DNA attenuates enterocyte Toll-like receptor 4-mediated intestinal mucosal injury after remote trauma

Chhinder Sodhi,1,2 Ryan Levy,2 Roop Gill,2 Matthew D. Neal,1,2 Ward Richardson,1,2 Maria Branca,1 Anthony Russo,1 Thomas Prindle,1 Timothy R. Billiar,2 and David J. Hackam1,2
1Division of Pediatric Surgery, Department of Surgery, Children’s Hospital of Pittsburgh, 2Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania
Submitted 3 August 2010; accepted in final form 12 January 2011

TRAUMA IS THE LEADING CAUSE of death among individuals up to the age of 45 and remains a major cause of morbidity in those who initially survive the early injury events (9). A significant cause of persistent morbidity in traumatized patients is the development of organ dysfunction at sites remote to the initially injured region (26). The development of intestinal dysfunction after remote trauma can have particularly significant physiological consequences, characterized by impaired nutrient absorption, and in select cases can cause barrier disruption and the translocation of enteric microbes (40, 42). Although the presence of intestinal injury is known to occur after remote trauma (8), the precise mechanisms that coordinate its development, and the mechanisms by which remote injury may be sensed and responded to at the level of the intestinal epithelium, remain incompletely understood.

Despite its established cytoprotective role when expressed on immune cells, emerging evidence has implicated Toll-like receptor 4 (TLR4) in the development of parenchymal organ dysfunction through its expression on epithelial cells. Using chimeric mice, we have shown that TLR4 on non-bone marrow-derived cells contributes to end organ injury and the systemic inflammatory response after trauma (32). There are several other instances where TLR4 signaling on parenchymal cells has proven to be deleterious to the host through its proinflammatory effects. Specifically, TLR4 activation in hepatocytes causes hepatic inflammation (41, 45), TLR4 signaling in the bronchial epithelium causes airway inflammation (31, 49), and TLR4 signaling in the gut causes intestinal inflammation (14–15, 17–19, 21, 23, 33). TLR4 is known to be activated primarily in response to bacterial LPS (3) but may also be triggered in response to endogenous molecules such as high-mobility group box-1 (HMGB1), a DNA-binding protein that is released from injured cells (48) whose circulating levels have been shown to be increased in both humans and animals after trauma (6, 35). Interest in a potential link between HMGB1 and intestinal dysfunction after trauma may be heightened by evidence showing that HMGB1 is involved in the determination of organ damage following hemorrhagic shock and femur fracture in mice (27, 47). Moreover, we have recently demonstrated that HMGB1 can activate TLR4 within the intestinal epithelium, in a manner that leads to intestinal barrier injury and impaired restitution (7). Taken together, these lines of reasoning raise the intriguing possibility that the release of HMGB1 after trauma within the intestinal epithelium may lead to TLR4-dependent intestinal dysfunction and bacterial translocation.

However, if TLR4 activation can lead to the development of intestinal dysfunction, then counterregulatory pathways must presumably exist to curtail the extent of TLR4 signaling and maintain intestinal mucosal homeostasis in the face of remote injury. In this regard, we (15) and others (25) have demonstrated that activation of the TLR4 homolog TLR9 with CpG-DNA limits TLR4 signaling in enterocytes and serves to reduce the extent of intestinal inflammation. These findings suggest the novel hypothesis that TLR4 activation within the intestinal epithelium after remote injury, perhaps by endogenous molecules such as HMGB1, could lead to intestinal dysfunction and bacterial translocation and that activation of TLR9 on the intestinal epithelium could reduce the degree of intestinal dysfunction that occurs.
In the present work, we now report that, in the setting of a clinically and physiologically relevant model of remote trauma, TLR4 activation within the intestinal epithelium leads to mucosal injury and bacterial translocation and that activation of TLR9 with CpG-DNA attenuates the extent of TLR4 signaling and reduces the degree of intestinal injury. The findings that HMGB1 levels are increased in the intestinal mucosa after trauma and that inhibition of HMGB1 reduces the extent of TLR4-signaling and bacterial translocation suggest a role for HMGB1 in mediating these effects. These findings provide new insights into the molecular mechanisms that mediate intestinal dysfunction after remote trauma and expand our understanding of the potentially deleterious effects that occur in response to activation of the innate immune system within the intestinal epithelium.

MATERIALS AND METHODS

Cell culture and reagents. Intestinal epithelial cell (IEC) enteroocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained as described (4, 16). Phosphothioated CpG-DNA, oligodeoxynucleotide (ODN) 1826 (TC-CATGACGTTCTCGAGCCT), was synthesized by the University of Pittsburgh DNA synthesis facility. ODNs were confirmed to be endotoxin free (<0.05 EU/ml) by Limulus assay. Antibodies were obtained as follows: TLR9: Imgenex, San Diego, CA; NF-κB (p65 subunit), TLR4 (L14), and pCNA: Santa Cruz Biotechnology, Santa Cruz, CA; cleaved caspase-3, phospho-p38-MAPK, phospho-ERK, total p38-MAPK, and total ERK: Cell Signaling Technology, Beverly, MA. Appropriate secondary antibodies for immunohistochemistry and SDS-PAGE were obtained from Molecular Probes (Eugene, OR) and Jackson ImmunoResearch (West Grove, PA), respectively, including Alexa488 (green) and Cy3 (red).

To isolate primary enteroocytes, samples of the small intestine were harvested from wild-type (strain Swiss Webster), TLR4-mutant, or TLR9-mutant mice that were generated by timed mating at ages e13.5–e15.5, considering noon on the day that a vaginal plug was observed as 0.5 days postconception. The ileum was dissected from frozen intestinal tissue was homogenized in buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 0.2 mM PMSF, and 0.5% NP-40] and incubated on ice for 15 min before being vigorously vortexed for 10 s at a maximum speed. Nuclear proteins were extracted by gently resuspending the nuclei with an appropriate volume of buffer B [20 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF] along with buffer D (identical to buffer C but has 1.6 M KCl). The ratio of buffer C to buffer D was 3 to 1. Buffer D was added in a dropwise fashion. After the nuclei were incubated with 50,000 cpm of 32P-labeled oligonucleotide for 30 min at room temperature in a reaction mixture containing 1 μg of poly(dI-dC), 10 mM Tris-HCl (pH 7.5), 10% glycerol, 1.0 mM EDTA, 1% NP-40, 1 mg/ml BSA, and 1.0 mM DTT (final volume 20 μl). The DNA protein complexes were resolved on a 4% nondenaturing polyacrylamide gel in 0.5 × Tris-borate-EDTA buffer. The gels were dried and then subjected to autoradiography.

Experimental models of trauma. All mice used in the experimental protocols were housed in accordance with University of Pittsburgh and National Institutes of Health (NIH) animal care guidelines in specific pathogen-free conditions. All experiments were approved by the Children’s Hospital of Pittsburgh Animal Care Committee and the Institutional Review Board of the University of Pittsburgh. The animals were maintained either in the University of Pittsburgh Animal Research Center and/or the Rangos Research Center of the Children’s Hospital of Pittsburgh of UPMC with a 12-h:12-h light/dark cycle and free access to standard laboratory feed and water. Male wild-type (i.e., C3H/HeJ) mice were obtained from Jackson Laboratories, Bar Harbor, ME. TLR9-mutant mice (CpG1) were generously provided by Dr. B. Beutler (The Scripps Research Institute, La Jolla, CA). All experiments were performed in 8–12- wk old males that weighted 20–30 g. All animals were fasted for ~12 h before experimental manipulation and were acclimatized for 7 days before being studied.

In all cases, mice were anesthetized, subjected to tissue trauma consisting of bilateral femur fracture in association with hemorrhagic shock, and euthanized 6 h later. Animals were anesthetized with intraperitoneal pentobarbitarial sodium (50 mg/kg) and inhaled isoflurane (Abbott Laboratories, Chicago, IL). Under sterile conditions, a left groin exploration was performed, and the left femoral artery was cannulated with tapered PE-10 tubing and connected to a blood pressure transducer (Micro-Med, Tustin, CA) for continuous mean arterial pressure monitoring for the duration of the experiment (6 h) as we have described (27). A bilateral, closed, midshaft femur fracture was then performed using two Hemostats applied to the hind-limb region. Where indicated, mice were also subjected to hemorrhagic shock as described (37). To do so, after mice recovered from the inhalational anesthesia for 10 min, blood was withdrawn to allow the mean arterial pressure to drop to 25 mmHg over 5 min, and the blood pressure was maintained at this level for 150 min. The mice were then resuscitated over 10 min with their remaining shed blood plus two times the maximal shed blood in lactated Ringer’s solution (Baxter, Deerfield, IL). Sham-operated mice underwent anesthesia and femoral cannulation only. All mice were reanesthetized with intraperitoneal pentobarbitarial sodium (20 mg/kg) as necessary throughout the experiment. At the end of 6 h (or after 4 h in cases in which mice underwent hemorrhage), mice were killed under inhalational anesthesia via cardiac puncture technique. Necropsy was performed to verify the presence of bilateral femur fractures and to ensure the absence of fracture-site hematomas. Serum from postmortem blood samples was obtained for cytokine and blood chemistry analysis. Where indicated, CpG DNA was administered 1 h before the initiation of the trauma/hemorrhagic shock model.

For analysis of bacterial translocation, mesenteric lymph nodes were harvested, weighed, and placed in a grinding tube containing 0.5 ml of ice-cold phosphate buffer saline, then homogenized with glass grinders, and a 250-μl aliquot of the homogenate was plated onto brain-heart infusion and MacConkey’s agar (Becton Dickinson, Sparks, MD). The plates were aerobically incubated at 37°C and examined at 24 h. The colonies were counted and results expressed as colony-forming units (CFU) per gram of tissue.

Where indicated, germ-free mice were purchased from Taconic (Germantown, NY; strain Swiss Webster Germ Free) at 9–10 wk of age and were housed within the germ-free shipper until use.

The extent of intestinal injury after trauma/hemorrhagic shock was quantified in histological sections of the terminal ileum by a pathologist who was blinded to the study groups and who assigned a score according to the following scale: 0, no evidence of histological injury; 1, mild injury (mild separation of enteroocytes from the lamina propria,
mild infiltration of leukocytes); 2, moderate injury (more severe epithelial disruption, edema of the lamina propria, ulceration); 3, severe injury (most severe epithelial disruption, areas of loss of villi integrity, leukocyte infiltration into lamina propria, villus separation from lamina propria). Slides were scored in triplicate; the average scores were calculated and analyzed by ANOVA with significance accepted at $P < 0.05$.

**Determination of enterocyte proliferation.** Enterocyte proliferation in vivo was measured as described (43). In brief, the terminal ilea of mice treated as described above was freshly harvested, fixed in formalin, and immunostained with antibodies to the proliferation marker pCNA as described (29). To quantify pCNA staining in the intestinal crypts, images of pCNA were digitized, regions of interest were drawn around the crypts, and the extent of pCNA expression was evaluated using ImageJ software (NIH).

**Generation and administration of adenoval constructs expressing GFP-tagged wild-type and dominant-negative TLR4.** Replication-deficient recombinant adenoviruses that express green fluorescent protein (GFP)-tagged mouse TLR4-cDNA, as well as the control adenovirus that expresses only GFP, were prepared using the Adeno-X Expression System2 kit (Clontech, Mountain View, CA) (15). In brief, the respective expression cassettes were cloned in frame with loxP sites into a donor vector, verified for the correct ligation by RT-PCR and Western blot analysis, and transferred into the E1a-deficient recombinant adenovirus that expresses GFP-tagged wild-type and dominant-negative TLR4.

The resulting adenovirus constructs were propagated into high-titer virus in permissive human embryonic kidney-293 cells. The high-titer viruses were verified again by RT-PCR and Western blot analysis also for the multiplicity of infection. Where indicated, adenoviral GFP, GFP-wild-type TLR4, or GFP-mutant TLR4 twice were administered daily for 3 days to mice before the induction of the trauma model (240 μl, 10^7 plaque-forming units). The expression of GFP in mucosal scrapings of the intestine, as well as the lung and liver, was assessed by RT-PCR and found to be restricted to the intestinal mucosa as described (15).

**Immunohistochemistry, immunofluorescence, and SDS-PAGE.** The immune analysis of cultured enterocytes of mouse and human intestine was performed as previously described (5, 24) and evaluated using an Olympus Fluoview 1000 confocal microscope under oil-immersion objectives. Images were assembled using Adobe Photoshop CS2 software (Adobe Systems, San Jose, CA). In parallel, Cryo-Gel (Cancer Diagnostics, Birmingham, MI) frozen sections of terminal ileum were sectioned (4 μm), rehydrated with PBS, and fixed with 2% paraformaldehyde. Nonspecific binding was blocked with 5% BSA. Sections were evaluated on an Olympus Fluoview 1000 confocal microscope using oil-immersion objectives as described above.

For assessment of NF-κB activation (translocation), primary enterocytes or IEC-6 cells were treated with LPS (50 μg/ml; Sigma-Aldrich, St. Louis, MO) and/or DNA (1 μM) either alone or in combination for 1 h and immunostained with antibodies against the p65 subunit of NF-κB. The extent of nuclear translocation was determined in an adaptation of the methodology of Yu and colleagues (48). In brief, a threshold limit was set on the basis of the emission signal for the nuclear stain DRAQ5, which therefore defined a nuclear signal for the nuclear stain DRAQ5, which therefore defined a nuclear region of interest, a circular region 12 pixels beyond the nucleus was stenciled upon each cell. The average integrated pixel intensity pertaining to the corresponding NF-κB expression within the cytoplasmic and nuclear regions was then determined for more than 200 cells per treatment group in at least four experiments per group, using MetaMorph software version 6.1 (Molecular Devices, Downingtown, PA).

Where indicated, the extent of apoptosis was quantified in vitro and in vivo as we have done previously (23) using the apoptosis marker cleaved caspase-3, then enumerating the number of caspase-3-positive cells as a percentage of the total number of cells present. At least 100 fields were assessed for each experimental group where indicated.

**RESULTS**

Remote trauma leads to TLR4 activation in the intestinal mucosa and mucosal disruption. In the present study, we seek to test the hypothesis that remote traumatic injury, as induced by the combination of femur fracture and hemorrhage-induced systemic hypotension, can lead to local injury to the intestinal mucosa in a TLR4-dependent manner, and furthermore, that the release of the endogenous TLR4 ligand HMGB1 may play a role in the induction of TLR4 signaling and intestinal mucosal dysfunction. To test this directly, adult male mice were subjected to a model of tissue trauma/hemorrhagic shock as described in MATERIALS AND METHODS, whereas control animals were exposed to the anesthetic agent and arterial cannulation alone. As is shown in Fig. 1, whereas the architecture of the terminal ileum is well preserved in control animals (Fig. 1, A and D), mice that were subjected to experimental trauma and hemorrhagic shock demonstrated a marked disruption of the small intestinal mucosa, characterized by the infiltration of inflammatory cells into the lamina propria, edema of the submucosa, and epithelial cell loss leading to mucosal disruption (Fig. 1, B and D). In seeking to define the mechanisms that could mediate this effect, we turned first to a potential role for TLR4, which has been implicated in the initiation of organ dysfunction after remote injury in other systems (28, 32).

Importantly, the expression of TLR4 protein was significantly increased in mucosal scrapings derived from the terminal ileal mucosa from injured compared with control animals (Fig. 1E). Where indicated, gene expression was assessed on 2.5% agarose gels using ethidium bromide staining. Images were obtained with a Kodak (New Haven, CT) Gel Logic 100 Imaging System using Kodak Molecular Imaging software. Where further indicated, expression of the gene of interest as a ratio of the housekeeping gene β-actin is demonstrated.

**Remote Trauma Blocks TLR4-Mediated Intestine Dysfunction After Trauma**

SDS-PAGE was performed as previously described (4). Blots were developed using the enhanced chemiluminescence reagent (ECL-Plus; Pierce, Rockford, IL) and developed on radiographic film.

**PCR.** Quantitative real-time PCR in cultured enterocytes and intestinal tissue using the Bio-Rad iCycler (Bio-Rad, Hercules, CA) was performed as previously described (23) using the following primer sequences: mouse IL-6: sense: CCAATTTTCAATCTGCTCCT, antisense: ACCA- CAGTGGAAAATGTCGA (182 bp); mouse TLR9: sense: TATCCAC- CACCTGACCAACT, antisense: TTCACTCTCCATCCGTAGAC (165 bp); intestinal alkaline phosphatase sense: TCGCCCCATCTA- CTCTCCAACA, antisense: ATGCTGAAATGACTGCTGATCGGT; villin: sense: AAATTTGAGCTCGCCGATCTAAG, antisense: GGCCAGGCTCAAGTGTTTCC. For studies in IEC-6 cells, the following rat primers were used: TLR9 (sense: CTACGCTTTGTCTG- GAGGA, antisense: AGACAAACAGAGACTCCTTGCG, 101 bp) and rat β-actin (sense: TGTGTCAAGCTAGTCAAGGAAAG, antisense: CAGT- GAGGCCCCAGATAGACG, 145 bp). Gene expression was normalized to β-actin expression (mouse-specific primer sequences: sense: CCA- CAGCTGAGGGGAAATC; antisense: TCTCCGGAGGAGAGAG- GAT). Where indicated, gene expression was assessed on 2.5% agarose gels using ethidium bromide staining. Images were obtained with a Kodak (New Haven, CT) Gel Logic 100 Imaging System using Kodak Molecular Imaging software. Where further indicated, expression of the gene of interest as a ratio of the housekeeping gene β-actin is demonstrated.

**Statistical analysis.** Data presented are means ± SE, and comparisons are by two-tailed Student’s t-test or ANOVA, with statistical significance accepted for $P < 0.05$. All experiments were repeated in at least triplicate, with at least five mice per group where indicated.
that bear an inhibitory mutation in TLR4 were subjected to experimental trauma and assessed for the degree of mucosal dysfunction, intestinal mucosal NF-κB activation, and bacterial translocation. As shown in Fig. 1, TLR4-mutant mice demonstrated histological evidence of reduced mucosal injury (Fig. 1, C and D), decreased NF-κB activation in the intestinal mucosa as determined by EMSA (Fig. 1G), and reduced bacterial translocation to mesenteric lymph nodes compared with wild-type mice (Fig. 1H). It is noteworthy that bacterial translocation was not observed in wild-type mice that were fed sterile food and maintained under conditions favoring a sterile gastrointestinal tract, suggesting the enteric source of the bacteria that were cultured from the mesenteric lymph nodes. Taken together, these findings strongly argue that remote trauma in association with hemorrhagic shock leads to TLR4 signaling in the intestinal mucosa, resulting in barrier dysfunction and bacterial translocation. On the basis of these findings, we therefore next sought to further define the cell types that mediated the TLR4 signaling response and to then explore the role, if any, of the endogenous ligand HMGB1 in mediating this effect.

Fig. 1. Remote trauma leads to Toll-like receptor 4 (TLR4) activation in the intestinal mucosa and mucosal disruption. A–C: hematoxylin and eosin micrographs showing the terminal ileum of wild-type mice subjected to either sham (A) or trauma (B), or TLR4-mutant C3H/HeJ mice subjected to trauma (C). Representative of 5 separate experiments with over 5 mice per experiment. Size bar = 100 μm. D: quantification of mucosal injury as described in MATERIALS AND METHODS. E and F: SDS-PAGE showing the expression of TLR4 in the intestinal mucosa of wild-type mice subjected to either sham or trauma conditions as described in MATERIALS AND METHODS and probed for the indicated protein. The quantification of protein density relative to β-actin is shown, *P < 0.05, representative of 5 separate experiments with over 5 mice per experiment. G: nuclear extracts obtained from small intestinal mucosa subjected to NF-κB-EMSA showing increased binding to NF-κB in C3H/HeOUJ (OUJ) mice after trauma compared with wild-type mice. H: extent of bacterial translocation to the mesenteric lymph nodes in wild-type, TLR4-mutant mice, and germ-free wild-type mice subjected to sham or trauma as described in MATERIALS AND METHODS. *P < 0.05 vs. control, **P < 0.005 vs. wild-type mice subjected to trauma; representative of 4 separate experiments with over 5 mice per group.
Trauma-induced TLR4 signaling in enterocytes leads to intestinal inflammation. We (23, 43) and others (11, 18, 22, 30, 46) have recently demonstrated that small intestinal epithelial cells express TLR4 and that TLR4 activation within enterocytes leads to epithelial barrier disruption. On the basis of this prior work and in view of the findings of Fig. 1, we next sought to determine whether TLR4 contributes to epithelial barrier injury after remote trauma by signaling on enterocytes or nonepithelial cells. To do so, we engineered and administered by enteral gavage adenoviruses that expressed either GFP alone, GFP-tagged wild-type, or dominant-negative TLR4 bearing the P712H mutation found in the C3H/HeJ mouse (36). When expressed in IEC-6 enterocytes in culture, the subcellular distribution of GFP-tagged wild-type TLR4 and dominant-negative TLR4 were found in a pattern that mimics endogenous TLR4 (34) as revealed by immunoconfocal microscopy (Fig. 2, A–C). Importantly, dominant-negative TLR4 inhibited TLR4 signaling in enterocytes, as demonstrated by the fact that IEC-6 cells infected with dominant-negative TLR4 showed reduced LPS-mediated phosphorylation of p38 (Fig. 2D) and markedly reduced LPS-mediated NF-κB translocation compared with IEC-6 cells that were infected with wild-type TLR4 (Fig. 2E) and adenoviral GFP. As shown in Fig. 2F, the in vivo expression of GFP after enteral administration of these adenoviruses was predominantly localized to the intestinal epithelium. Strikingly, the ectopic expression of dominant-negative TLR4 in the small intestine led to a significant reduction in the extent of IL-6 (Fig. 2G) and inducible nitric oxide synthase (iNOS) (Fig. 2H) expression in the intestinal mucosa of mice subjected to trauma/hemorrhagic shock compared with control mice subjected to trauma and administered adenoviral GFP. These two molecules were measured because they are proinflammatory cytokines whose expression levels correlate with the degree of mucosal injury in a variety of trauma models (13, 14). It is noteworthy that the administration of adenoviral wild-type TLR4 resulted in a marked increase in the expression of the proinflammatory molecules IL-6 and iNOS compared with GFP, providing further evidence for the role of enterocyte TLR4 in mediating the proinflammatory response that occurs in the intestine after remote trauma. There was no significant effect of transfection of GFP, wild-type, or dominant-negative TLR4 on baseline NF-κB activation (see solid bars in Fig. 2, G and H). On the basis of these findings, we next sought to determine the potential signaling molecules involved and focused on the endogenous TLR4 ligand HMGB1.

Intestinal mucosal TLR4 signaling in response to HMGB1 leads to intestinal barrier disruption after remote trauma. Having demonstrated that enterocyte TLR4 signaling is required for the initiation of intestinal epithelial disruption after remote trauma, we next sought to determine potential endogenous ligands involved. To do so, we explored a role for HMGB1, a DNA-binding protein that may be released from injured cells and that has been shown to be a potent activator of TLR4 (10, 48). Although a role for HMGB1 in the pathogenesis of intestinal injury after trauma remains unexplored, we have recently demonstrated that HMGB1 may activate TLR4 in enterocytes leading to decreased restitution via increased RhoA/focal adhesion kinase signaling (7). As is shown in Fig. 3A, remote trauma led to an increase in the expression of HMGB1 protein in the small intestinal mucosa in wild-type mice compared with uninjured animals. Such an increase in HMGB1 occurred to a reduced degree in TLR4-mutant mice, suggesting that TLR4 signaling may be required for the release of HMGB1 in the intestinal mucosa (Fig. 3A, see densitometry). To confirm the HMGB1 expression data that were obtained by SDS-PAGE, we harvested samples from the ileum of TLR4 wild-type and TLR4-mutant mice and subjected the tissues to immunoconfocal microscopy using antibodies against HMGB1. As shown in Fig. 3B, after trauma there was a significant increase in the expression of HMGB1 in the intestinal mucosa (green) that was not seen in mice that do not undergo trauma, nor at any time in TLR4-mutant mice. These data confirm the results from SDS-PAGE and indicate that trauma results in the expression of HMGB1 in the intestinal trauma in a TLR4-dependent manner. There was no induction of HMGB1 in the intestinal mucosa of wild-type mice subjected to shock alone (not shown). Importantly, HMGB1 was found by our group recently to contribute to intestinal epithelial barrier dysfunction after remote trauma via TLR4 signaling, as the administration of inhibitory antibodies to HMGB1 significantly attenuated after pretreatment with inhibitory antibodies to HMGB1 the extent of remote trauma-induced NF-κB activation in the ileal mucosa in TLR4-expressing mice, yet had no effect in TLR4-mutant mice (28). The physiological relevance of these findings is seen in the fact that remote trauma led to an increase in bacterial translocation to mesenteric lymph nodes in wild-type mice that was significantly attenuated after pretreatment with inhibitory HMGB1 antibodies, which did not occur in TLR4-mutant mice (Fig. 3C). Taken together, these findings raise the possibility that HMGB1 signaling may lead to NF-κB activation and mucosal disruption leading to bacterial translocation after trauma, in part by signaling through TLR4. On the basis of these findings, we next considered the potential mechanisms that could limit the extent of TLR4 signaling in the small intestine after remote trauma.

DNA activation of TLR9 inhibits TLR4 signaling in primary enterocytes and reduces intestinal mucosal injury after remote trauma. To investigate potential factors that could limit the extent of TLR4-induced intestinal dysfunction after remote trauma, we next turned our attention to a potential role for DNA, which we have recently shown to limit TLR4 signaling in the newborn intestinal mucosa when administered exogenously (15). Evidence that DNA activation of TLR9 limits TLR4 signaling in primary enterocytes is shown in Fig. 4. In wild-type primary IEC compared with untreated cells (Fig. 4A), LPS significantly increased the translocation of NF-κB from the cytoplasm to the nucleus (Fig. 4B), which was markedly reduced after treatment with the TLR9 ligand CpG-DNA (Fig. 4C) and did not occur in primary enterocytes from TLR4-mutant mice (Fig. 4D). It is noteworthy that CpG-DNA did not prevent the LPS-mediated translocation of NF-κB in cells derived from TLR9-mutant mice (Fig. 4E), confirming that TLR9 is the receptor on these primary enterocytes that mediates the inhibitory effects of CpG-DNA. In additional controls, the addition of DNA did not affect the extent of bacterial translocation in enterocytes from TLR4-mutant mice in which there was no translocation of NF-κB (noted in Fig. 4F). Furthermore, in enterocytes obtained from the TLR9 wild-type mouse (i.e., C57BL6), LPS-treated enterocytes show marked nuclear translocation of NF-κB, whereas the addition of DNA significantly reversed the extent of NF-κB translocation in this
Fig. 2. Trauma-induced TLR4 signaling in enterocytes leads to intestinal inflammation. A–C: confocal micrographs showing green fluorescent protein (GFP) emission in intestinal epithelial cell (IEC)-6 cells that were infected with adenoviruses expressing either GFP (A), GFP-wild-type (wt) TLR4 (B), or GFP-mutant TLR4 (C). Size bar = 10 μm. D, i: SDS-PAGE of IEC-6 cells infected with GFP, wild-type or mutant TLR4 and treated with LPS, then blotted with antibodies to phospho-p38, then stripped and reprobed with antibodies to F-actin. Representative of 3 separate experiments. Densitometry is shown (D, ii), *P < 0.05 vs. control in each case shown. E: extent of NF-κB translocation as quantified in MATERIALS AND METHODS in IEC-6 cells expressing either GFP, wild-type, or dominant negative (dn) TLR4, then treated with either medium (ctrl) or LPS. *P < 0.05 vs. control-treated wild-type TLR4-transfected cells; **P < 0.01 vs. LPS-treated wild-type TLR4-transfected cells. Representative of 5 separate experiments with over 100 cells per high-power field examined. These cells were infected with 25 μl of the indicated adenovirus in each single well of 6-well plates (35-mm2 surface area) at a titer of 1×1012 for 48 h before analysis F: confocal micrographs of the terminal ileum of mice that had been treated with saline (i) administered by gavage adenoviruses expressing either GFP (ii), GFP-dominant-negative TLR4 (iii), or GFP-wild-type TLR4 (iv). Green = GFP, blue = nuclei, red = actin. Size bar = 100 μm. Representative of over 100 similar images in 3 separate experiments. Note the predominantly enterocyte expression of the GFP. In these studies, mice were administered by enteral gavage the indicated adenovirus (250 μl per 30-g mouse, twice a day for 4 days, at 1×1012 for 72 h before induction of the trauma model). G and H: expression by RT-PCR of IL-6 (G) or inducible nitric oxide synthase (iNOS) (H) in the small intestinal mucosa of mice that were administered the indicated adenovirus corresponding to F, then subjected to either sham (solid bars) or trauma (hatched bars) as indicated. *P < 0.05 vs. control; †P < 0.001 vs. GFP; ‡P < 0.005 vs. TLR4-wild-type. Representative of 4 separate experiments with over 4 mice per experiments.
wild-type strain (Fig. 4, G–I). These findings, which are quantified in Fig. 4J, are consistent with our recent report showing that DNA can inhibit TLR4 signaling in the nontransformed enterocyte cell line IEC-6 (15) and therefore led us to investigate whether DNA activation of TLR9 could regulate TLR4-induced intestinal mucosal injury after remote trauma. Of note, whereas TLR4 expression was increased in the intestinal mucosa after remote trauma (see Fig. 1D), the expression of TLR9 was significantly reduced (Fig. 5A), suggesting the possibility that a reduction in TLR9 signaling may be associated with the development of intestinal mucosal injury after remote trauma. In support of this possibility, mice with mutations in TLR9 (CpG1 mice) demonstrated significantly increased intestinal inflammation after remote trauma compared with wild-type mice, as illustrated by more severe histological disruption of the ileal mucosa (Fig. 5C) and increased release of the proinflammatory cytokine IL-6 (Fig. 5B), two findings that both were reversed in TLR4-mutant mice (Fig. 5, B and C). Taken along with the findings of Fig. 4, these results strongly argue in favor of a salutary role for TLR9 activation in maintaining mucosal homeostasis of the small intestine after remote trauma. There was no effect on the expression of TLR4 or TLR9 in the intestinal mucosa, or of mucosal histology, after hemorrhagic shock alone for the time period studied (6 h). In additional controls, it can be seen that the intestinal mucosa in TLR4-mutant and TLR9-mutant mice before trauma appears similar to the respective wild-type mice (Fig. 5C).

DNA activation of TLR9 reverses the deleterious effects of TLR4 activation on small intestinal mucosal injury and repair after remote trauma. We (23) and others (20, 39) have recently shown that activation of TLR4 on the small intestine leads to the induction of enterocyte apoptosis and to reduced enterocyte proliferation and restitution, which in aggregate lead to intestinal mucosal injury. On the basis of these findings, we next sought to investigate in greater detail the mechanisms contributing to TLR4-induced dysfunction of the intestinal mucosa after remote trauma and to define whether TLR9 activation could reverse such processes. As shown in Fig. 6, compared with control mice (Fig. 6A), the exposure of animals to remote...
trauma and hemorrhage led to an increase in enterocyte apoptosis in the terminal ileum (Fig. 6B) that was significantly reduced after treatment with DNA (Fig. 6C). The increase in enterocyte apoptosis that was induced in response to remote trauma required TLR4 signaling in enterocytes, as the extent of enterocyte apoptosis after remote trauma was markedly reduced after expression of dominant-negative TLR4 in enterocytes and increased after the expression of wild-type TLR4 in enterocytes (Fig. 6G). The finding that TLR4 activation in the intestine after remote trauma led to an increase in mucosal injury suggested the possibility that TLR4 activation could also adversely affect mucosal repair, which typically occurs through the combined effects of enterocyte proliferation and migration (13). In support of this possibility, compared with control mice (Fig. 6D), remote injury caused a significant decrease in the extent of enterocyte proliferation (Fig. 6E) that could be reversed by the administration of CpG-DNA (Fig. 6F) and required TLR4 activation in enterocytes (Fig. 6H). Furthermore, remote injury also caused a marked decrease in enterocyte migration, which was reversed after the administration of CpG-DNA (Fig. 6I). Finally, by reducing the extent of TLR4-mediated enterocyte apoptosis, as well as restoring enterocyte proliferation and migration to baseline levels, the administration of exogenous DNA to mice subjected to remote trauma was found to maintain the histological appearance of the small intestine to levels that resembled that of uninjured mice (Fig. 6, J–L). Taken together, these findings demonstrate that remote trauma leads to intestinal dysfunction through the activation of TLR4 on enterocytes that can be reversed through the activation of TLR9 with DNA.
DISCUSSION

In the present study, we provide evidence that remote trauma leads to activation of TLR4 within enterocytes and that this activation leads to mucosal injury. We also provide evidence that HMGB1, known to be released from injured cells and a potent endogenous TLR4 ligand (13), may contribute to this response, as levels of HMGB1 were elevated in the intestinal mucosa after trauma, and inhibition of HMGB1 in vivo using selective antibodies attenuated the extent of NF-κB activation and bacterial translocation after trauma. Furthermore, and perhaps most significantly, we now provide evidence that exogenous DNA can attenuate the effects of TLR4-mediated intestinal dysfunction by reversing the extent of TLR4 signaling and thus restoring intestinal barrier integrity. Taken together, these findings provide insights into the mechanisms by which the host seeks to maintain intestinal integrity after remote injury.

What is the potential teleological basis to explain how activation of TLR4 on the intestinal epithelium can have such deleterious consequences to the host? We have sought to address this important issue in broad phylogenetic terms. We now presume that the presence of TLRs on all multicellular organisms, from worms through flies to humans, confirms the simple efficiency with which this family of receptors can protect the host from potential pathogens. Such protection is achieved through the recognition of discrete molecular components of microbial agents, rather than through the identification of particular virulent properties. However, in this simplicity lies the stark vulnerability of the system, as demonstrated by the activation of TLRs by endogenous molecules such as HMGB1 in an apparent example of molecular mimicry. Such a theme, in which the host inexplicably turns against itself under pathological states, is consistent with the development of various autoimmune diseases, some of which have been linked to early injury (ex lupus, arthritis). We now submit that, although the development of an aberrant “autoimmune” response at the level of the intestinal mucosa to remote injury represents a novel finding, it is not without precedent in other disease states that have been ascribed to aberrant activation of the host immune system.

A major finding of the present study involves the observation that exogenous DNA serves a protective role in maintaining intestinal epithelial barrier integrity in the face of remote trauma by inhibiting TLR4 signaling in enterocytes. This finding both supports and extends our previous observation that the reciprocal nature of TLR4/TLR9 expression and signaling combine to determine that extent of TLR4 signaling that occurs in the neonatal intestine (15) and provides insights into the mechanisms by which the host seeks to maintain intestinal...
mucosal homeostasis after remote injury. It is noteworthy that, although Zhang’s group (50) have shown that levels of mitochondrial DNA are in fact increased in the circulation after trauma, it is not clear as to whether levels of CpG-DNA are also increased or indeed whether such molecules could serve a potential beneficial role. The fact that the systemic administration of CpG-DNA has been shown by ourselves and others to lead to the activation of macrophages leads us to speculate that circulating DNA may in fact serve two roles: as a proinflammatory stimuli to alert the host immune cells to the presence of injury, and as an anti-inflammatory agent that acts to limit the extent of TLR4-mediated bacterial translocation and prevent the otherwise unbridled release of additional microbial DNA. It is noteworthy that both the expression and function of TLR9 were found to be increased in B cells of severely injured patients compared with controls (1), whereas the inhibition of TLR9 conferred protection from liver injury in a model of ischemia/reperfusion (2). We have recently shown that TLR9 contributes to liver damage in experimental hemorrhagic shock and tissue trauma, supporting the notion that there are organ-specific roles for TLR9 (R. Gill and T. Billiar, unpublished observations). This suggests that the ongoing surveillance of DNA by the host leads to deleterious effects that must be circumvented within the organ that is exposed to the greatest concentration of microbial products, namely the intestinal epithelium. Although we now demonstrate that the administration of exogenous DNA leads to intestinal mucosal protection after remote injury, we cannot be completely certain of the site at which this protective action occurs, i.e., enterocytes vs. intestinal macrophages. Further studies using targeted deletion of TLR9 from intestinal macrophages vs. enterocytes will be required to resolve this issue and are now ongoing.

We readily acknowledge that, although the present studies provide evidence that TLR4 activation in the intestinal epithelium after remote trauma has deleterious effects on enterocyte
migration, proliferation and barrier integrity, previous authors have shown a requirement for enterocyte TLR4 in the maintenance of intestinal mucosal homeostasis (2, 38). In addressing this apparent controversy, prior works that have led to the conclusion that TLR4 is required for mucosal homeostasis have largely been performed in models of colonic inflammation involving the local administration of epithelial disrupting agents, in which no attempt to selectively delete TLR4 from the enterocytes has been previously made (12). It is therefore reasonable to conclude that the protective effects attributed to TLR4 signaling in the gut by others may in fact reflect in part the mitigating effects of TLR4 signaling on other cells. In support of this possibility, we note that Fukata et al. (12) have used chimeric mice to demonstrate that TLR4 activation in colonic epithelial cells worsened intestinal inflammation, whereas at least three groups have shown that at least four different TLR4 antibodies can suppress the extent of DSS-induced colitis (11, 30, 46). Although we do not fully understand the physiological consequences of the bacterial translocation that we measure and report in the present study, in as much as bacterial translocation provides a measure of impaired epithelial barrier integrity, it is apparent from the present work that TLR4 activation within the intestine is deleterious to the host after trauma.

On the basis of these findings, we now propose the following model to explain the mechanisms leading to intestinal dysfunction after trauma/hemorrhagic shock. As shown in Fig. 1, remote injury leads to activation of TLR4 within the intestine and release of HMGB1 within the intestinal mucosa. These have the combined effects of leading to reduced proliferation and migration, resulting in bacterial translocation, effects that can be restored in TLR4-mutant mice or with anti-HMGB1 antibodies. In parallel, the release of exogenous DNA (shown to be increased after trauma, unpublished observations) leads to activation of TLR9 and a protection from TLR4-induced injury, leading to a restoration of the mucosal barrier. It remains an open question as to whether the release of HMGB1 after remote trauma may serve to bind DNA in the circulation as much as it does within cells, and if so, whether a HMGB1-DNA complex could alter the interaction with TLR9, although we note that such HMGB1-DNA complexes have been identified in models of inflammation with deleterious effects on host immunity (44). We also readily acknowledge that TLR4 activation on enterocytes is likely to occur in association with TLR4 activation on other cells and that the combined downstream signaling responses of various TLR4-expressing cells are likely to determine the nature and extent of intestinal mucosal injury that occurs in the setting of remote trauma. It is now our hope that these molecular insights will provide the rationale for potential therapeutic approaches in the care of injured patients, with a focus on restoring the intestinal barrier and protecting against intestinal-mediated systemic sepsis that causes long-term morbidity in the injured patient.

ACKNOWLEDGMENTS

The authors acknowledge the technical expertise of Debra Williams, who assisted greatly in the performance of the trauma models.

GRANTS

This work was supported by 1R01-GM078238-01 (to D. Hackam), RO1DK083752 (to D. Hackam), D43TW007560 (to T. Billiar), RO1GM050441 (to T. Billiar), and P50GM053789 (to T. Billiar and D. Hackam) from the National Institutes of Health and The Hartwell Foundation, Memphis, Tennessee to D. Hackam. M. Neal is supported by the American College of Surgeons Research Fellowship.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


