Depletion of gut commensal bacteria attenuates intestinal ischemia/reperfusion injury

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Yoshiya K, Lapchak PH, Thai T-H, Kannan L, Rani P, Lucca JJ, Tsokos GC. Depletion of gut commensal bacteria attenuates intestinal ischemia/reperfusion injury. Am J Physiol Gastrointest Liver Physiol 301: G1020–G1030, 2011. First published September 8, 2011; doi:10.1152/ajpgi.00239.2011.—Gut commensal bacteria play important roles in the development and homeostasis of intestinal immunity. However, the role of gut commensals in intestinal ischemia/reperfusion (I/R) injury is unclear. To determine the roles of gut commensal bacteria in intestinal IR injury, we depleted gut microbiota with a broad-spectrum antibiotic cocktail and performed mesenteric I/R (M/I/R). First, we confirmed that antibiotic treatment completely depleted gut commensal bacteria and diminished the size of secondary lymphoid tissues such as the Peyer’s patches. We next found that antibiotic treatment attenuated intestinal injury following M/I/R. Depletion of gut commensal bacteria reduced the expression of Toll-like receptor (TLR)2 and TLR4 in the intestine. Both are well-known receptors for gram-positive and -negative bacteria. Decreased expression of TLR2 and TLR4 led to the reduction of inflammatory mediators, such as TNF, IL-6, and cyclooxygenase-2. Intestinal I/R injury is initiated when natural antibodies recognize neo-antigens that are revealed on ischemic cells and activate the complement pathway. Thus we evaluated complement and immunoglobulin (Ig) deposition in the damaged intestine and found that antibiotic treatment decreased the deposition of both C3 and IgM. Interestingly, we also found that the deposition of IgA also increased in the intestine following M/I/R compared with control mice and that antibiotic treatment decreased the deposition of IgA in the damaged intestine. These results suggest that depletion of gut commensal bacteria decreases B cells, IgA, and TLR expression in the intestine, inhibits complement activation, and attenuates intestinal inflammation and injury following M/I/R.

TLRs in the intestine. Therefore, we hypothesized that antibiotic treatment would decrease TLR expression in the intestine, leading to an attenuation of intestinal inflammation.

Several molecules and cells have been implicated in I/R injury, such as reactive oxygen species (ROS), cytokines and chemokines (28), adhesion molecules (23, 30), complement (13, 26, 33, 65), natural antibodies (27, 67, 70, 71), B cells (14), T cells (19, 54), neutrophils (31), and platelets (45). Among these molecules, natural antibodies are known to be central in the pathogenesis of M/I/R injury and initiate I/R-induced tissue injury (67, 70). Brief periods of ischemia could lead to an alteration in surface epitopes, and these changes result in binding by natural immunoglobulin M (IgM) and activation of the complement system. In addition to natural antibodies, autoantibodies, which recognize self-antigen, could cause tissue injury after I/R (24, 25, 36). Complement activa-
tion is also an essential step in the initiation and amplification of the inflammatory response in I/R injury. Deficiency of complement factors has been shown to be protective against I/R injury (26, 67), and inhibition of the complement pathway through various interventions has been reported to attenuate I/R-induced damage (13, 65). Here we evaluated the influence of antibiotic treatment on the deposition of IgG and complement in the damaged intestine after M I/R.

IgA is considered to be the principal Ig in intestinal secretions and functions to prevent commensals and pathogenic microorganisms from gaining access to epithelial cell surfaces (40). However, IgA can also generate immune complexes and cause tissue injury in some conditions such as IgA nephropathy (51). In this study, depletion of gut commensals diminished PPs, which are the major sites of intestinal IgA production. Reduced IgA deposition in the intestine may influence intestinal I/R injury. Therefore, we also evaluated the deposition of IgA after M I/R in both control and antibiotic-treated mice. We provide the first evidence that IgA is deposited in the small intestine following M I/R and that antibiotic treatment decreased IgA deposition and reduced intestinal damage.

In this study, we administered broad-spectrum antibiotics to deplete gut commensal bacteria and investigated the role of commensal bacteria in acute inflammation following M I/R. Depletion of gut commensal bacteria decreased complement, IgM, and IgA deposition in gut, attenuated inflammation, and protected the gut from M I/R injury.

MATERIALS AND METHODS

Mice. Specific pathogen-free male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained in the specific pathogen-free animal facility of Beth Israel Deaconess Medical Center (BIDMC). All mice were housed in a 12-h:12-h light/dark, temperature-controlled room, allowed food and water ad libitum, and kept in specific pathogen-free conditions. Mice were acclimated for 1 wk before experiments were conducted. All experiments were performed in accordance with the guidelines and approval of the Institutional Animal Care and Use Committee of the BIDMC.

Depletion of gut commensal bacteria. Animals were provided ampicillin (1 g/l, Sigma, St. Louis, MO), vancomycin (500 mg/l, Sigma), neomycin sulfate (1 g/l, Sigma), and metronidazole (1 g/l, Sigma) in drinking water for 4 wk, from 4 to 8 wk of age, based on the protocol of Fagarasan et al. (20) and Rakoff-Nahoum et al. (49). Four-week-old control mice were also housed for 4 wk and provided with standard water. After 4 wk of antibiotic treatment, fecal matter was removed from colons using sterile technique and placed in thioglycolate. Both aerobic and anaerobic cultures were performed, and the bacterial species were determined at the Charles River Diagnostic Laboratory (Flamingham, MA).

Flow cytometry. After 4 wk of treatment with antibiotics (n = 6) or standard water (n = 6), lymphoid cells were isolated from PPs and mesenteric lymph nodes (mLNs) from mice. The cells were stained with antibodies conjugated with fluorescent dye for 30 min at room temperature, washed, and fixed with 0.3% paraformaldehyde, and subsets were determined by flow cytometry. The analysis was performed on the LSRII flow cytometer (BD Biosciences, San Jose, CA). In all experiments, at least 10,000 events were recorded and analyzed using FlowJo software ver. 7.6.1 (TreeStar, Ashland, OR).

The following antibodies were used for staining: PAC anti-mouse B220, APC anti-mouse CD38, and PE anti-mouse FAS (BioLegend, San Diego, CA).

Experimental protocol. After 4 wk of antibiotic or standard treatment, mice were prepared for surgery. Anesthesia was induced with 72 mg/kg of pentobarbital (Nembutal; Lundbeck, Deerfield, IL) and maintained with 36 mg/kg of pentobarbital by intraperitoneal injection. All procedures were performed on spontaneously breathing animals. Body temperature was maintained at 37°C using a water-circulating heating pad throughout the experiment.

Animals were subjected to I/R as previously described (46). Briefly, a midline laparotomy was performed before a 30-min equilibration period. The superior mesenteric artery was identified and isolated, and a small nontraumatic vascular clamp (Roboz Surgical Instruments, Rockville, MD) delivering ∼85 g of pressure was applied for 30 min. After this ischemic phase, the clamp was removed and the intestine was allowed to reperfuse for 4 h. Sham-operated mice were subjected to the same surgical intervention, except that they did not undergo superior mesenteric artery occlusion. The laparotomy incision was sutured with 4–0 Sofsilk (Strynture, Mansfield, MA), and mice were resuscitated with 1.0 ml of prewarmed sterile PBS subcutaneously and monitored during the reperfusion period. At the conclusion of the reperfusion period, mice were euthanized with carbon dioxide asphyxiation following the BIDMC IACUC Guidelines. After euthanasia, a 15-cm segment of the small intestine was harvested for further evaluation.

Histology. Tissues from the small intestine were promptly fixed in 10% buffered formalin phosphate after euthanasia. Formalin-fixed tissues were then embedded in paraffin, sectioned transversely in 6-μm sections, stained with hematoxylin and eosin, and examined by light microscope. For each section, 50–100 villi were put on a six-tiered scale as previously described, and mean score was recorded and graded in a third-party blinded fashion (16). Briefly, a score of 0 was assigned to a normal villus; villi with tip distortion were scored as 1; villi lacking goblet cells and containing Gruenhagen’s spaces were scored as 2; villi with patchy disruption of the epithelial cells were scored as 3; villi with exposed but intact lamina propria and epithelial cell sloughing were assigned a score of 4; villi in which the lamina propria was exuding were scored as 5; and finally, villi displaying hemorrhage or denuded villi were scored as 6.

Immunohistochemistry. To perform immunohistochemistry (IHC) staining, formalin-fixed paraffin sections of small intestine were subjected to rehydration, and endogenous peroxidase activity was quenched with 3% H2O2. Then antigen retrieval was performed using Retrieval Solution A (BD Pharmingen, San Jose, CA) according to the manufacturer’s directions. The sections were blocked with 10% BSA/PBS containing the serum from host species of secondary antibody. Primary antibodies prepared in 10% BSA/PBS were applied overnight at 4°C. The slides were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 60 min at room temperature, developed with NovaRED (Vector Laboratories, Burlingame, CA), counterstained with hematoxylin, and dehydrated. The sections were mounted in mounting medium (Thermo Scientific, Waltham, MA) and evaluated with Nikon eclipse 80i microscope. Images were analyzed using Nikon NIS-Elements software (Nikon, Melville, NY).

For IHC studies, the following reagents were used: goat anti-mouse IgM (Abcam, Cambridge, MA), goat anti-mouse IgA (Invitrogen, Carlsbad, CA), mouse anticomplement 3 (C3) (Santa Cruz Biotechnology, Santa Cruz, CA), HRP-conjugated goat anti-mouse IgG (Jack- son ImmunoResearch, West Grove, PA), HRP-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology). Consecutive tissue sections were stained with appropriate isotype controls.

Real-time quantitative RT-PCR. Tissues from the small intestine were immediately snap frozen after harvest and stored at −80°C until RNA purification. Total RNA was isolated from the small intestine using TRIzol reagent (Invitrogen), and cDNA was synthesized using the high-capacity cDNA reverse-transcription kit (Applied Biosystems, Carlsbad, CA) according to manufacturer’s protocol. Real-time RT-PCR was performed with the LightCycler 480 System (Roche, South San Francisco, CA) using TaqMan gene expression Master Mix and predesigned TaqMan probes for mouse TLR2, TLR4, TNF, IL-6, cyclooxygenase (Cox)-2, and GAPDH as recommended by Applied
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Biosystems. The averaged cycle threshold values of each reaction derived from the target gene, determined with LightCycler 480 System software (Roche), were normalized to GAPDH levels. Cycle threshold values were used to calculate relative mRNA expression by the ΔΔCt relative quantification method.

Protein extraction and Western blotting. Tissues from the small intestine were immediately snap frozen after harvest and stored at −80°C. Small intestine from individual mice were homogenized and lysed in freshly prepared RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) (Boston Bioloaboratories, Boston, MA) containing a complete protease inhibitor cocktail (Roche). Concentrations of protein extracts were determined by the Bradford protein assay using Coomassie Brilliant Blue. Protein extracts were separated by gradient SDS-PAGE gels (Invitrogen) under reducing conditions and subjected to standard Western blot analysis. Blots were incubated with primary antibodies and followed by HRP-conjugated secondary antibodies. Specific bands were visualized with enhanced chemiluminescence kit (Pierce, Rockford, IL), and images were obtained using a Fujifilm LAS-4000 luminescent image analyzer (Fujifilm, Valhalla, NY). The density of each band was calculated with Multi Gauge software (Fujifilm, Valhalla, NY) and normalized to actin levels. For Western blotting, the following antibodies were used: goat anti-mouse IgM (Abcam), goat anti-mouse IgA (Invitrogen), goat anti-actin (Santa Cruz Biotechnology), and HRP-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology).

Statistical analysis. Numeric data are presented as means ± SD. Statistical analyses were performed using the unpaired Student’s t-test. SPSS version 17.0 for Windows (SPSS, Chicago, IL) was used for all statistical analyses. A value of P < 0.05 was considered statistically significant.

RESULTS

Depletion of gut commensal bacteria diminished intestinal lymphoid tissues and dilated cecum of treated mice. To address the role of gut commensal bacteria in M I/R injury, we depleted gut microbiota with a broad-spectrum antibiotic cocktail for 4 wk before M I/R. First we evaluated the effects of antibiotic treatment after 4 wk of treatment. We performed both aerobic and anaerobic bacterial cultures on fecal matter from colon and confirmed the complete depletion of both aerobic and anaerobic bacteria in fecal matter from antibiotic-treated mice (data not shown). We also evaluated the number of lymphocytes in PPs and mLNs. In antibiotic-treated mice the number of B cells (B220+) and germinal center cells (GC cells: B220+ CD38- FAS+) in PPs was significantly decreased (Fig. 1, A and B, *P < 0.05) but not in mLNs (data not shown). In addition, the number and size of PPs were macroscopically smaller in antibiotic-treated mice than control mice (data not shown), and the size of spleen was also diminished significantly in antibiotic-treated mice (Fig. 1C, *P < 0.05). Antibiotic-treated mice also displayed enlarged ceca compared with control mice (Fig. 1D). These phenotypes are consistent with germ-free mice (52, 57). These results suggest that antibiotic treatment leads to the reduction of intestinal inflammation and thus less damage after M I/R.

Depletion of commensal bacteria attenuated intestinal injury after M I/R. To determine whether the reduction of GC B cells and the abrogation of commensal bacteria would mitigate intestinal injury, we performed M I/R after antibiotic treatment and evaluated intestinal injury. Histological analysis showed that antibiotic treatment significantly decreased intestinal injury in antibiotic-treated mice compared with nonantibiotic experimental mice in small intestine (Fig. 2, *P < 0.005, **P < 0.01). Villi injury in antibiotic-treated mice occurred mainly in the tips, was infrequent, and was apparently less than in nonantibiotic experimental mice (Fig. 2A). We concluded that depletion of gut flora led to the attenuation of intestinal injury and that gut commensal bacteria played a causative role in intestinal injury after M I/R.

Antibiotic treatment decreased the expression of TLR2 and TLR4 in intestine. It is known that gut commensal bacteria and their products interact and signal through TLRs. TLR2 and TLR4 are especially important for signaling of gram-positive and -negative bacteria (35). Thus we evaluated the effect of antibiotic treatment on TLR2 and TLR4 expression in the intestine following M I/R. The mRNA levels of both TLR2 and TLR4 in the intestine increased significantly after M I/R but remained unchanged in antibiotic-treated mice (Fig. 3, A and B, *P < 0.05, **P < 0.01, ***P < 0.001). These results demonstrated a direct correlation between the presence of commensal bacteria and the expression of TLRs.

Gut commensal bacteria were required for the induction of intestinal inflammation after M I/R. To confirm the role of gut commensal bacteria in the inflammatory response after M I/R, we used quantitative RT-PCR to measure inflammatory mediators in the intestine. In accordance with the results of TLR expression, M I/R increased TNF and IL-6 expression in the intestine, and antibiotic treatment decreased their expression (Fig. 4, A and B, *P < 0.05, **P < 0.01, ***P < 0.005). Previous studies indicated that Cox-2 expression was also elevated in response to M I/R and that it was mediated through TLR4 (44). Thus we also evaluated Cox-2 mRNA expression in the intestine following M I/R as an indicator of tissue damage. We observed a significant increase in Cox-2 expression in the intestine after M I/R, and antibiotic treatment reduced this expression in M I/R group (Fig. 4C, *P < 0.05, ***P < 0.005). These data demonstrated that depletion of gut commensal bacteria decreased the inflammatory response in intestine after M I/R. They also suggested that the change in TLR2 and TLR4 expression after antibiotic treatment was responsible for the reduction of intestinal inflammation after M I/R.

Depletion of gut flora led to reduced deposition of complement in the damaged intestine. Previous studies showed that complement played a key role in initiating intestinal I/R injury (26). To determine whether antibiotic treatment reduced complement activation and deposition in the damaged intestine, we performed IHC. M I/R increased C3 deposition in the intestine compared with sham-operated mice, and antibiotic treatment decreased their expression (Fig. 5, A and B, *P < 0.05, ***P < 0.005), Villi injury in antibiotic-treated mice occurred mainly in the tips, was infrequent, and was apparently less than in nonantibiotic experimental mice (Fig. 2A). We concluded that depletion of gut flora led to the attenuation of intestinal injury and that gut commensal bacteria played a causative role in intestinal injury after M I/R.

IgM deposition in intestine was decreased by antibiotics treatment. Natural antibodies are known to be central to the pathogenesis of mesenteric I/R-mediated injury (67). We previously reported that Igs were key players that triggered I/R-mediated injury. Ig-deficient animals were resistant to I/R-induced injury, and administration of Igs into Ig-deficient animals resulted in intestinal injury after M I/R (25). Thus we evaluated the deposition of natural IgM antibodies in our animal models by IHC. As previously reported, IgM deposition was increased in damaged intestine, and we found intense IgM

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deposition in the tips of the damaged villi (Fig. 6A, top, red staining and arrows). In contrast, depletion of gut microbiota decreased IgM deposition in intestines in both sham and M I/R groups (Fig. 6A, bottom). We also confirmed these results by Western blotting (Fig. 6B and C, *P < 0.05). From these results, we concluded that depletion of gut commensals could prevent overt intestinal I/R injury through the reduction of IgM deposition.

M I/R increased IgA deposition in the intestine and depletion of gut flora reduced IgA deposition in the damaged intestine. GC cells in PPs are chronically induced by commensal bacteria and are the major sites for the generation of gut IgA-mediated immune responses (22). In this study, antibiotic treatment decreased the number of GC cells in PPs. Therefore, we evaluated IgA deposition in the intestines of both control and antibiotic-treated mice after M I/R. IHC staining for IgA revealed that intestinal I/R increased IgA deposition in the intestine compared with sham-operated mice, and antibiotic treatment reduced IgA deposition in intestine following M I/R (Fig. 7A, red staining and arrows). We also performed Western blot analysis to confirm these results (Fig. 7B and C, *P < 0.05). These data implied that IgA also played an important role in intestinal I/R injury.

**DISCUSSION**

The gut is a critical target organ after severe insult such as shock, trauma, sepsis, and surgery. During these severe stress states, it is known that blood flow to the gut is disproportionately decreased to preserve central hemodynamic stability and
that it could cause intestinal I/R, leading to the loss of gut barrier function (17). Dysfunction of the gut barrier after severe insults has been implicated as a major contributor to systemic inflammatory response syndrome, resulting in acute respiratory distress syndrome or multiple-organ dysfunction syndrome (18). Gut commensal bacteria are known to play important roles in maintaining the gut mucosal barrier and the intestinal immune system (32). Gut microbiota stimulate the normal development of the humoral and cellular mucosal immune system during neonatal life and maintain homeostasis of intestinal immune system thereafter (11, 61). Under normal conditions, the gastrointestinal tract provides resistance to both beneficial commensals and potentially pathogenic microorganisms. Perturbations in the composition of gut microbiota in patients or animal models, known as dysbiosis, are associated with multiple diseases such as inflammatory bowel disease (IBD) (41), diabetes (66, 72), allergy (47), autoimmune diseases (6, 38, 68), and obesity (39), and dysbiosis can greatly influence the pathogenesis and severity of inflammatory diseases. Some inflammatory diseases have been reported to be clearly linked to certain gut bacteria or their factors (29, 43, 47, 68). Animal models of IBD have provided additional insights into the role of intestinal bacteria in the pathogenesis of IBD. IL-2-deficient mice, which are regarded as a model of chronic, immune-mediated colitis with dysregulated T cell function, have a delayed and milder intestinal inflammation when raised under germ-free conditions (56). IL-10-deficient mice, which develop inappropriate inflammatory responses and spontaneous colitis when raised under conventional conditions, have no evidence of colitis and immune system activation when maintained under germ-free conditions (53). These findings suggest that signals from gut microbiota play essential roles to drive pathogenic inflammatory responses in these models. On the contrary, in the dextran sulfate sodium-induced chronic colitis model, commensal depletion was reported to increase injury and TLR-dependent signaling from commensal bacteria was
Gut commensal bacteria are protective against colitis (49). In acute intestinal inflammatory states, there are some reports that implicate a role for gut commensal bacteria. Clinically, abnormal composition of gut flora has been considered to have major influences on the severity and prognosis of critically ill patients in acute states (18, 34, 55, 62). In laboratory animals, consistent with our data, Souza et al. (59) reported that there was no local, remote, or systemic inflammatory response in germ-free mice after intestinal I/R and concluded that the lack of intestinal microbiota is accompanied by a state of active IL-10-mediated inflammatory hyporesponsiveness that leads to decreased injury. However, Chen et al. (15) showed that depletion of commensal bacteria augmented intestinal injury after M I/R and that TLR ligand administration decreases M I/R injury-induced gut damage through TNF signaling (15). The possible reasons of the disparate outcomes from these studies, which involved antibiotic treatment, may relate to difficulties in depleting gut microbiota by oral antibiotic administration and diversity of gut commensal bacteria. As Reikvam et al. (50) previously mentioned, depletion of gut commensal bacteria by administering antibiotics in drinking water is technically difficult, and it will change from facility to facility depending on its environment.
Our animal facility is specific pathogen free-certified, and we treated mice aseptically as much as possible, using filtered antibiotic cocktail and irradiated food and changing cages every day aseptically to avoid contamination by fecal matter. We could decrease and deplete gut microbiota as much as possible and confirmed by bacterial cultures. However, mice could still have a few bacteria that could not be detectable by culture. Therefore, we confirmed that our antibiotic-treated mice had the phenotype close to germ-free mice (Fig. 1). The antibiotic-treated mice in Chen’s study and ours might have less bacteria, and the remaining species of bacteria could be different depending on the environment and the vendor source. This difference could also affect the extent of inflammation and injury following M I/R. The role of gut commensal bacteria in acute intestinal injury can differ depending on various factors such as environment, age, composition of gut microbiota, anatomical location in intestine, and type of injury.

TLR signaling is also important to understand the relationship between gut flora and intestinal I/R injury. Innate immune responses against microorganisms are mediated by innate TLRs, nucleotide-binding oligomerization domain-like receptors, and G protein-coupled receptors (1, 10, 32). Bacterial products released during I/R could be detected by the immune system through TLRs. This TLR-mediated recognition leads to the production of proinflammatory cytokines and chemokines. Recently TLR activation has been demonstrated to play an essential role in I/R injury in the liver, kidney, and gut (4, 5). In the gut, M I/R was reported to elevate TLR2 and TLR4 expression in intestine (64). In a M I/R model of adult mice, Victoni et al. (63) demonstrated that acute intestinal and lung injury decreased in MyD88-deficient mice, was associated with TNF and IL1-β production, and led to enhanced survival. Moses et al. (44) demonstrated that the absence of TLR4 signaling attenuated intestinal mucosal damage with significantly decreased cytokines and eicosanoid secretion including prostaglandin E2 production. However, in a M I/R model of neonatal mice, TLR2- or TLR4-deficient mice had increased intestinal injury (3, 60). The role of TLR signaling in acute intestinal inflammation remains controversial.

In this study, we demonstrated that depletion of gut commensal bacteria resulted in attenuation of intestinal I/R injury. In our animal model, we depleted gut commensal bacteria with a broad-spectrum antibiotic cocktail for 4 wk resulting in diminished PPs and spleen size, both of which are major sites of inflammatory responses, and led to reduced intestinal inflammation and injury following M I/R. Here we also addressed the reduction of TLR2 and TLR4 expression and the downstream proinflammatory mediators in damaged intestine. Although these mRNA data were from multiple cell lineages in the intestine, they demonstrated that reduced TLR signaling and diminished inflammatory response is a plausible outcome for the effects of the antibiotic treatment. Further analyses using single cell populations from intestinal tissues are essential to clarify the detailed mechanisms of TLR signaling in this model.

Natural antibodies and complement have been considered as key contributors in initiating intestinal I/R injury (26, 27, 67, 71). Hill et al. (33) were the first to report that inhibition of the
complement pathway by soluble complement receptor 1 ameliorated intestinal and remote lung injury after I/R. The importance of natural antibody in intestinal I/R injury has emerged from the study by Williams et al. (67). On the basis of these observations, it has been proposed that intestinal I/R injury is initiated by natural antibodies binding to neo-antigens that are exposed in ischemic intestine. This hypothesis has been confirmed by many studies (27, 37, 67, 69, 71). These natural antibodies are mainly produced by B1 and marginal zone B cells, which reside primarily within the peritoneum and the spleen, respectively, and are known to act as the first line of host defense against pathogens (7, 8, 21). B1 B cells are thought to act against I/R-related neo-antigens in intestinal I/R injury (26, 27, 67, 71). Recent studies identified specific self-reactive natural antibodies associated with intestinal I/R injury, such as CM22, an antibody that recognizes the cryptic neo-antigen, annexin IV (37, 70). Zhang et al. (69) identified a natural IgM that targeted another ischemia-induced antigen, nonmuscle myosin heavy chain II, which is expressed on the surface of ischemic tissue and was a target of CM22. Similar to natural antibodies, autoantibodies have also been reported to induce intestinal I/R injury in I/R-resistant mice (24, 25, 36). In this study, we evaluated complement and IgM deposition in damaged intestine to confirm the protective effect of antibiotic treatment on intestinal I/R injury. M I/R increased complement and IgM deposition in control mice. As expected, depletion of gut flora decreased complement and IgM deposition in intestine and attenuated intestinal I/R injury. These data suggest that...
depletion of commensal bacteria decrease intestinal injury through the reduction of complement and IgM deposition in intestine.

IgA is considered to be protective in intestine under normal conditions. It binds to pathogens and prevents them from entering through the epithelial barrier. Interaction of commensal bacteria with IgA-producing plasma cells triggers the production of IgA from these cells (12, 40). In this study, we found elevated levels of IgA in intestine following M I/R (Fig. 7). This is the first evidence that suggests that IgA expression may contribute to intestinal I/R injury. However, it is still unclear whether increased IgA protects the intestine or whether IgA plays a role in increasing intestinal injury as well, similar to other Ig isotypes (24, 25, 27). We also showed that antibiotic treatment decreased IgA expression both in sham and M I/R mice, which eventually led to attenuation of intestinal injury. Together these results suggest that IgA, similar to IgM, is involved in exacerbating intestinal injury following M I/R. However, further studies are needed to determine the precise role of IgA in acute inflammation after M I/R.

In conclusion, our results clearly demonstrate that gut commensal bacteria contribute to intestinal I/R injury. In intestinal I/R injury, ischemia can lead to alteration in surface epitopes of cells subjected to ischemia, and natural antibodies bind to these epitopes and subsequently activate the complement system. These changes can activate and attract inflammatory cells such as neutrophils and monocytes. The complement system amplifies the inflammatory response, leading to further tissue damage.

**Fig. 7.** IgA deposition in small intestine following M I/R. We evaluated the deposition of IgA by IHC and Western blots. A: representative IgA staining in each group. B: representative Western blots of IgA and actin from each group. C: quantitation of IgA signal measured by densitometry from Western blots normalized to actin (n = 3 per each group). All photomicrographs were ×200 magnification. Brown staining shows IgA deposition, and blue staining was nuclear staining with hematoxylin (*P < 0.05). Results are representative of 2 experiments for Western blots and 2 experiments for IHC.
as neutrophils, macrophages, and lymphocytes. These inflammatory cells cause inflammation in the intestine and augment I/R-induced injury. Depletion of the gut flora diminished lymphoid tissues, decreased deposition of immunoglobulins and complement, led to attenuation of intestinal inflammation, and improved intestinal integrity. Further studies are needed to determine the mechanisms that regulate gut commensal bacteria and intestinal inflammatory responses in intestinal I/R injury. Comprehensive understanding of these mechanisms will provide new therapeutic possibilities, such as manipulation of gut flora with probiotics, antibiotics, or other interventions, to improve prognosis of critically ill patients with intestinal I/R injury.

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DISCLOSURES
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