Activation of the MyD88 signaling pathway inhibits ischemia-reperfusion injury in the small intestine

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Watanabe T, Kobata A, Tanigawa T, Nadatani Y, Yamagami H, Watanabe K, Tominaga K, Fujiwara Y, Takeuchi K, Arakawa T. Activation of the MyD88 signaling pathway inhibits ischemia-reperfusion injury in the small intestine. Am J Physiol Gastrointest Liver Physiol 303: G324–G334, 2012. First published May 23, 2012; doi:10.1152/ajpgi.00075.2012.—Toll-like receptors (TLRs) recognize microbial components and trigger the signaling cascade that activates innate and adaptive immunity. Recent studies have shown that the activation of TLR-dependent signaling pathways plays important roles in the pathogenesis of ischemia-reperfusion (I/R) injuries in many organs. All TLRs, except TLR3, use a common adaptor protein, MyD88, to transduce activation signals. We investigated the role of MyD88 in I/R injury of the small intestine. MyD88 and cyclooxygenase-2 (COX-2) knockout and wild-type mice were subjected to intestinal I/R injury. I/R-induced small intestinal injury was characterized by infiltration of inflammatory cells, disruption of the mucosal epithelium, destruction of villi, and increases in myeloperoxidase activity and mRNA levels of TNF-α and the IL-8 homolog KC. MyD88 deficiency worsened the severity of I/R injury, as assessed using the histological grading system, measuring luminal contents of hemoglobin (a marker of intestinal bleeding), and counting apoptotic cells using the histological grading system, measuring luminal contents of hemoglobin (a marker of intestinal bleeding), and counting apoptotic cells. MyD88 deficiency increased enterocolitis and mesenteric ischemia (30). Reperfusion after ischemic events in the small intestine causes a progressive loss of mucosal structure with epithelial cell death and reduced epithelial function. The resultant intestinal mucosal barrier dysfunction causes transmucosal movement of bacteria and various hydrophilic solutes (41), resulting in high morbidity and mortality.

The innate immune system detects the invasion of microorganisms through Toll-like receptors (TLRs), which recognize microbial components, known as pathogen-associated molecular patterns (PAMPs), and trigger inflammatory responses. To date, 10 and 12 functional TLRs have been identified in humans and mice, respectively (24). Each TLR detects distinct PAMPs derived from viruses, bacteria, mycobacteria, fungi, and parasites. These include lipoproteins (recognized by TLR1, TLR2, and TLR6), double-stranded RNA (TLR3), LPS (TLR4), flagellin (TLR5), single-stranded (ss) RNA (TLR7 and TLR8), and DNA (TLR9) (2). Ligand binding to TLRs leads to the activation of downstream signaling pathways, including NF-κB, MAPKs, and type I interferon pathways, resulting in the induction of proinflammatory cytokines and chemokines (8). In addition to the recognition of PAMPs, TLR2, TLR4, and TLR9 have also been shown to recognize endogenous ligands, which have been termed danger-associated molecular patterns (DAMPs). TLR2 and TLR4 are extraacellular TLRs and have a wide range of putative endogenous ligands, including heat shock proteins, high-mobility group box 1 (HMGB1) and breakdown products of fibronectin and hyaluronic acid (9).

Recent studies have shown that the activation of TLR-dependent signaling pathways by DAMPs and PAMPs plays important roles in the pathogenesis of I/R injuries in many organs, including the heart, brain, kidney, liver, and gut (4, 6, 12), while evidence regarding the roles of TLRs is conflicting. Some studies have reported that inhibition of signaling pathways via TLRs, especially TLR2 and TLR4, ameliorates deleterious inflammatory responses and is protective against I/R injuries (5, 13, 27, 36, 45, 51); other studies reported protection against I/R injuries by TLR activation (16, 29).

The TLR-induced inflammatory response is dependent on a common signaling pathway that is mediated by the adaptor molecule MyD88. MyD88 is utilized by all TLRs, with the exception of TLR3, and drives the activation of NF-κB and MAPKs to control inflammatory responses (23). However, several studies indicated crucial roles of the TLR-dependent/MyD88-independent pathway in I/R injuries (40, 54). In this study, we investigated the role of MyD88 in I/R injury of the small intestine in mice. We also examined the role of MyD88 in the expression of cyclooxygenase (COX)-1 and COX-2, important cytoprotective factors in the gastrointestinal tract, during the development of intestinal I/R injury.

MATERIALS AND METHODS

Animals and induction of I/R injury of the small intestine. MyD88 knockout (KO) mice that were backcrossed eight times on the C57BL/6 background, originally generated by Dr. S. Akira (22) (Osaka University, Osaka, Japan), were obtained from Oriental Bio-service (Kyoto, Japan). COX-2 KO mice (C57BL/6) were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were purchased from Charles River Japan (Atsugi, Japan) and used as the...
control strain for MyD88 and COX-2 KO mice. All animals were housed in polycarbonate cages with paper chip bedding in an air-conditioned biohazard room with a 12:12-h light-dark cycle. All animals had free access to food and water.

To induce injury, the superior mesenteric artery was clamped (ischemia) under urethane anesthesia, and reperfusion was established 45 min later by removal of the clamp. After a 60-min reperfusion period, the intestine was excised. Intestinal injury after reperfusion was evaluated. To investigate the role of PGE2, some animals were intraperitoneally given 16, 16-dimethyl PGE2 (Cayman Chemical, Ann Arbor, MI) at a dose of 100 μg/kg 5 min before reperfusion. Furthermore, to investigate the enterobacteria in the injury, wild-type mice were orally given an antibiotic, ampicillin (800 mg/kg; Sigma-Aldrich, St. Louis, MO) twice, 24 h and 1 h before induction of I/R injury. All experimental procedures were approved by the Animal Care Committee of the Osaka City University Graduate School of Medicine.

Assessment of intestinal mucosal injury induced by I/R. The animals were killed after reperfusion by CO2 inhalation, and the intestine was removed and submitted for examination. Intestinal injury after reperfusion was evaluated by measuring luminal contents of hemoglobin (a marker of intestinal bleeding), histological examination, and counting apoptotic cells. After making a 20-cm intestinal loop, saline (1 ml) was injected into the loop. The luminal contents were aliquoted after centrifugation. Intestinal bleeding was quantified indirectly as hemoglobin concentration in the luminal lavage fluid using a kit (Wako Pure Chemical Industries, Osaka, Japan) and following the manufacturer’s protocol.

For the histological evaluation, segments of the ileum were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde and embedded in OCT compound (Miles, Elkhart, IN). Cryostat sections were then stained with hematoxylin-eosin and subjected to analysis. The pathological changes observed by semiquantitative histological evaluation were graded from 0 to 4. In grade 0, no specific pathological changes are observed, i.e., normal architecture of the gut wall, including villi, crypts, lamina propria, and muscularis externa. In grade 1, mild mucosal damage is observed, i.e., denudation of villi epithelium, otherwise normal structure. In grade 2, moderate damage occurs, i.e., loss of villus length and epithelial sloughing with evidence of congestion, hemorrhage, and inflammation in the mucosa, but no change in the submucosa or muscularis externa. In grade 3, extensive damage is observed, i.e., loss of a large number of villi, including denudation, sloughing, and the presence of granulomatous tissue; the damage is localized to submucosa and muscularis. In grade 4, there is severe damage and necrosis, i.e., inflammation and necrosis in areas throughout the thickness of the intestinal wall.

We also evaluated the intestinal I/R injury by counting apoptotic epithelial cells using immunohistochemistry with an antibody against COX-2 (CytoTech Biotechnology, Santa Cruz, CA). Counterstaining was performed with methyl green. The apoptotic index was defined as the percentage of labeled epithelial cells in a total count of > 500 epithelial cells.

Determination of mRNA levels by real-time quantitative RT-PCR. Total RNA was extracted from small intestinal tissue using an ISOGENT kit (Nippon Gene, Tokyo, Japan). Real-time quantitative RT-PCR analyses were performed as previously described (50). Expression of mRNAs for TNF-α, the mouse IL-8 homolog KC, COX-1, and COX-2 was normalized for the expression of GAPDH mRNA and expressed as ratio to the mean value for small intestinal tissue of sham-operated mice. The sequences of PCR primers and TaqMan probes are shown in Table 1.

Western blot analysis. Small intestinal tissues were homogenized and lysed on ice in a buffer containing 0.5% NP-40, 40 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1 mM PMSF, and 10 μg of leupeptin per milliliter. The protein of the lysate was measured with a modified bichinchoninic acid method. Proteins were denatured with SDS sample buffer, subjected to 10% SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in Tris-buff ered saline (TBS) containing 5% BSA and then incubated overnight with rabbit polyclonal antibodies against COX-1 and COX-2 (both from Santa Cruz Biotechnology). The bound antigen-antibody complexes were detected with an anti-rabbit IgG-horseradish peroxidase-conjugated antibody (Amersham, Arlington Heights, IL) using enhanced chemiluminescence in accordance with the manufacturer’s instructions.

Immunofluorescent study. To evaluate colocalization of MyD88 and COX-2, double labeling by immunofluorescence was performed using confocal scanning microscopy. The sections were then reacted with an Alexa Fluor 488-conjugated donkey anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA) or an Alexa Fluor 594-conjugated donkey anti-goat IgG antibody (Invitrogen, respectively). Samples were examined with a confocal microscope (model TC41D/DMDRBB; Leica, Heidelberg, Germany) equipped with argon and argon-krypton laser sources.

Assay of the PGE2 concentration in small intestinal tissue. Small intestinal tissue was removed, weighed, and placed in a tube containing 100% methanol and 1 mM indomethacin (Wako Pure Chemical Industries). The tissues were then homogenized by using a Polytron homogenizer (IKA, Tokyo, Japan) and centrifuged at 12,000 rpm for 10 min at 4°C. After the supernatant of each sample had been evaporated with N2 gas, the residue was resolved in assay buffer and used for determination of PGE2. The concentrations of PGE2 were measured by using an enzyme immunoassay (PGE2 ElA kit; Cayman Chemical).

Measurement of myeloperoxidase activity. Myeloperoxidase (MPO) activity in small intestinal tissue, a marker of neutrophil infiltration, was assayed as previously described (49). One unit of MPO activity was defined as that degrading 1 μmol of peroxide per min at 25°C. Results were measured as units per gram tissue protein.

Effect of ampicillin on small intestinal bacterial flora. Mice were orally given ampicillin (800 mg/kg) or vehicle twice (24 h and 1 h before they were killed), and the small intestinal contents were obtained. Then the bacteria flora was subjected to analysis. The methods of isolation and identification of each bacterium have been described.

### Table 1. PCR primers and TaqMan probes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer and Probe</th>
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<tbody>
<tr>
<td>TNF-α</td>
<td>Primer (forward) 5’-CGAGAGGGAGCGATCAGCTCTC-3’;</td>
</tr>
<tr>
<td></td>
<td>Primer (reverse) 5’TCACTACCGGCGGCTACTTC-3’;</td>
</tr>
<tr>
<td>KC</td>
<td>Primer (forward) 5’TCTGAGTTTCTCCGGTGGGTA-3’;</td>
</tr>
<tr>
<td></td>
<td>Primer (reverse) 5’TCTGAGTTTCTCCGGTGGGTA-3’;</td>
</tr>
<tr>
<td>COX-1</td>
<td>Primer (forward) 5’-TCTAAGGGTCCGCGGATGAT-3’;</td>
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<tr>
<td></td>
<td>Primer (reverse) 5’TAAAGGCTCCGCAAGGAAACCTG-3’</td>
</tr>
<tr>
<td>COX-2</td>
<td>Primer (forward) 5’-AGGCGCACTGATCAGTGAAGTT-3’</td>
</tr>
<tr>
<td></td>
<td>Primer (reverse) 5’TAAAGGCTCCGCAAGGAAACCTG-3’</td>
</tr>
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</table>

KC, an IL-8 homolog; COX, cyclooxygenase.

**Table 1.** PCR primers and TaqMan probes

*Note:* Primer sequences were designed using Primer3 software (http://www.bioinformatics.nl/cgi-bin/Primer3/Primer3Plus.cgi) by AIP-Gastrointest Liver Physiol. doi:10.1152/ajpgi.00075.2012 • www.ajpgi.org
described in detail elsewhere (20). The number of viable bacteria was expressed as log colony-forming units per gram small intestinal content. The lower limit of bacterial detection with this procedure was $2 \times 10^2$ colony-forming units per gram intestinal content.

Statistical analysis. Values are means ± SD. One-way ANOVA was used to test for significance of differences among treatment group means, and results were examined by Fisher’s protected least significant difference test. Differences with $P$ values < 0.05 were considered significant.

RESULTS

I/R-induced small intestinal injury. Histological analyses showed that reperfusion after clamping of the superior mesenteric artery caused small intestinal damage, characterized by infiltration of inflammatory cells, disruption of the mucosal epithelium, and destruction of villi in wild-type mice (Fig. 1B), while mucosal damage was not evident in the small intestine of the sham operation group (Fig. 1A). I/R increased MPO activity (Fig. 1C) and induced overexpression of mRNAs or inflammatory cytokines such as TNF-α and KC (Fig. 1, D and E) in the small intestine.

Effect of MyD88 deficiency on severity of I/R injury of the small intestine. Compared with wild-type mice, MyD88 KO mice exhibited more severe I/R-induced intestinal injury (Fig. 2, A and B). Statistical evaluation of the histological grading scores showed that MyD88 deficiency worsened the severity of I/R-induced intestinal injury (Fig. 2C). The mean value of luminal hemoglobin concentrations in MyD88 KO mice was significantly higher than that in wild-type mice (Fig. 2D). Furthermore, I/R treatment resulted in induction of apoptosis of epithelial cells labeled with an antibody to ssDNA in the injured mucosa in wild-type mice (Fig. 2E), and such induction was more prominent in MyD88 KO mice (Fig. 2F). The mean apoptotic index in MyD88 KO mice was significantly higher than that in wild-type mice (Fig. 2G).

Effect of MyD88 deficiency on inflammatory responses in I/R-induced intestinal injury. MyD88 deficiency markedly inhibited the I/R-induced increase in expression levels of mRNAs for TNF-α (Fig. 3A) and KC (Fig. 3B) in the small intestine, whereas it did not affect the I/R-induced increase in intestinal MPO activities. The mean value of MPO activity in MyD88 KO mice was similar to that in wild-type mice (Fig. 3C).

Effect of MyD88 deficiency on expression of COX-1 and COX-2 and PGE2 synthesis in I/R-induced intestinal injury. I/R did not affect intestinal COX-1 expression at both mRNA (Fig. 4A) and protein (Fig. 4C) levels in wild-type and MyD88 KO mice. On the other hand, I/R markedly increased intestinal COX-2 expression in wild-type mice (Fig. 4B and 4C). This increase in

Fig. 1. Ischemia/reperfusion (I/R)-induced small intestinal injury. Mice were subjected to 45 min of ischemia followed by 60 min of reperfusion. After removal of the small intestine, histological examination, an assay of myeloperoxidase (MPO) activity (a marker of neutrophil infiltration), and determination of mRNA levels of inflammatory cytokines were performed. A and B: histological findings in the small intestine. I/R induced infiltration of inflammatory cells, disruption of the mucosal epithelium, and destruction of villi in wild-type (WT) mice (B), while such mucosal damages were not evident in the small intestine of the sham operation group (A). C: effect of I/R on neutrophil infiltration. MPO activity in the small intestine of mice that were subjected to I/R injury and sham operation were assayed. Each column represents the mean ± SD. N = 6. *$P < 0.05$ vs. sham operation group. D and E: effect of I/R treatment on the expression of inflammatory cytokines. The mRNA levels of TNF-α (D) and the mouse IL-8 homolog KC (E) in the small intestine were determined by quantitative RT-PCR. Each column represents the mean ± SD. N = 6. **$P < 0.01$, *$P < 0.05$ vs. sham operation group.
COX-2 expression was inhibited by MyD88 deficiency (Fig. 4, B and C).

I/R also increased the PGE2 concentration in the small intestine of wild-type mice. MyD88 deficiency inhibited the increase in the intestinal PGE2 concentration following I/R by 38% (Fig. 4D). Expression levels of the mRNAs for COX-1 and COX-2 and PGE2 concentration were not different between sham-operated wild-type mice and sham-operated MyD88 KO mice.

Effect of COX-2 deficiency on severity of I/R injury of the small intestine. Similar to the finding in MyD88 KO mice, COX-2 KO mice exhibited severe intestinal I/R-induced damage with massive necrosis of epithelial cells (Fig. 5, A and B). Histological grading score, luminal hemoglobin concentration, and apoptotic index in COX-2 KO mice were significantly higher compared with wild-type mice (Fig. 5, D–F). Treatment with 16, 16-dimethyl PGE2 reduced the severity of intestinal I/R damage in COX-2 KO mice, as assessed by the histological grading score, luminal hemoglobin concentration, and apoptotic index (Fig. 5, C–F).

We also examined the effect of 16, 16-dimethyl PGE2 on intestinal I/R injury in MyD88 KO mice. Exogenous PGE2 prevented exacerbation of intestinal I/R injury in MyD88 KO mice. The histological grading score, luminal hemoglobin concentration, and apoptotic index in COX-2 KO mice were reduced to the levels of wild-type mice that were subjected to I/R injury (Fig. 6, A–C).

Localization of MyD88 and COX-2 in the small intestine. Both MyD88 (Fig. 7A) and COX-2 (Fig. 7B) were expressed...
mainly on inflammatory cells in the injured intestinal mucosa and on some epithelial cells at the tips of the villi. Double staining showed colocalization of COX-2 with MyD88 in many inflammatory cells in the lamina propria (Fig. 7C).

Effect of ampicillin on the damage and inflammatory responses induced by I/R. Treatment with ampicillin inhibited intestinal I/R injury, as assessed by using the histological grading system, measuring luminal hemoglobin concentration, and calculating the apoptotic index (Fig. 8, A–C). It also inhibited increases in MPO activities and expression of mRNA for TNF-α, but did not affect the expression of mRNA for COX-2 (Fig. 8, D–F).

We next examined the effect of ampicillin on small intestinal bacterial flora. Treatment with ampicillin significantly decreased the number of total bacteria and Enterobacteriaceae (Table 2). It also decreased the number of Bifidobacterium, but the difference between ampicillin group and the vehicle group did not reach a statistical significance ($P = 0.1$).

DISCUSSION

In this study, we demonstrated that MyD88 deficiency aggravated intestinal I/R injury and inhibited increases in COX-2 expression and PGE$_2$ synthesis during the development of injury.

Since COX-2 KO mice also exhibited severe intestinal I/R injury with significant reduction of PGE$_2$ synthesis and exogenous PGE$_2$ abolished the sensitivity to injury in both MyD88 KO and COX-2 KO mice, these results suggest that MyD88 may play a protective role in intestinal I/R injury via stimulation of COX-2-mediated PGE$_2$ synthesis.

Multiple studies have shown that TLRs protect from I/R-induced tissue damage via a MyD88-dependent mechanism. Wang et al. (47) demonstrated that TLR4 activation conferred a potent cardiac protection against I/R injury via a MyD88-dependent pathway. Iwata et al. (21) also reported that extracellular BCL2, which can act as a DAMP, protected mouse hind limb skeletal muscle from I/R injury via a MyD88-dependent pathway. On the other hand, Hua et al. demonstrated that blocking the MyD88-dependent signal protected the myocardium of rats from I/R injury (18). Furthermore, Moses et al. (31) reported that MyD88 KO mice exhibited less severe intestinal injury induced by 30 min ischemia followed by 120 min of reperfusion. These results conflict with our finding that MyD88 KO mice were highly susceptible to I/R-induced in-


intestinal damage. Although reasons for this discrepancy are unknown, different periods of ischemia and reperfusion may provide conflicting results. In this study, we evaluated I/R-induced intestinal damage by using three different methods (i.e., histological grading system, luminal hemoglobin concentration, and the apoptotic index), and all of these parameters of tissue damage were much higher in MyD88 KO mice than in wild-type mice. These findings strongly suggest that activation of the MyD88-dependent pathway prevents intestinal I/R injury.

Since MyD88 is an adaptor molecule essential for signaling via TLRs, our results on the protection of intestinal I/R injury via a MyD88-dependent pathway lead to the hypothesis that TLR signals also protect from intestinal I/R injury. In fact, animal studies have shown that activation of the TLR-dependent signal prevents intestinal I/R injury, although conflicting evidence exists regarding the role of TLR signaling in I/R injury (6). A recent study by Chen et al. (10) showed that LPS, a ligand for TLR4, decreased mesenteric I/R-induced gut damage, whereas blocking TLR signals by commensal flora depletion enhanced I/R-induced gut damage. The protective roles of TLR4 have also been observed in neonatal murine intestinal I/R injury (42). Similar to TLR4, TLR2 prevented intestinal I/R injury. Aprahamian et al. (3) reported that after I/R, TLR2 KO mice had increased jejunal mucosal injury compared with wild-type animals with dysregulated mucosal immune response. Thus, intestinal I/R injury may trigger TLR/MyD88-dependent pathways, resulting in amelioration of the damage.

The preventive effects of TLRs and MyD88 on tissue injuries are thought to be due to modulation of the expression of several molecules, such as tight junction proteins (7, 15), heat-shock proteins (37), and COX-2 (11, 14), which play important roles in the maintenance of intestinal mucosal integrity. In this study, we investigated the role of MyD88 in the expression of COX-1 and COX-2 during the development of intestinal I/R injury. As expected, MyD88-dependent signals enhanced the expression of COX-2, an inducible cytoprotective factor, confirming previous findings demonstrated in other models of intestinal injuries (11, 14). We also investigated whether COX-2 acts as a protective factor during the development of intestinal I/R injury, since the role of COX-2 in I/R-induced tissue injuries is conflicting and inconclusive (25, 33–35, 39). Our findings that COX-2 KO mice exhibited more
severe intestinal damage compared with wild-type mice and that exogenous PGE2 attenuated the damage in COX-2 KO mice to the level of wild-type mice clearly suggest that COX-2-derived PGE2 is protective against I/R injury of the small intestine. However, the role of COX-2 in the injury may differ between different organs or under different experimental conditions.

Apoptosis was suggested to be a major mode of cell death in the destruction of rat small intestinal epithelial cells induced by I/R (19). In this study, both MyD88 KO and COX-2 KO mice, which were susceptible to intestinal I/R injury, exhibited increased epithelial apoptosis with reduction of PGE2 synthesis, and PGE2 reduced apoptosis in MyD88 KO and COX-2 KO mice to the level noted in wild-type mice that were subjected to I/R injury. Furthermore, consistent evidence has been reported that misoprostol, a PGE1 analog, prevents I/R-induced intestinal injury with reduction of apoptosis (43), suggesting that PGE2 prevented intestinal I/R-injury via inhibition of apoptosis of intestinal cells. Many lines of evidence support an antiapoptotic role of COX-2 and PGE2 (26, 28, 38, 44), although the precise mechanism is still unclear. Induction or modulation of the expression of antiapoptotic proteins, such as BCL-2 and survivin, is thought to be involved in the inhibition of apoptosis by PGE2.

Commensal bacteria play a crucial role in the pathogenesis of many types of small intestinal injuries, such as nonsteroidal anti-inflammatory drugs-induced enteropathy (49), but the role of commensals in I/R injury is unclear and conflicting results have been reported. Chen et al. (10) reported that depletion of gut commensal bacteria by antibiotics for 4 wk enhances
I/R-induced intestinal damage, whereas Yoshiya et al. (53) demonstrated attenuation of the injury and expression of inflammatory cytokines induced by I/R with commensal depletion by antibiotics for the same period. Since long-term depletion of commensal bacteria modulates the expression of several molecules such as TLRs (10) and heat-shock proteins (37) that play important roles in maintaining the intestinal homeostasis, we investigated the effect of short-term depletion of commensal bacteria on intestinal I/R injury using ampicillin, an antibiotic, to clarify the direct role of commensal bacteria in the injury. Depletion of commensal bacteria by ampicillin resulted in inhibition of injury, neutrophil infiltration, and TNF-α expression.

**Fig. 6.** Effect of exogenous PGE2 on intestinal I/R injury in MyD88 KO mice. WT mice and MyD88 KO mice were subjected to 45 min of ischemia followed by 60 min of reperfusion. Some MyD88 KO mice were intraperitoneally given 16,16-dimethyl PGE2 at a dose of 100 μg/kg 5 min before reperfusion. After removal of the small intestine, intestinal injuries were assessed by using the histological grading system (A), measuring the luminal hemoglobin concentration (B), and counting apoptotic epithelial cells with an antibody to ssDNA (C). Each column represents the mean ± SD. N = 6. *P < 0.01 vs. sham operation group, #P < 0.01 vs. WT mice.

**Fig. 7.** Localization of MyD88 and COX-2 in the small intestine of mice subjected to I/R injury. Both MyD88 (A) and COX-2 (B) were expressed mainly on inflammatory cells (arrows) in the injured intestinal mucosa and on some epithelial cells (arrowheads) at the tips of the villi. Double staining showed colocalization of COX-2 with MyD88 in inflammatory cells (C).
pression, suggesting that bacterial PAMPs may trigger inflammatory responses via activation of TLR/MyD88 signaling, leading to intestinal I/R injury. However, treatment with ampicillin failed to affect COX-2 expression. Since an increase in COX-2 expression by I/R was markedly inhibited in MyD88 KO mice, TLR ligands other than bacterial PAMPs may induce COX-2 expression in a MyD88-dependent manner. Recent studies have demonstrated that DAMPs, such as HMGB1, play an important role in the pathogenesis of I/R injury (6, 12, 46). Taken together, these results suggest that DAMPs may activate inflammatory cells in a MyD88-dependent manner during the development of intestinal I/R injury, leading to overexpression of COX-2 in such cells. In addition, these results also suggest that activation of the MyD88-dependent pathway elicited by DAMPs may be protective for intestinal I/R injury, whereas that elicited by PAMPs may be deleterious. Our hypothesis is supported by studies that showed that immune-neutralization of HMGB1 worsened I/R injuries of the retina (52) and heart (32), whereas HMGB1 treatment improved myocardial functional recovery and decreased infarct size after I/R injury (1).

In conclusion, our results suggest that a MyD88-dependent signaling pathway may inhibit I/R injury in the small intestine via induction of COX-2 expression. Further studies aimed at understanding the mechanisms underlying the protective effects of MyD88 signaling in intestinal I/R injury are warranted.
identifying specific pathways that are responsible for COX-2 induction are needed to develop useful therapies for intestinal I/R injury.

ACKNOWLEDGMENTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: T.W., K. Takeuchi, and T.A. edited and revised manuscript; T.W. approved final version of manuscript; T.W. and Y.N. prepared figures; T.W. drafted manuscript; T.W., K. Takeuchi, and T.A. edited and revised manuscript; T.W. approved final version of manuscript.

REFERENCES


