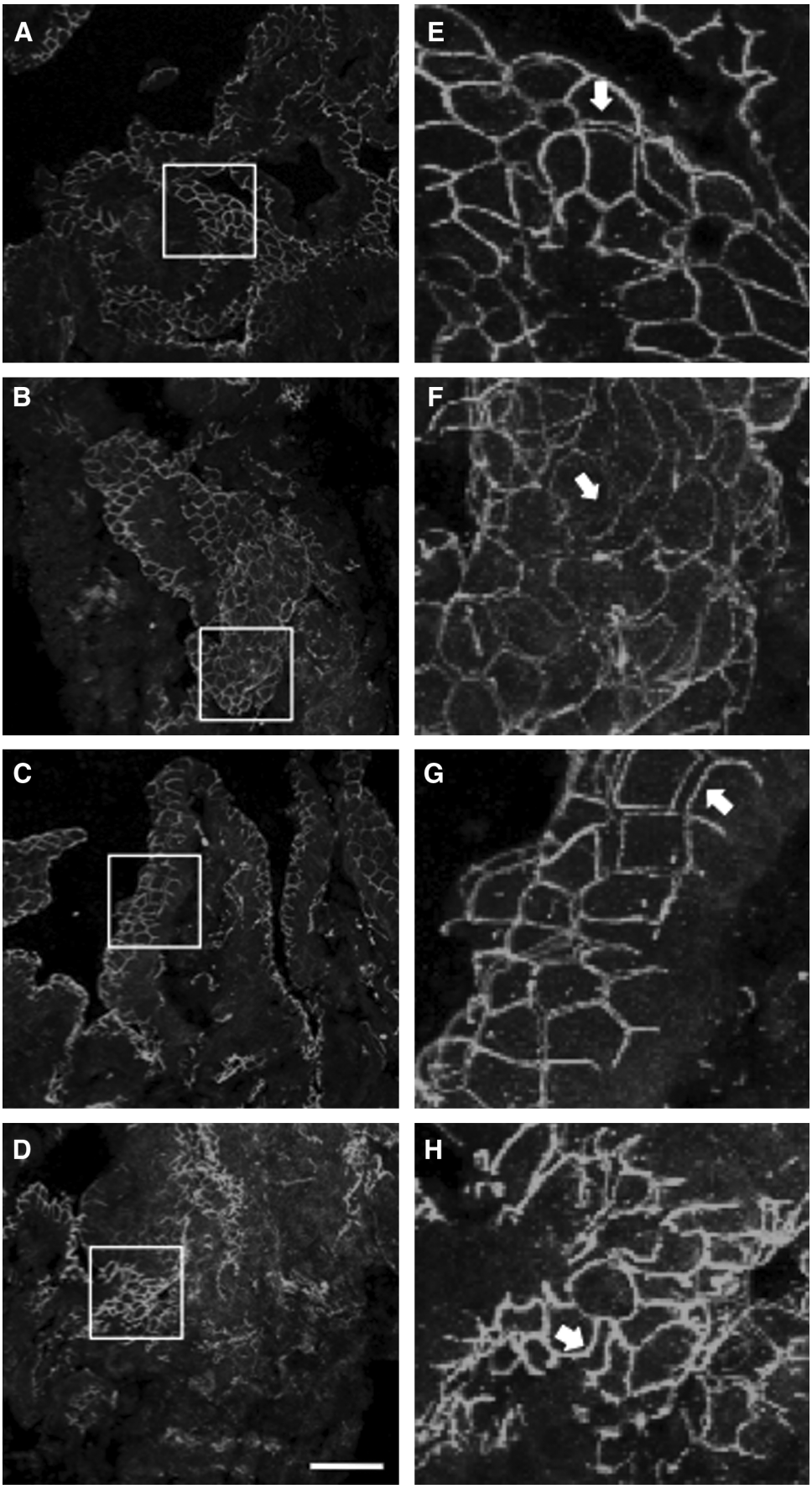


FIG. 3. Intestinal concentrations of tight junction protein occludin are not decreased in TLR-4 knockout (KO) animals after thermal injury. (**A**) Western blot analysis of total intestinal occludin demonstrated that the protein was decreased in TLR-4 wild-type (WT) animals (dark bars) after 30% total body surface area steam burn, but not significantly decreased in TLR-4 KO animals (light bars) after thermal injury (*$p < 0.01$ compared with TLR-4 WT sham-treated animals). (**B**) Representative images of Western blots of occludin and β-actin bands.

FIG. 4. The TLR-4 knockout (KO) mice do not have alterations in epithelial occludin protein after thermal injury. Confocal images of ileum stained for occludin protein taken at 600× magnification (**A–E**, size bar = 30 micrometers). White box denotes area of image to the right (**E-H**). (**A,** E) Images of ileum from TLR-4 wild type (WT) sham-treated animals showing smooth, even outline of protein at the plasma membrane where adjacent cells come into contact. (B, F) Ileum from TLR-4 WT animal with 30% total body surface area steam burn. Note disordered appearance of occludin, which is no longer located distinctly at the cell membrane, resulting in loss of intercellular architecture. (C, G) Ileum from sham-treated TLR-4 KO animal with normal occludin localization and architecture. (D, H) Ileum from burn-injured TLR-4 KO animal. Note distinct localization of occludin to plasma membrane and the clear architecture. White arrows highlight areas of adjacent cell membranes demonstrating that both TLR-4 WT (F) and TLR-4 KO (H) ileum from thermally injured animals has larger intercellular spaces.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to thermal injury was significantly lower than that in the thermally injured WT animals ($p < 0.05$).

Intestinal concentrations of occludin protein after burn injury

The intestinal concentration of occludin in the TLR-4 WT mice was decreased by nearly half compared with WT sham-treated animals (0.63 ± 0.17 vs. 1.01 ± 0.13 fold change over sham; $p < 0.01$; Fig. 3). The TLR-4 KO animals exposed to thermal injury did not have a significant decrease in occludin protein after thermal injury and were similar to TLR-4 KO sham-treated animals (0.87 ± 0.17 vs. 1.00 ± 0.15 fold change over sham).

Cellular location of occludin protein after thermal injury

Confocal imaging of sections stained for occludin demonstrated localization to the cell membrane of intestinal epithelial cells in the TLR-4 WT sham-treatment ileum, resulting in a smooth, even outline of the plasma membrane where adjacent cells come into contact (Fig. 4A and E). In TLR-4 WT ileum from animals exposed to thermal injury, the occludin protein was no longer distinctly located at the cell membrane, resulting in a loss of intercellular architecture and a widening of the intercellular spaces (Fig. 4B and F). The TLR-4 KO sham-treated (Fig. 4C and G) and TLR-4 KO burn (Fig. 4D and H) mice did not show this loss of architecture and disordered appearance of occludin. Interestingly, both TLR-4 WT and TLR-4 KO ileum taken from thermally injured animals appeared to have larger intercellular spaces (white arrows in Fig. 4).

Tumor necrosis factor-α concentrations

Tumor necrosis factor-α is an early mediator of the inflammatory cascade, becoming elevated within hours after injury. Plasma concentrations of TNF-α were measured 2 h after thermal injury to assess the extent of systemic inflammation mediated by TLR-4. The burned TLR-4 WT animals showed a trend toward elevated TNF-α compared with TLR-4 WT sham-treated animals (2.91 ± 1.96 vs. 1.13 ± 0.74 pg/mL, respectively; $p = 0.3$). More importantly, TLR-4 KO animals in both the thermal-injury and the sham-injury groups had undetectable plasma TNF-α concentrations. Intestinal TNF-α was not detectable at 2 h in any of the four groups.

Discussion

Accidental injury remains the number one cause of death in persons age 1–44 years and the fifth leading cause of death overall [30]. Individuals who survive the original insult may go on to develop SIRS, sepsis, and MSOF, all of which are major causes of late deaths after trauma and burns [31]. It is postulated that the gastrointestinal system is central to the development of SIRS and MSOF after burns because mucosal injury leads to bacterial translocation and incites an inflammatory response [32]. Continuing research into the etiology and pathophysiology of these disease processes may ultimately lead to the development of therapeutics for this widespread problem.

Our study demonstrated that TLR-4 mediates greater intestinal barrier breakdown after severe thermal injury. The TLR-4 KO mice had preservation of normal ileal histology after burn injury, and they did not have a decrease in the quantity or redistribution of the TJ protein occludin. The intestinal histologic abnormalities seen after burns are well documented by our laboratory and others, including loss of villus structure and height, abnormal nuclear polarity, and mucosal necrosis [16, 33, 34], and our results in TLR-4 WT mice after severe thermal injury are congruent with previous literature. Additionally, we demonstrated that TLR-4 KO mice were protected from increased intestinal epithelial permeability to FITC-dextran after burn injury and that TLR-4 activation after burn injury is associated with a decrease in occludin, leading to a breakdown in intracellular adhesion as well as to barrier dysfunction and greater mucosal permeability to intraluminal contents. To our knowledge, this is the first such study to demonstrate the importance of TLR-4 in mediating intestinal barrier breakdown after thermal injury.

The TJ proteins hold adjacent cells together and regulate paracellular permeability in the intestinal epithelium. Occludin is a transmembrane protein in the TJ complex that, along with claudin, interacts with adjacent cells. Occludin and claudin are connected to the actin cytoskeleton through intermediary adaptor proteins such as ZO-1 [35, 36]. Myosin light chain kinase phosphorylates the myosin light chain and alters the localization of F-actin and the structure of TJ proteins [37]. Furthermore, the addition of TNF-α to epithelial cell cultures increases the expression of MLCK through activation of nuclear factor (NF)-κB, resulting in increased epithelial permeability [38, 39]. Our laboratory has shown previously that severe thermal injury increases the amount of TNF-α, upregulates NF-κB, and activates the expression of MLCK in the intestine, resulting in a decrease and redistribution of occludin and ZO-1 [25, 27].

The family of Toll-like receptors functions as pattern recognition molecules that recognize evolutionarily conserved markers on exogenous pathogens, known as pathogen-associated molecular patterns (PAMPs), setting off the innate immune response [1, 3]. The TLR-4 protein initiates an inflammatory response after activation by endotoxin, a known PAMP [2, 17, 40, 41]. A paradigm has emerged recently proposing that TLR-4 also functions as a damage recognition receptor that binds endogenous danger signals, such as high-mobility group box (HMGB)-1 and heat shock proteins, and activates the innate immune system [4–6]. In this study, we demonstrated that TLR-4 activation is critical in mediating the breakdown of the intestinal barrier after thermal injury. The receptor may become activated after burns by DAMPs released after the injury, leading to the induction of an inflammatory response in the intestine, production of TNF-α, activation of MLCK, and breakdown of the TJ proteins, causing intestinal barrier dysfunction.

These results help to illuminate the course of intestinal barrier breakdown after thermal injury by demonstrating the critical role TLR-4 plays in mediating TJ breakdown, intestinal injury, and inflammation. How TLR-4 becomes activated after an injury is an area of growing research. Several possible hypotheses exist regarding the activation of TLR-4 and the resulting intestinal inflammation.

Activation of TLR-4 is required for production of TNF-α from a cutaneous wound [42]. Furthermore, mice without a functional TLR-4 receptor have lower concentrations of inflammatory cytokines in the serum after sterile injuries, such

as hemorrhagic shock and femoral fracture [41, 43]. These studies indicate that a functional TLR-4 receptor in the local tissue is critical to a systemic inflammatory response. In contrast, recent reports conclude that Kupffer cells are responsible for the production of serum cytokines after sterile injury in a TLR-4-dependent manor [44, 45]. Our study specifically assessed TNF-α concentrations in the serum and intestinal tissue. Concentrations of TNF-α were unmeasurable in TLR-4 KO animals, suggesting the importance of an intact TLR-4 receptor for TNF-α synthesis after injury.

The gastrointestinal system is unique in that it is continually exposed to potential PAMPs and other instigators of the inflammatory response. Abreu et al. [46, 47] have shown that intestinal epithelial cells downregulate TLR-4 and its co-receptor MD-2 and that activation by LPS in the basolateral membrane is proportional to the expression of these receptors, thus providing a mechanism for the intestinal epithelial cells to be tolerant to native LPS within the intestinal lumen. Our study did not specifically evaluate the ligand that activates TLR-4 in this model. The TLR-4 on the epithelial cells may become hypersensitive to intraluminal LPS after an injury, so that even minute quantities of the protein produce a meaningful response. Additionally, epithelial TLR-4 may become activated preferentially by systemically released DAMP, which may not require the same co-receptors as LPS or have the same magnitude of response. Lastly, the mucosal breakdown seen after thermal injury may result in LPS traversing the epithelial barrier and activating other cells within the bowel wall. The current research is unclear about exactly which cell type is key in signaling intestinal inflammation via TLR-4 after injury. Expression of TLR-4 in the epithelial cell layer increases after a systemic physiologic stressor, thus priming the intestinal mucosa to respond to intraluminal endotoxin and triggering the inflammatory cascade [48]. Alternatively, severe thermal injury primes macrophages and dendritic cells, resulting in overproduction of inflammatory cytokines after LPS exposure [49], which may indicate that intestinal barrier breakdown results from hyperactive, primed leukocytes. Future studies will be needed to delineate the role of TLR-4 in each cell type, as well as the specific ligands required for activation, and how this affects the response to injury and inflammation.

In summary, we have demonstrated that TLR-4 activation mediates intestinal barrier breakdown after burn injury. Mice lacking a functional TLR-4 receptor were protected from burn-induced intestinal histologic abnormalities and increased intestinal permeability though preservation of the TJ protein occludin. Toll-like receptor 4 may prove to be a target for the treatment or amelioration of intestinal inflammatory diseases, sepsis, SIRS, and MSOF.

Author Disclosure Statement

No competing financial interests exist. No financial support was received for this study.

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