TLR4-mediated Cox-2 expression increases intestinal ischemia/reperfusion-induced damage

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ABSTRACT

Mesenteric IR induces significant inflammation and immune-mediated mucosal damage. TLR4 is a critical receptor in the induction of the inflammatory response and plays a role in intestinal homeostasis. To determine the role of TLR4 in IR-induced epithelial damage, we performed IR studies using TLR4lox/lox and TLR4lox/− mice and analyzed mucosal damage and inflammation. We found that the absence of TLR4 or TLR4-induced signaling attenuated local mucosal damage with significantly decreased cytokine and eicosanoid secretion including PGE2 production. Similar results were seen in MyD88−/− mice. Wild-type mice treated with NS-398 (a Cox-2 inhibitor) not only decreased PGE2 production but also attenuated tissue damage. In contrast, PGE2 was not sufficient to induce damage in the TLR4lox/lox mice. Together, these data indicate that TLR4 stimulation of Cox-2 activation of PGE2 production is necessary but not sufficient for intestinal IR-induced damage and inflammation. J. Leukoc. Biol. 86: 971–980; 2009.

Introduction

Ischemia, the lack of blood and oxygen to an organ, and the subsequent return of oxygenated blood during reperfusion result in significant pathology in clinical conditions including myocardial infarction and stroke. Cellular alterations, some of which may have occurred during ischemia, are recognized by the innate immune system during the reperfusion phase, resulting in significant damage to the organ. Although less well known, mesenteric IR-induced injury remains an inflammatory condition with high mortality rates, likely as a result of a lack of early diagnosis [1–3]. Sequelae of intestinal IR not only include local tissue damage but frequently progress to systemic inflammatory responses and subsequent multiple organ failure [4].

Under normal conditions, the intestinal epithelium forms a barrier preventing pathogenic bacterial infections, while allowing the presence of commensal bacteria. The mechanism of this distinction is unknown but may involve TLRs. As pattern recognition receptors, TLRs recognize specific bacterial components to induce an inflammatory response. Specifically, in conjunction with CD14 and myeloid differentiation protein 2, TLR4 recognizes LPS in the cell wall of gram-negative bacteria [5–8]. Most TLRs, including TLR4, initiate intracellular signaling pathways involving MyD88 to induce NF-κB translocation, resulting in the up-regulation of proinflammatory cytokine production [5, 6]. Expressed by epithelial cells and intestinal macrophages, tight regulation of TLR4 expression is critical to maintaining the intestinal epithelium’s tolerance to commensal LPS [9–11]. Endogenous ligands can also activate TLR4 to induce inflammation in the absence of a pathogen. Cellular debris and extracellular matrix degradation products such as hyaluronic acid, fibronectin, fibrinogen, and heparin sulfate can induce “sterile inflammation” through TLR4 [12, 13], and TLR4 activation by an endogenous ligand appears to have a role in heart, kidney, and liver IR events (reviewed in ref. [12]). It is unknown whether TLR4 has a role in mesenteric IR.

The intestine also produces significant quantities of the eicosanoid, PGE2. Produced following Cox conversion of arachidonic acid, fibronectin, fibrinogen, and heparin sulfate can induce PGE2. Produced following Cox conversion of arachidonic acid to PGG2, PGE2 is a vasodilator, which causes edema in intestinal IR. Constitutively active, Cox-1 maintains mucosal homeostasis within the intestine, and Cox-2 is up-regulated during inflammation [14]. However, a deficiency of either Cox gene does not result in mucosal damage, suggesting redundancy between Cox-1 and Cox-2 activity [15]. Although the Cox-mediated production of PGG2 leads to PGE2 during intestinal IR, PGG2 also results in production of the pulmonary vasoconstrictor TxA2, which may increase tissue damage [16]. Thus, during tissue damage, Cox-2 activation may induce the vasodilating effects of PGE2 and/or increased vasoconstriction as a result of TxA2.
Recently, in a long-term mouse model of inflammatory bowel disease, TLR4 was found to mediate PGE2 production by regulation of Cox-2 [10, 11]. In addition, TLR4 is critical for colonic epithelial repair through PGE2 production [10, 11, 17, 18]. In contrast, TLR4 is instrumental in the induction of IR-induced kidney and heart damage [19–22]. However, these studies did not examine the involvement of PGE2 in the tissue damage. Therefore, we hypothesized that TLR4 and Cox-2-mediated production of PGE2 may be responsible for the induction and/or repair of intestinal IR-induced mucosal damage. To test this hypothesis, TLR4lps-def, TLR4lps-n (B10/ScN/J and C3H/HeJ, respectively), MyD88−/−, Trif−/−, and appropriate wild-type control mice were subjected to ischemia and subsequently, reperfused for 2 h prior to evaluating the extent of mucosal injury and PGE2 production. We show that compared with wild-type control animals, the intestinal mucosal damage is attenuated in both strains of TLR4-altered mice and compared with wild-type control animals, the intestinal mucosal damage is attenuated in both strains of TLR4-altered mice and in MyD88−/− mice in response to IR with a corresponding decrease in Cox-2-mediated PGE2 production. However, PGE2 is not sufficient to restore mucosal injury in TLR4lps-def mice.

MATERIALS AND METHODS

Mice

C3H/HeJ, C3HlscN/J (TLR4lps-def), C57Bl/10, B10/ScN/J (TLR4lps-def), Rag-1−/−, Trif−/−, and C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) or bred at KSU (Manhattan, KS, USA) and housed in the KSU Division of Biology rodent facility. MyD88−/− and C3H/HeJ, respectively), MyD88−/−/H11002, Trif−/−/H11002, and appropriate wild-type control mice were subjected to ischemia and subsequently, reperfused for 2 h prior to evaluating the extent of mucosal injury and PGE2 production. We show that compared with wild-type control animals, the intestinal mucosal damage is attenuated in both strains of TLR4-altered mice and in MyD88−/− mice in response to IR with a corresponding decrease in Cox-2-mediated PGE2 production. However, PGE2 is not sufficient to restore mucosal injury in TLR4lps-def mice.

Experimental protocol

Animals were subjected to IR, as described previously [23]. Briefly, a midline laparotomy was performed on anesthetized mice, the superior mesenteric artery was identified and isolated, and a small vascular clamp (Roboz Surgical Instruments, Gaithersburg, MD, USA) was applied for 30 min. Sham animals were subjected to the same surgical intervention without superior mesenteric artery occlusion. After clamp removal, the incisions were sutured, and animals were monitored during the 2-h reperfusion period. Additional ketamine and xylazine were administered by I.P. injection immediately before euthanasia. After euthanasia, the small intestine, 10–20 cm distal to the gastroduodenal junction, was removed for subsequent analysis. Intestinal sections were snap-frozen in liquid nitrogen and stored at −80°C until homogenized in Trizol (Invitrogen, Carlsbad, CA, USA) and RNA, extracted using the manufacturer’s protocol. RNA concentration was quantified by spectrophotometry, and 1 ug RNA was reverse-transcribed with the Superscript III First-Strand synthesis kit (Invitrogen). The cDNA was subjected to PCR with the primers listed in Table 1 [24]. Each sample was normalized to the expression of GAPDH in that sample. The mean fold increase and SEM were calculated from Image J (National Institutes of Health, Bethesda, MD, USA) analysis.

Histology and injury scoring

Immediately after euthanasia, 2 cm segments of small intestine specimens were fixed in 10% buffered formalin embedded in paraffin, and 8 μm sections were cut transversely and H&E-stained. Mucosal injury (mucosal injury score) was graded on a six-tiered scale adapted from Chiu et al. [25], as described previously [23]. Briefly, the average damage score of an ~2-cm section of mid-jejunum intestine (75–150 villi) was determined after grading each villus from 0 to 6. Normal villi were assigned a score of zero; villi with tip distortion were assigned a score of 1; score 2 was assigned when Guggenheims’ spaces were present; villi with patchy disruption of the epithelial cells were assigned a score of 3; score 4 was assigned to villi with exposed but intact lamina propria with epithelial sloughing; a score of 5 was assigned when the lamina propria were exuding; and last, villi, which displayed hemorrhage or were denuded, were assigned a score of 6. Using the same slides, the ratio of the villus height and the crypt depth was determined using Metavis software and a 20X objective, which had been calibrated with a slide micrometer.

Ex vivo peroxidase and eicosanoid determination

Intestinal ex vivo eicosanoid generation was determined, as described previously [25]. Briefly, immediately after collection, 2 cm mid-jejunum sections were minced, washed, and resuspended in 37°C-oxygenated Tyrode’s buffer (Sigma-Aldrich, St. Louis, MO, USA). Supernatants were collected after incubating for 20 min at 37°C and stored at −80°C. The concentration of

Cox-1 and Cox-2 mRNA expression

Intestinal sections were snap-frozen in liquid nitrogen and stored at −80°C until homogenized in Trizol (Invitrogen, Carlsbad, CA, USA) and RNA, extracted using the manufacturer’s protocol. RNA concentration was quantified by spectrophotometry, and 1 ug RNA was reverse-transcribed with the Superscript III First-Strand synthesis kit (Invitrogen). The cDNA was subjected to PCR with the primers listed in Table 1 [24]. Each sample was normalized to the expression of GAPDH in that sample. The mean fold increase and SEM were calculated from Image J (National Institutes of Health, Bethesda, MD, USA) analysis.

NS-398 and PGE2 treatments

In some experiments, mice were treated with an inhibitor of Cox-2 activation or with exogenous PGE2. A total of 20 mg/kg Cox-2 inhibitor, NS-398, was dissolved in DMSO, diluted appropriately, and administered in two doses at 18 h and 4 h prior to ischemia. Control mice received DMSO diluted similarly in the absence of NS-398. The final concentration of the inhibitor in the mouse was ~0.8 μM, which is well within the Cox-2-specific range of 0.1–1 μM. As expected, treatment did not alter Cox-1 expression, which requires 75–220 μM (data not shown). PGE2 (40 ng/kg; Cayman Chemical, Ann Arbor, MI, USA) was diluted and injected intraluminally into the lower duodenum 15 min prior to ischemia.

TABLE 1. PCR Primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Temp</th>
<th>Cycles</th>
<th>Sequence (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>Cox-2</td>
<td>54°C</td>
<td>40</td>
<td>Forward ATC CTG CCA GCT CCA CCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse TGG TCA AAT CCT GTG CTC ATA CAT</td>
</tr>
<tr>
<td>Cox-1</td>
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<td>35</td>
<td>Forward AAG GAG TCT CTC GCT GTG TGT T</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse TCT GAG GGA TGG TAC AGT TGG G</td>
</tr>
<tr>
<td>GAPDH</td>
<td>54°C</td>
<td>32</td>
<td>Forward CCA TGG AGA AGG CCG GGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reverse CAA AGT TGT CAT GGA TGA CC</td>
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total peroxidase was determined by 3,4,5-trimethoxybenzoic acid using HRP as a standard. In addition, LTβ4 and PGE2 concentrations were determined using enzyme immunoassay kits (Cayman Chemical). All secreted concentrations were standardized to the total tissue protein content determined by bicinchoninic acid (Pierce, Rockford, IL, USA), adapted to microtiter plates.

Cytokine/chemokine analysis

Cytokines and chemokines released into serum samples were detected by the Milliplex MAP kit (Millipore, Bedford, MA, USA), according to the manufacturer’s instructions. The samples were processed on a Luminex 100, and results were analyzed with MasterPlexQT software (MiraiBio, South San Francisco, CA, USA).

Antibody preparation

Protein L bead columns (Pierce) were used to purify Ig from C57Bl/10, C3HeB/FeJ, C3H/HeJ, and B10/ScNJ sera. Briefly, Protein L bead slurry was packed into a 0.5-ml bead bed, and 0.5 ml sera was applied to each column. Eluted Ig fractions that contained the highest absorbance were pooled and dialyzed at 4°C overnight in PBS with two changes: The dialyzed fractions were concentrated to 1 mg/ml using Centriplus Microcon concentrators (Millipore). In some experiments, prior to clamp application, 100 µl purified antibody was administered i.v. to Rag1-/- mice. To control for the effects of antibody injection, some mice received saline only. Other control mice included sham-treated mice. Intestinal sections from wild-type mice were swollen and edematous with regions of mild to moderate hemorrhage. Microscopically, the intestinal villi were shortened and exhibited significant lifting and sloughing, as well as exuding lamina propria and hemorrhage (Fig. 1, A–C). In contrast, intestinal damage was reduced significantly in the absence of TLR4; however, the damage remained significantly higher than sham-treated mice. Intestinal sections from B10/ScNJ mice had significantly taller villi (Fig. 1B), decreased epithelial lifting and sloughing, and limited hemorrhage (Fig. 1, A and D) compared with wild-type control mice (Fig. 1, A–C). To verify this data, TLR4+/- mutant mice (C3H/HeJ) and appropriate wild-type controls, C3HeB/FeJ mice, were also subjected to IR. C3H/HeJ mice express TLR4 with a point mutation that prevents TLR4 signaling [26, 27]. Although the villi from C3H/HeJ mice were significantly shorter than similarly

RESULTS

The absence of TLR4 signals attenuates intestinal damage and systemic inflammation

While maintaining a barrier in the presence of commensals, intestinal epithelial cells must also alert the immune response to bacterial infections. As TLR4 is critical to the inflammatory response and to maintaining intestinal homeostasis [17], it is likely that during IR, TLR4 expression may play a role in epithelial damage. To test this hypothesis, we subjected wild-type (C57Bl/10) and B10/ScNJ (TLR4+/-) mice to 30 min ischemia, followed by 2 h reperfusion, and evaluated intestinal epithelial damage. Macroscopically, intestinal sections from wild-type mice were swollen and edematous with regions of mild to moderate hemorrhage. Microscopically, the intestinal villi were shortened and exhibited significant lifting and sloughing, as well as exuding lamina propria and hemorrhage (Fig. 1, A–C). In contrast, intestinal damage was reduced significantly in the absence of TLR4; however, the damage remained significantly higher than sham-treated mice. Intestinal sections from B10/ScNJ mice had significantly taller villi (Fig. 1B), decreased epithelial lifting and sloughing, and limited hemorrhage (Fig. 1, A and D) compared with wild-type control mice (Fig. 1, A–C). To verify this data, TLR4+/- mutant mice (C3H/HeJ) and appropriate wild-type controls, C3HeB/FeJ mice, were also subjected to IR. C3H/HeJ mice express TLR4 with a point mutation that prevents TLR4 signaling [26, 27]. Although the villi from C3H/HeJ mice were significantly shorter than similarly

Figure 1. Histological analysis indicates intestinal IR-induced damage is attenuated in TLR4-altered mice. (A) H&E-stained, formalin-fixed tissue sections were scored for intestinal epithelial damage (0–6), as described in Materials and Methods. (B) Villus height/crypt depth (V/C) of individual villi were measured using Metavue microscopic software calibrated with a slide micrometer. Each bar is the average ± SEM with five to 10 mice/group. The one-way ANOVA with the Newman-Keuls post-hoc test determined a significant difference (P<0.05) compared with similarly treated wild-type controls and is indicated by * . (C–F) Representative H&E-stained sections of each treatment are presented. (C) C57Bl/10 IR (wild-type); (D) B10/ScNJ (TLR4+/-); (E) C3HeB/FeJ (wild-type); (F) C3H/HeJ (TLR4+/-). Original magnification for all photomicrographs and measurements was obtained at ×100.
treated B10/ScN mice (Fig. 1, B and F), the intestinal damage and villus height/crypt depth were attenuated in response to IR when compared with similarly treated, TLR4-sufficient C3HeB/FeJ mice (Fig. 1, A, B, and E). Nevertheless, the intestinal damage was significantly higher than sham treatment, as also seen in the B10/ScNJ mice. No significant intestinal damage was visible in any of the sham-treated animals (Fig. 1A). Thus, a lack of TLR4 expression (B10/ScN) or a lack of TLR4 signaling capability (C3H/HeJ) decreased mucosal damage.

To confirm the role of TLR4 in intestinal IR-induced damage, systemic cytokine/chemokine production was determined in TLR4-sufficient and -deficient mice. In wild-type mice, IR induced significant sera concentrations of the inflammatory chemokines, CXCL10 (IP-10), CCL5 (RANTES), and CXCL1 (KC; Fig. 2, A–C). In addition, multiple cytokines, including IL-10, IL-6, and TNF-α (Fig. 2, D–F), were also elevated significantly after IR treatment compared with sham treatment. In contrast, after IR, all of these chemokines and cytokines were decreased significantly in B10/ScN mice (Fig. 2). Additionally, we examined bacterial translocation at this acute injury time-point. Despite the decreased cytokine production and epithelial damage, there was no significant bacterial translocation of aerobic or anaerobic bacteria at 2 h postischemia in wild-type or mutant mice (data not shown). Together with the intestinal injury, these data indicate a role for TLR4 in intestinal IR-induced damage.

**The absence of TLR4 decreases intestinal eicosanoid production**

IR-induced intestinal damage is accompanied by inflammation with significantly elevated concentrations of intestinal peroxidase and LTB4 and PGE2 [23, 28, 29]. As TLR4 activation generally induces inflammation, we hypothesized that total tissue peroxidase LTB4 and PGE2 production would be decreased in a TLR4-altered mouse, similar to the cytokine production. As expected in response to IR, total peroxidase was increased significantly in both strains of wild-type control mice (Fig. 3A). Despite a decrease in overall intestinal damage after IR treatment, TLR4lps-def and TLR4lps-n mice produced increased quantities of intestinal peroxidase, which were similar to those found in IR-treated wild-type mice (Fig. 3A). Therefore, the induction of total peroxidase is independent of TLR4 signaling.

The inflammatory eicosanoids, LTB4 and PGE2, are also up-regulated in response to IR in wild-type mice [23, 29].

**Figure 2. TLR4 is required for IR-induced chemokine and cytokine production.** Sera were collected from wild-type, TLR4lps-n, and TLR4lps-def mice at 2 h postischemia and evaluated in duplicate on a Luminex 100, followed by analysis with MasterPlexQT software. Chemokines (A) CXCL10 (IP-10), (B) CCL5 (RANTES), and (C) CXCL1 (KC) and cytokines (D) IL-10, (E) IL-6, and (F) TNF were determined. Each bar is the mean ± sem with three to six animals/group. *, Significant difference from sham treatment; †, significant difference from wild-type treatment (P≤0.05), as determined by one-way ANOVA with Newman-Keuls post-hoc analysis.
Again, both strains of wild-type mice released significant amounts of LTB4 and PGE2 in intestines from IR-treated mice compared with sham-treated mice (Fig. 3B and C). In contrast, IR-induced production of LTB4 and PGE2 was significantly lower in TLR4<sup>−/−</sup> and TLR4<sup>−/+</sup> mice compared with appropriate wild-type controls (Fig. 3B and C). Therefore, TLR4 appears to be required for IR-induced LTB4 and PGE2 production, which correlates with intestinal damage in wild-type mice.

### The naturally occurring antibody repertoire of TLR4<sup>−/−</sup> mice is sufficient to induce injury

Previous studies indicated that the inflammatory response to IR involves naturally occurring antibodies binding to ischemic tissue [23, 30–32]. Therefore, it was possible that the lack of TLR4 altered the naturally occurring antibody repertoire. To test this hypothesis, total antibody was purified from each mouse strain and injected into Rag-1<sup>−/−</sup> mice. To test this hypothesis, total antibody was purified from each mouse strain and injected into Rag-1<sup>−/−</sup> mice to determine if the lack of TLR4 altered the naturally occurring antibody repertoire. To test this hypothesis, total antibody was purified from each mouse strain and injected into Rag-1<sup>−/−</sup> mice. To test this hypothesis, total antibody was purified from each mouse strain and injected into Rag-1<sup>−/−</sup> mice.

Figure 3. Intestinal IR-induced PGE2 is TLR4-dependent. Ex vivo peroxidase (A), LTB4 (B), and PGE2 (C) production by tissue sections from each treatment group at 2 h postischemia was determined by enzyme immunoassay, as described in Materials and Methods. Each bar is the mean ± SEM with five to 10 animals/group. *, Significant difference from sham; #, significant difference from wild-type treatment (P<0.05), as determined by one-way ANOVA with Newman-Keuls post-hoc analysis.

Figure 4. Antibodies from TLR4-altered mice are sufficient for IR- induced damage and PGE2 production in Rag-1<sup>−/−</sup> mice. One hour after injection of antibodies from wild-type or TLR4-altered mice, Rag-1<sup>−/−</sup> mice were subjected to mesenteric IR or sham treatment. (A) Immediately after 2 h reperfusion, intestinal sections were formalin-fixed, paraffin-embedded, and H&E-stained. The stained tissues were scored for mucosal injury (0–6), as described in Materials and Methods. (B) Additional tissue sections were collected, and ex vivo PGE2 production was determined. Each bar is the average ± SEM with six to 10 animals/group. Compared with sham control mice, a significant difference (P<0.05) was determined by ANOVA with Newman-Keuls post-hoc test and is indicated by *.

Studies [33, 34], Rag-1<sup>−/−</sup> mice did not sustain intestinal damage in response to IR, but when antibodies from wild-type mice (C57BL/10 or C3HeB/FeJ) were injected into Rag-1<sup>−/−</sup> mice, the intestine sustained significant damage in response to IR treatment (Fig. 4A). Interestingly, administration of antibodies from C3HeB/FeJ mice resulted in significantly more damage than antibodies from C57Bl/10 mice. When Rag-1<sup>−/−</sup> mice were administered antibodies from TLR4-altered mice (B10/ScNJ or C3H/HeJ), intestinal damage also ensued. In addition, administration of antibodies did not alter the intestinal pathology of sham-treated mice. Correlating with the mucosal damage, intestinal tissues from Rag-1<sup>−/−</sup> mice-administered antibodies from wild-type or TLR4<sup>−/−</sup> mice secreted increased levels of PGE2 in response to IR (Fig. 4B) as well as peroxidase and LTB4 (data not shown). This indicates that the repertoire of naturally occurring antibodies is not altered by the lack of TLR4. In addition, administration of these antibodies to Rag-1<sup>−/−</sup> mice is sufficient to induce normal quantities of PGE2 and other inflammatory products in response to treatment.

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The absence of MyD88 but not Trif attenuates IR-induced intestinal damage and inflammation

After TLR4 and coreceptors are activated by ligand binding, an intermediate adaptor molecule, MyD88 or Trif, is recruited to the complex, resulting in additional recruitment and phosphorylation of downstream molecules leading to the activation of NF-κB. We hypothesized that the absence of MyD88 or Trif may also limit IR-induced intestinal damage. In response to IR, the Trif−/− mice sustained intestinal damage and inflammation similar to wild-type mice (Fig. 5, A–C). In contrast, when MyD88−/− mice were subjected to intestinal IR, the intestinal damage was attenuated (Fig. 5A), as determined by injury scores, which were approximately half of that seen in C57BL/6 control mice. This decrease in injury score was comparable with the attenuation of injury observed in B10/ScNj (TLR4lps-def) and C3H/HeJ (TLR4lps-n) mice, 36% and 55%, respectively (Fig. 1A). Importantly, in these three strains lacking the ability to signal through TLR4, the damage remained two- to fivefold higher than sham-treated mice (Figs. 1A and 5A). Similar to that of the TLR4-altered strains, the IR-induced intestinal damage and inflammation similar to wild-type mice (Fig. 5A), as determined by injury scores, which were approximately half of that seen in C57BL/6 control mice. This decrease in injury score was comparable with the attenuation of injury observed in B10/ScNj (TLR4lps-def) and C3H/HeJ (TLR4lps-n) mice, 36% and 55%, respectively (Fig. 1A). Importantly, in these three strains lacking the ability to signal through TLR4, the damage remained two- to fivefold higher than sham-treated mice (Figs. 1A and 5A). Similar to that of the TLR4-altered strains, the IR-induced intestinal damage and inflammation were attenuated significantly, although not to sham-treatment levels. However, the decreased PGE2 production by wild-type animals was comparable with that of untreated B10/ScN mice (Fig. 5C). These data indicate that IR-induced LTB4 and PGE2 production is TLR4- and MyD88-dependent.

The absence of TLR4-mediated Cox-2 activation attenuates intestinal damage and inflammation in response to IR

Previous studies indicated that TLR4 regulation of PGE2 production is Cox-2-dependent [10]. Therefore, we examined mid-jejunum Cox-1 and Cox-2 expression in response to sham and IR treatment in wild-type and TLR4-altered mice. As indicated in Figure 6, A and B, Cox-1 was not significantly different between sham and IR treatments. However, when compared with sham treatment, IR increased Cox-2 mRNA expression significantly in wild-type (C57BL/6, C57Bl/10, or C3HeB/FeJ) mice (Fig. 6, A and C). In contrast, in response to IR, there was no increase in Cox-2 mRNA in either of the TLR4-altered mouse strains or MyD88−/− mice (Fig. 6, A and C). However, the basal or sham levels of Cox-2 expression were also elevated slightly compared with the wild-type controls (Fig. 6, A and C). To verify that Cox-2-mediated PGE2 production played a role in intestinal IR-induced tissue damage, mice were treated with or without the Cox-2-selective inhibitor NS-398, resuspended in DMSO, and diluted in PBS. As indicated in Figure 7, the IR-induced tissue damage and PGE2 production in wild-type mice were attenuated significantly, although not to sham-treatment levels. However, the decreased PGE2 production by wild-type animals was comparable with that of untreated B10/ScN mice (Fig. 6, A and C). To verify that Cox-2-mediated PGE2 production played a role in intestinal IR-induced tissue damage, mice were treated with or without the Cox-2-selective inhibitor NS-398, resuspended in DMSO, and diluted in PBS. As indicated in Figure 7, the IR-induced tissue damage and PGE2 production in wild-type mice were attenuated significantly, although not to sham-treatment levels. However, the decreased PGE2 production by wild-type animals was comparable with that of untreated B10/ScN mice (Fig. 6, A and C).

Figure 5. At 2 h postischemia, MyD88−/− mice lack inflammatory markers and have decreased intestinal IR-induced damage. MyD88−/−, Trif-deficient, or wild-type C57Bl/6 mice were subjected to mesenteric IR or sham treatment. (A) Immediately after 2 h reperfusion, intestinal sections were formalin-fixed, paraffin-embedded, and H&E-stained. The stained tissues were scored for mucosal injury (0–6), as described in Materials and Methods. Additional tissue sections were collected, and ex vivo (B) LTB4 and (C) PGE2 production was determined. Representative H&E-stained, formalin-fixed intestinal sections from IR-treated MyD88−/− (D) or Trif-deficient (E) mice are shown. Each bar is the average ± SEM with three to five animals/group. * Significant difference from sham treatment of similar strains of mice (P<0.05), as determined by one-way ANOVA with Newman-Keuls post-hoc test or unpaired t-test. Significant difference compared with IR-treated wild-type controls is indicated by †. Photomicrographs were obtained at ×200 original magnification.
Moses et al. TLR4 alters intestinal IR-induced injury and repair

Figure 6. TLR4 mediates IR-induced Cox-2 mRNA expression. Wild-type, TLR4^{lps-}, and TLR4^{lps-def} mice were subjected to intestinal ischemia followed by 2 h reperfusion. (A) Intestinal sections were analyzed for Cox-1, Cox-2, and GAPDH mRNA expression by RT-PCR using primers and conditions indicated in Materials and Methods. Representative samples are shown. (B and C) Intensity of the Cox-1 (B) and Cox-2 (C) PCR bands was quantitated using Image J and normalized to GAPDH of the same sample. Each bar is the average ± SEM with three to 10 animals/group. *, Significant difference from sham treatment of similar strains of mice (P<0.05), as determined by one-way ANOVA with Newman-Keuls post-hoc test or unpaired t-test. Significant difference compared with IR-treated wild-type controls is indicated by φ.

Figure 7. Cox-2 inhibition attenuates IR-induced injury. Wild-type and TLR4^{lps-} mice were subjected to sham or intestinal IR after administration of diluted DMSO (control) or Cox2 inhibitor NS-398 (A and B). At 2 h postreperfusion, intestinal sections were removed and analyzed for injury (A) or PGE2 production (B), as described in Materials and Methods. Representative H&E-stained intestinal sections from C57Bl/10 (C and D) or B10/ScN (E and F) mice subjected to IR treatment after administration of DMSO (C and E) or NS-398 (D and F) are shown. Each bar is the average ± SEM with four to eight animals/treatment group. *, Significant difference from sham treatment of similar strains of mice (P<0.05), as determined by one-way ANOVA with Newman-Keuls post-hoc test or unpaired t-test. Significant difference compared with IR-treated wild-type controls is indicated by φ. Photomicrographs were obtained at ×200 original magnification.

This hypothesis, TLR4 mutant mice were administered PGE2 intraluminally immediately prior to reperfusion. Although administration of intestinal PGE2 resulted in concentrations similar to that found in IR-treated wild-type mice (Fig. 8B), intestinal injury remained significantly lower (Fig. 8A). Even the administration of PGE2 at concentrations that were tenfold higher than those seen in wild-type mice was not sufficient to restore damage in MyD88^{−/−} mice (data not shown). Thus, although Cox-2-mediated PGE2 appears to have a role in IR-induced intestinal damage, it did not alter the limited damage or lower production of PGE2 (Fig. 7). These data indicate that in response to IR, Cox-2 regulation of PGE2 is TLR4-mediated.

Restoring PGE2 concentrations is not sufficient for IR-induced damage or inflammation in TLR4^{lps-def} mice

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Restoring PGE2 concentrations is not sufficient for IR-induced damage or inflammation in TLR4^{lps-def} mice

It was possible that the mutant mice did not secrete sufficient PGE2 concentrations for IR-induced intestinal damage. To test this hypothesis, TLR4 mutant mice were administered PGE2 intraluminally immediately prior to reperfusion. Although administration of intestinal PGE2 resulted in concentrations similar to that found in IR-treated wild-type mice (Fig. 8B), intestinal injury remained significantly lower (Fig. 8A). Even the administration of PGE2 at concentrations that were tenfold higher than those seen in wild-type mice was not sufficient to restore damage in MyD88^{−/−} mice (data not shown). Thus, although Cox-2-mediated PGE2 appears to have a role in IR-induced intestinal damage, it did not alter the limited damage or lower production of PGE2 (Fig. 7). These data indicate that in response to IR, Cox-2 regulation of PGE2 is TLR4-mediated.
responsive to LPS in the absence of commensal depletion. As these studies were conducted in animals depleted of commensal organisms, it is likely that the natural expression of TLR4 was altered. With the initially low endotoxin concentrations in the commensally depleted animals, followed by LPS administration, it is possible that endotoxin tolerance played a role in these studies [41]. In models of colitis, including dextran sulfate sodium chemical induction and IL-10-deficient mice, the absence of TLR4 resulted in increased damage and bacterial translocation without a concurrent increase in neutrophilic inflammation [11, 17, 42]. One explanation for the difference in the current results and these data is that these studies were colon-specific with a high bacterial load. In comparison, the necrotizing enterocolitis studies and the current studies focused on the jejunum, which normally has an extremely low bacterial load compared with the colon. Another difference between the studies is the type of injury; in the colonic studies, chronic damage was induced over a much longer time course, whereas the current studies focus on acute injury. Therefore, a second possibility is that multiple mechanisms are involved in distinct types of injury. This explanation seems likely, as multiple types of acute IR (myocardial and intestinal) appear to use similar mechanisms. The two possibilities are not mutually exclusive, as the heart also has low bacterial loads [19, 35].

TLR4 can be activated by exogenous and endogenous ligands [12, 13]. The specific TLR4 ligand inducing injury in intestinal IR is currently unknown. However, there are at least two possibilities that may be interacting. The first possibility is that exogenous ligands, including commensals, which have crossed the epithelial barrier, are the activating ligand. This can be supported by a recent study showing that commensal-depleted animals have exacerbated IR-induced damage, whereas administration of LPS to these mice prior to IR decreased the degree of intestinal permeability to that seen in normal mice after IR but not to the level of sham treatment [41]. However, our studies at this acute time-point indicate no significant difference in aerobic or anaerobic bacterial translocation to the mesenteric lymph nodes, liver, or spleen (data not shown). This suggests that in the presence of commensal bacteria, other ligands may be initiating TLR4 activation. The second possibility is that endogenous ligands, including HMGB-1, are the culprits that activate TLR4. This would be similar to a liver IR

**DISCUSSION**

In this study, we assessed the role of TLR4 activation and signaling through MyD88 and Trif in intestinal IR-induced damage at 2 h postischemia. At this time-point, a lack of TLR4 or MyD88 attenuated intestinal damage to ~50% of that seen in wild-type animals. The attenuated injury was accompanied by decreased, typical proinflammatory mediators, including chemokines, cytokines, and eicosanoid LT4 and PGE2 production. The decreased PGE2 appears to be mediated by Cox-2 activation, as inhibition of the Cox-2 in wild-type animals reduced tissue damage and PGE2 production to levels comparable with TLR4<sup>−/−</sup> and MyD88<sup>−/−</sup> mice. Thus, these studies support the hypothesis that TLR4 expression affects the extent of intestinal damage by altering Cox-2-mediated PGE2 production. However, PGE2 alone is not sufficient to restore damage in the TLR4-altered mice, implicating additional mechanisms of TLR4-mediated damage.

In other models of IR, TLR4 has been implicated in increased damage. TLR4<sup>−/−</sup> mice subjected to 60 min of regional myocardial ischemia followed by up to 24 h of reperfusion showed reduced damage and inflammation [19, 35]. The decreased inflammation was characterized by reduced NF-kB translocation and subsequent cytokine production. However, the role of PGE2 in this model is unknown, as its production was not analyzed in these studies. Similar to our results, protection from myocardial IR-induced damage appears to be MyD88-dependent [36]. In hepatic IR, TLR4 mutation resulted in decreased cellular damage, although this protection was MyD88-independent [37–39]. In addition, another model of small intestinal ischemic damage, necrotizing enterocolitis, showed reduced injury in the absence of TLR4 as well [40].

In contrast, Chen et al. [41] showed that depletion of intestinal commensals increased IR-induced intestinal permeability to small molecules (dextran), suggesting a protective role for TLR4. The addition of orally administered LPS attenuated the commensal-depleted, IR-treated animals to untreated IR levels but not sham-treatment levels. However, these studies did not treat with LPS in the absence of commensal depletion. As
model, in which anti-HMGBl attenuated liver damage in wild-type mice but not in TLR4 mutants [37]. Further studies are required to determine definitively the ligand or ligands responsible for TLR4 activation in intestinal IR.

Our studies showed that inhibition of Cox-2 results in decreased intestinal IR-induced damage in the mouse. These results are similar to intestinal IR studies in the rat, which showed that tissue damage was decreased after administration of Celebrex (Cox-2 inhibitor) but not Flunixin (Cox-1 inhibitor) [28]. However, administration of Celebrex did not decrease 2 h postischemic injury, polymorphonuclear neutrophil infiltration, or edema to the degree of sham treatment. Similarly, inhibition of Cox-2 with NS-398 attenuated IR-induced PGE2 production and intestinal damage but not to background levels (Fig. 7). The low levels of PGE2 and intestinal damage may be a result of constitutive Cox-1 activation (Fig. 6). Interestingly, administration of PGE2 intraluminally was not sufficient to induce damage in response to IR (Fig. 8).

This correlates with multifactorial damage in which inhibition of one system (TLR4 and Cox-2 in this case) may attenuate damage and inflammation, but administration of an end-product (PGE2) is not sufficient to induce damage. Together, these data further indicate that IR-induced damage is mediated by multiple factors.

Our studies show that TLR4 expression induces Cox-2 activation and PGE2 production in response to intestinal IR, resulting in local tissue damage. Although inhibition of Cox-2-mediated, TLR4- and MyD88-dependent PGE2 production attenuates damage, PGE2 alone is not sufficient to induce damage in MyD88- or TLR4-deficient mice. Thus, inhibition of TLR4-stimulated, Cox-2-induced PGE2 production may provide beneficial results, despite the involvement of additional mechanisms of damage.

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DISCLOSURE

Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Institutes of Health.

REFERENCES


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