Critical role of interleukin-17A in murine intestinal ischemia-reperfusion injury

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LEET, T. M. KIM, S. T. BROWN, M. HAMB, V. D. D’AGATI, AND Y. MORI-AKIYAMA. Critical role of interleukin-17A in murine intestinal ischemia-reperfusion injury. Am J Physiol Gastrointest Liver Physiol 304: G12–G25, 2013. First published November 1, 2012; doi:10.1152/ajpgi.00201.2012.—Intestinal ischemia-reperfusion (I/R) injury causes severe illness frequently complicated by remote multiorgan dysfunction and sepsis. Although the exact barrier necrosis and dysfunction with increased bacterial translocation and systemic inflammation and sepsis. Recent studies implicated interleukin-17A (IL-17A) in regulating inflammation, autoimmunity, and I/R injury. Here, we determined whether IL-17A is critical for generation of intestinal I/R injury and subsequent liver and kidney injury. Mice subjected to 30 min of superior mesenteric artery ischemia not only developed severe small intestinal injury (necrosis, apoptosis, and neutrophil infiltration) but also developed significant renal and hepatic injury. We detected large increases in IL-17A in the small intestine, liver, and plasma. IL-17A is critical for generating these injuries, since genetic deletion of IL-17A- or IL-17A-neutralizing antibody treatment markedly protected against intestinal I/R injury and subsequent liver and kidney dysfunction. Intestinal I/R caused greater increases in portal plasma and small intestine IL-17A, suggesting an intestinal source for IL-17A generation. We also observed that intestinal I/R caused rapid small intestinal Paneth cell degranulation and induced murine α-defensin cryptdin-1 expression. Furthermore, genetic or pharmacological depletion of Paneth cells significantly attenuated the intestinal I/R injury as well as hepatic and renal dysfunction. Finally, Paneth cell depletion significantly decreased small intestinal, hepatic, and plasma IL-17A levels after intestinal I/R. Taken together, we propose that Paneth cell-derived IL-17A may play a critical role in intestinal I/R injury as well as extraintestinal organ dysfunction.

INTESTINAL ISCHEMIA-REPERFUSION (I/R) injury is a serious and frequent clinical problem after aortic surgery, trauma, liver, and intestinal transplantation (15). In addition, acute mesenteric ischemia is a dire surgical emergency with a mortality rate exceeding 50% (3, 38). Intestinal I/R leads to gut epithelial barrier necrosis and dysfunction with increased bacterial translocation and proinflammatory cytokine dissemination, resulting in systemic inflammation and sepsis. Although the exact mechanisms leading to remote organ injury after intestinal I/R remain obscure, direct portal delivery of proinflammatory mediator(s) from injured small intestine may cause hepatic injury and systemic inflammation.

Recent studies identified a critical role for interleukin (IL)-17A in several autoimmune diseases, systemic inflammation, and I/R injury (10, 32, 40, 41). However, it remains to be determined whether IL-17A is critical in generating intestinal injury with subsequent remote multiorgan dysfunction. Furthermore, although previous studies implicated T lymphocytes as the major source of IL-17A after I/R injury (8, 40), we previously showed that small intestine generated a robust amount of IL-17A after acute injury to the liver or kidney (29, 32). Therefore, the source of IL-17A in generating intestinal I/R needs to be elucidated.

Paneth cells located in the base of intestinal crypts contain large eosinophilic granules and provide mucosal immunity against pathogens by actively secreting several antimicrobial peptides (e.g., α-defensins) (16, 27, 28). We previously showed that hepatic I/R caused Paneth cell degranulation and increased secretion of Paneth cell-derived IL-17A (32). Furthermore, Paneth cells produce and release IL-17A to mediate tumor necrosis factor (TNF)-α-induced shock (8, 41). Depletion of Paneth cells not only provided significant hepatic protection but also attenuated systemic generation of IL-17A after liver I/R. These findings suggest that small intestinal Paneth cells may serve as a source and reservoir of proinflammatory IL-17A to initiate intestinal injury as well as systemic inflammation and remote organ injury after intestinal I/R.

In contrast, Grootjans et al. recently described that intestinal I/R injury causes rapid Paneth cell apoptosis with subsequent bacterial translocation to the liver and spleen (13). Furthermore, Paneth cell granule depletion exacerbated bacterial translocation and systemic inflammation after hemorrhagic shock in rats, implicating a protective role of this cell type against organ injury. Therefore, the exact role of small intestinal Paneth cells in remote organ dysfunction and systemic inflammation after intestinal I/R remains unclear. In this study we tested the hypothesis that IL-17A plays a critical role in a murine model of warm intestinal I/R injury and aimed to identify the source of IL-17A critical for generating intestinal I/R injury utilizing genetic and pharmacological approaches.

MATERIALS AND METHODS

Murine model of intestinal I/R. C57BL/6 mice [20–25 g, wild-type (WT) control mice for IL-17A-deficient mice] were obtained from Harlan (Indianapolis, IN). IL-17A-deficient mice on a C57BL/6 background were obtained from Dr. Yojiro Iwakura (University of Tokyo, Tokyo, Japan) through Dr. Jay K. Kolls (Louisiana State University, New Orleans, LA) (24). Paneth cell-deficient mice (Sox9Δbox/Δfox/Villin Cre+/−) were generated as described (22). Sox9 transcription factor is required for the differentiation of Paneth cells,

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and intestinal-specific deletion of Sox9 results in near absence of Paneth cells. Sox9flox/+;Villin Cre−/− littermate mice were used as WT controls. After Columbia University Institutional Animal Care and Use Committee approval, male mice were subjected to sham surgery or 30 min of superior mesenteric artery (SMA) ischemia. After unclamping of the SMA, 0.5 ml warm saline was administered subcutaneously. Sham-operated mice underwent identical abdominal manipulations as mice subjected to intestinal I/R (laparotomy, intestinal retraction, and positioning). Samples (including systemic and portal plasma, small intestine, liver, and kidney) were collected 5 h after sham operation or intestinal I/R.

To deplete Paneth cell granules, mice were treated with dithizone (100 mg/kg iv; Sigma, St. Louis, MO) 6 h before SMA occlusion as described (37, 39). Dithizone (10 mg/ml) was dissolved in saturated lithium carbonate (1 g/100 ml). To neutralize IL-17A, mice were injected intravenously with 200 μg of anti-mouse IL-17A antibody (eBioscience, San Diego, CA) immediately before SMA ischemia.

**Plasma alanine aminotransferase activity and creatinine levels after intestinal I/R.** Plasma alanine aminotransferase (ALT) activity and creatinine (Cr) levels were measured using the Infinity ALT assay kit and an enzymatic Cr reagent kit, respectively, according to the manufacturer’s instructions (Thermo Fisher Scientific, Waltham, MA).

**Histological analysis of intestine I/R injury.** Small intestine (jejunum and ileum) was washed in ice-cold PBS and fixed overnight in 10% formalin. After automated dehydration through a graded alcohol series, tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin (H&E). Intestinal H&E sections were graded for intestinal I/R-induced mucosal injury using the Chiu score (7) by a pathologist (D’Agati) blinded to the samples as described previously (18). We also graded the degree of Paneth cell degranulation after SMA I/R with a semiquantitative score (0 –3 in ×200 field).

**Determination of apoptosis after intestinal I/R.** Apoptosis was detected in the small intestine with terminal deoxynucleotidyl transferase nick end-labeling (TUNEL) staining of fragmented DNA using a commercially available in situ cell death detection kit (Roche, Indianapolis, IN) according to the instructions provided by the manufacturer as described previously (29). TUNEL-positive cells per 200 field were counted to quantify apoptosis (10 random fields were counted/slide).

**Assessment of small intestine neutrophil infiltration after intestinal I/R.** Neutrophil infiltration in the small intestine after SMA I/R was determined with immunohistochemistry as described previously with a monoclonal antibody against polymorphonuclear neutrophils (PMN) (clone 7/4; AbD Serotec, Raleigh, NC) (29). A primary antibody that recognized IgG2a (MCA1212; AbD Serotec) was used as a negative isotype control in all experiments.

**Small intestine immunofluorescence staining for IL-17A, PMN, and CD11b.** Small intestines were embedded in Tissue-Tek oxytetracycline compound (Fisher Scientific, Pittsburgh, PA) and cut into 5-μm sections. After fixation in 4% paraformaldehyde overnight, sections were blocked in 10% BSA in PBS for 1 h at room temperature and incubated with CD3 (MCA771G) or PMN (MCA500G) primary antibody (1:100 dilution; Serotec) in a humidified chamber for 16 h at 4°C. Secondary antibody (green fluorescent donkey anti-rat immunoglobulin G, 1:200 dilution) was then applied at room temperature for 1 h. Subsequently, IL-17A primary antibody (1:100 dilution, 16 h at 4°C; sc-6077; Santa Cruz Biotechnology) and red fluorescent secondary antibody (donkey anti-goat immunoglobulin G) were applied. Sections were imaged with an Olympus IX81 epifluorescence microscope (Tokyo, Japan) and captured and stored using SlideBook 4.2 software (Intelligent Imaging Innovations, Denver, CO).

**Measurement of proinflammatory mRNA expression after intestinal I/R.** Intestinal, renal, and hepatic inflammation after SMA I/R in mice were additionally determined by measuring mRNA encoding markers of inflammation, including IL-17A, intercellular adhesion molecule 1 (ICAM-1), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 2 (MIP-2), and TNF-α and IL-6 (liver and kidney only) (Table 1). RT-PCR was performed as described (29). We also performed quantitative real-time RT-PCR (Q-RTPCR) with the MxIQ Real Time Detection System (Bio-Rad, Hercules, CA) using SYBR Green I Brilliant Mastermix (Stratagene, La Jolla, CA) to detect relative IL-17A expression in jejunum, liver, and kidney. The Ct values were determined by using Mx3000P software. Values were normalized for GAPDH mRNA, and relative expression of IL-17A mRNA in each tissue was calculated with the ΔΔCt method.

**Enzyme-linked immunosorbent assay for plasma IL-17A.** Five hours after intestinal I/R, plasma (portal and systemic) and tissue (small intestine, liver, and kidney) IL-17A levels were measured with a mouse-specific IL-17A enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions (eBioscience) as described (29). To test whether the neutralizing antibody given 5 h before sample collection interfered with IL-17A ELISA, we injected naive mice with 200 μg of anti-mouse IL-17A antibody, or with an equal dose of isotype antibody (n = 3 in each group, eBioscience). Five hours after antibody injection, we collected their plasma and jejunum for IL-17A ELISA. Before performing ELISA, plasma and jejunum samples were spiked with increasing concentrations (0–1,000 pg/ml) of recombinant mouse IL-17A. Our ELISA results show

<table>
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<th>Primers</th>
<th>Accession No.</th>
<th>Sequence (sense/antisense)</th>
<th>Product size, bp</th>
<th>Cycle No.</th>
<th>Annealing Temperature, °C</th>
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<td>5′-GAGAGCGTCTGCTGTTAAGG-3′</td>
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bp, Base pairs; TNF-α, tumor necrosis factor-α; ICAM-1, intercellular adhesion molecule 1; MCP-1, monocyte chemotactic protein 1; MIP-2, macrophage inflammatory protein 2; IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
that the antibody used to neutralize the IL-17A in vivo does not interfere with the quantification of plasma and tissue IL-17A.

Detection of small intestinal cryptdin-1 mRNA expression. Five hours after sham operation or intestinal I/R, RT-PCR was performed to analyze the expression of cryptdin-1 (as a marker for mature murine Paneth cells as described) (6, 30, 31) (Table 1).

Statistical analysis. The data were analyzed with a two-tailed Student’s *t*-test when comparing means between two groups. One-way ANOVA plus Tukey’s post hoc multiple-comparison test was used when comparing multiple groups. The ordinal values of the Chiu scores were analyzed by the Mann-Whitney nonparametric test. In all cases, a probability statistic <0.05 was taken to indicate significance. All data are expressed throughout the text as means ± SE.

RESULTS

Intestinal I/R causes acute liver and kidney injury in mice. We first determined whether intestinal I/R injury led to remote liver and kidney injury in mice. IL-17A WT mice subjected to 30 min of SMA occlusion and 5 h of reperfusion developed severe necrotic damage to the intestinal mucosa with sloughing of the villous tips, lifting of the epithelium from the lamina propria, and development of subepithelial Gruenhagen’s spaces (IL-17A WT I/R, H&E staining, Fig. 1A). Intestinal I/R significantly increased the Chiu intestine injury score compared with sham-operated mice (Fig. 1B). Furthermore, small intestinal I/R in IL-17A WT mice led to liver dysfunction with significantly higher plasma ALT (>6-fold) and acute kidney injury (AKI) with significant rises in plasma Cr (~3-fold) 5 h after injury compared with sham-operated mice (IL-17A WT I/R, Fig. 2).

Intestinal I/R increases plasma and tissue IL-17A production. Next we determined whether plasma and tissue IL-17A levels increased after intestinal I/R injury. Five hours after small

Fig. 1. Critical role of interleukin (IL)-17A in intestinal ischemia-reperfusion (I/R) injury. A: representative photomicrographs of jejunum from 4–5 experiments (hematoxylin and eosin staining, magnifications of ×200). Compared with sham-operated IL-17A wild-type (WT) mice, intestinal I/R followed by 5 h reperfusion led to severe damage to the intestinal mucosa, sloughing of the villous tips, and the development of subepithelial Gruenhagen’s spaces. In contrast, the intestines of IL-17A-deficient mice (IL-17A KO I/R) or IL-17A WT mice treated with IL-17A neutralizing antibody (IL-17A AB I/R) were protected from small intestinal injury. SMA, superior mesenteric artery. B: small intestinal histology was evaluated with Chiu scores (scale 0–5) in the jejunum. Tissues were collected 5 h after intestinal I/R. *P < 0.05 vs. IL-17A WT sham group. #P < 0.05 vs. IL-17A WT I/R group. Data are presented as means ± SE.
IL-17A plays a critical role in intestinal, renal, and hepatic injury after intestinal I/R in mice. Induction of IL-17A after intestinal I/R plays a critical role in generating intestinal mucosal injury, since mice treated with IL-17A neutralizing antibody (IL-17A AB I/R) had better preserved intestinal histology and significantly lower Chiu scores compared with IL-17A WT I/R mice (Fig. 1). IL-17A WT mice treated with IL-17A antibody also had significantly reduced hepatic and renal injury after intestinal I/R (Fig. 2). In addition, mice deficient in IL-17A (IL-17A KO I/R) were protected against intestinal, hepatic, and renal injury after intestinal I/R compared with IL-17A WT I/R mice (Figs. 1 and 2). Systemic and portal vein plasma, jejunum, and liver IL-17A levels were significantly reduced in mice treated with IL-17A antibody and in IL-17A-deficient mice (Fig. 3, A and B) after intestinal I/R compared with IL-17A WT I/R mice. Kidney IL-17A levels, most likely due to smaller increases in IL-17A, were not affected by IL-17A neutralizing antibody treatment. These data also suggest that the reduction in renal injury with IL-17A modulation after intestinal I/R is due to reduced intestinal and/or hepatic injury rather than due to the reduction in kidney IL-17A levels.

As T lymphocytes as well as neutrophils can express IL-17A (8), we also determined whether increased intestinal IL-17A induced by I/R is actually localized in Paneth cells. We performed immunohistochemistry for IL-17A and PMN as well as IL-17A and CD3 in jejunum of mice subjected to sham surgery or to SMA I/R. Figure 3C shows that IL-17A stain (red) was localized in small intestinal crypts (Paneth cells) in sham-operated mice (representative of 3 experiments). Five hours after small intestinal I/R, IL-17A staining increased in Paneth cells. We also detected increased IL-17A staining in intestinal epithelia of mice subjected to SMA I/R. In contrast, we determined that most CD3-positive cells (green, Fig. 3, top) or PMNs (green, Fig. 3, bottom) did not express IL-17A after small intestinal I/R.

IL-17A neutralization or deficiency reduces small intestinal neutrophil infiltration after I/R. Thirty minutes of SMA ischemia and 5 h of reperfusion resulted in neutrophil recruitment into the small intestine in IL-17A WT mice (IL-17A WT I/R, jejunum shown, ×200, Fig. 4). Neutrophil infiltration occurred heavily at the base of the small intestine and in the necrotic intestinal epithelial villi area (representative of 4 experiments). Neutralization of IL-17A (IL-17A KO I/R) or deficiency in IL-17A (IL-17A KO I/R) reduced the neutrophil infiltration in the small intestine after I/R (Fig. 4).

IL-17A neutralization or deficiency reduces proinflammatory gene expression in the intestine, kidney, and liver after intestinal I/R. We also measured the expression of proinflammatory cytokine mRNAs in the intestine (TNF-α, ICAM-1, MCP-1, and MIP-2), liver, and kidney (TNF-α, ICAM-1, MCP-1, MIP-2, and IL-6) 5 h after small intestinal I/R with RT-PCR (primer sequences listed in Table 1). IL-17A WT mice subjected to small intestinal I/R (IL-17A WT I/R) significantly increased the expression of all proinflammatory mRNAs examined in all three organs compared with the sham-operated IL-17A WT mice (Fig. 5). However, neutralization of IL-17A (IL-17A AB I/R) or deficiency in IL-17A (IL-17A KO I/R) significantly reduced the proinflammatory mRNA expression in all three organs.

IL-17A neutralization or deficiency reduces small intestinal apoptosis after I/R. Small intestinal I/R caused massive small intestinal epithelial apoptosis 5 h after injury in IL-17A WT mice (examined with TUNEL staining, jejunum shown, ×100,
30 min SMA ischemia and 5 hr reperfusion

**A**

Systemic Plasma IL-17A (pg/mL)

- IL-17A WT Sham
- IL-17A KO Sham
- IL-17A WT IR
- IL-17A AB IR
- IL-17A KO IR

Portal Vein Plasma IL-17A (pg/mL)

- IL-17A WT Sham
- IL-17A KO Sham
- IL-17A WT IR
- IL-17A AB IR
- IL-17A KO IR

**B**

Intestine IL-17A (pg/mg protein)

- IL-17A WT Sham
- IL-17A WT IR
- IL-17A AB IR
- IL-17A KO IR

Liver IL-17A (pg/mg protein)

- IL-17A WT Sham
- IL-17A WT IR
- IL-17A AB IR
- IL-17A KO IR

Kidney IL-17A (pg/mg protein)

- IL-17A WT Sham
- IL-17A WT IR
- IL-17A AB IR
- IL-17A KO IR

**C**

200X

IL17A (Sham) -> SMA IR

- Lumen
- IL17A
- CD3
- Merged

Sham-operation or 30 min SMA ischemia and 5 hr reperfusion
jejum confirmed significant reductions in the number of TUNEL-positive cells in IL-17A antibody-treated mice and IL-17A-deficient mice subjected to small intestinal I/R (Fig. 6B).

Pharmacological depletion or genetic deletion of Paneth cells attenuates intestinal, hepatic, and renal injury after intestinal I/R. The next series of experiments were performed to determine the role of small intestinal Paneth cells in generating intestinal I/R injury. We first demonstrated that Paneth cells rapidly (~<5 h) degranulated after small intestinal I/R (Fig. 7A). We also determined that intestinal I/R significantly upregulated cryptdin-1 mRNA (a murine Paneth cell defensin and a specific marker for Paneth cells) (Fig. 7B).

Subsequently, we tested the hypothesis that pharmacological depletion of Paneth cell granules or genetic deletion of Paneth cells attenuates intestinal I/R injury. Previous studies have shown that zinc depletion with dithizone treatment rapidly results in the loss of Paneth cell secretory granules in mice (37, 39). Accordingly, we subjected dithizone- or vehicle (LiCO3)-treated mice to SMA I/R to test the effect of pharmacological Paneth cell ablation on the response to intestinal I/R injury. We also performed SMA I/R in mice genetically deficient in the Paneth cell lineage (with intestine-specific Sox9 gene deletion in Sox9floxflox Villin Cre+/- mice) (2, 22).

Our studies show that small intestinal Paneth cells play a critical role in generating intestinal mucosal injury after intestinal I/R. Mice treated with dithizone (dithizone I/R) or mice deficient in Paneth cells (Sox9 KO I/R) had better preserved intestinal histology and significantly lower Chiu scores compared with vehicle-treated or Sox9 WT mice subjected to intestinal I/R (Fig. 8). In addition, Paneth cell depletion or deletion also reduced hepatic (ALT) and renal (Cr) injury after intestinal I/R in mice (Fig. 9). Furthermore, plasma, jejunum, and liver IL-17A levels were significantly reduced in Paneth cell-deficient mice after intestinal I/R (Fig. 10). Paneth cell deficiency resulted in >50% reduction in systemic IL-17A and >80% reduction in portal plasma IL-17A levels 5 h after SMA I/R (Fig. 10). Paneth cell-deficient mice also had >70% reduction in jejunum IL-17A and >60% reduction in liver IL-17A levels 5 h after SMA I/R. Kidney IL-17A, was not affected by Paneth cell deficiency, most likely due to a small increase in IL-17A.

Pharmacological depletion or genetic deletion of Paneth cells attenuates intestinal apoptosis and inflammation after I/R. We also determined that Paneth cell depletion or Paneth cell deficiency markedly attenuated small intestinal apoptosis (TUNEL staining, Fig. 11) after SMA I/R (jejumon shown, representative of 4 experiments). Vehicle (LiCO3)-treated or Paneth cell WT mice (Sox9floxflox Villin Cre+/-, Sox9 WT)
Fig. 5. IL-17A neutralization or deficiency reduces proinflammatory gene expression in the intestine, liver, and kidney after intestinal I/R. A: representative gel images (top) of RT-PCR and densitometric quantification of relative band intensities normalized to GAPDH (bottom) of proinflammatory markers tumor necrosis factor-α (TNF-α), intercellular adhesion molecule 1 (ICAM-1), monocyte chemotactic protein 1 (MCP-1), and macrophage inflammatory protein 2 (MIP-2) from jejunum of mice subjected to SMA I/R (n = 4–5/group). B–E: representative gel images (B and D) of RT-PCR and densitometric quantification of relative band intensities normalized to GAPDH (C and E) of proinflammatory markers TNF-α, ICAM-1, MCP-1, MIP-2, and IL-6 from liver (B and C) and kidney (D and E) of mice subjected to SMA I/R (n = 4–5/group). IL-17A WT mice subjected to intestinal I/R (IL-17A WT I/R) significantly increased the expression of all proinflammatory mRNAs examined compared with the sham-operated IL-17A WT mice. However, neutralization of IL-17A (IL-17A AB I/R) or deficiency in IL-17A (IL-17A KO I/R) significantly reduced the proinflammatory mRNA expression after intestinal I/R. *P < 0.05 vs. sham-operated mice. #P < 0.05 vs. IL-17A WT I/R. Error bars represent 1 SE. Data analyzed with 1-way ANOVA plus Tukey’s post hoc multiple-comparison test.
observed in mice genetically deficient in Paneth cells and subjected to intestinal I/R (data not shown). We again determined increased cryptdin-1 mRNA after SMA I/R in vehicle-treated mice (Fig. 12, A and B). Depletion of Paneth cells with dithizone significantly attenuated this increase in cryptdin-1 expression after SMA I/R.

**DISCUSSION**

Intestinal I/R injury is a serious and frequent clinical problem in the perioperative period. Many major surgical procedures, including aortovascular surgery, intestinal or liver transplantation, as well as hepatic and pancreatic resections, require an obligate period of intestinal ischemia that is an important determinant of short- and long-term outcomes (3, 9, 14). Furthermore, acute thrombotic or embolic mesenteric ischemia is associated with an exceedingly high mortality rate despite appropriate surgical intervention (3, 9, 33). After intestinal I/R, significant mucosal injury occurs during the reperfusion phase (3, 9). Furthermore, intestine-derived cytotoxic factors (e.g., free radicals, cytokines, high-mobility group protein 1) cause systemic inflammation and remote multiorgan dysfunction after intestinal I/R (19, 42). Therefore, the intestine has been referred to as “the motor of multiorgan failure” (21, 42). Unfortunately, strategies to diminish the cascade of events leading to intestinal and multiorgan injury during and after intestinal I/R are lacking. Our studies suggest that modulation of small intestinal Paneth cell IL-17A release may improve outcome from the intestinal I/R injury.

We show in this study a critical role of IL-17A in generating intestinal I/R injury and subsequent remote liver and kidney dysfunction. IL-17A has been shown to play a critical role in inflammation as well as host immune defense (8, 41). Indeed, IL-17A is upregulated in a variety of inflammatory conditions, including rheumatoid arthritis, systemic lupus erythematosus, and sepsis (20, 43). Furthermore, IL-17A induces expression of several additional proinflammatory genes, including IL-8, TNF-α, and IL-1 (43). Although IL-17A was originally detected from the Th17 subset (5, 12), subsequent studies have demonstrated that many other cell types, including NK T cells, neutrophils, and Paneth cells, can produce IL-17A (8). Surprisingly, intestinal lamina propria was shown to produce IL-17A in naive animals (17). IL-17A has been detected in the intestinal lamina propria but not in the spleen, mesenteric lymph nodes, or Peyer’s patches of a naive mouse (1). Our findings suggest intestinal Paneth cells as an important source of initial IL-17A upregulation after intestinal I/R injury. We believe that Paneth cell-derived IL-17A drives intestinal inflammation. We also propose that Paneth cell-derived IL-17A propagates to the liver via portal drainage with subsequent induction of IL-17A from other cell types, including Th17 T cells.

We implicate in this study a critical role for small intestinal Paneth cell-derived IL-17A in directly causing intestinal injury after I/R. Pharmacological Paneth cell depletion or genetic

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**Fig. 6. IL-17A neutralization or deficiency reduces small intestinal apoptosis after I/R.** A: representative fluorescent photomicrographs (of 4–5 experiments, magnifications of ×100) of TUNEL staining illustrating severe jejunal apoptosis after intestinal I/R injury. Thirty minutes of SMA ischemia and 5 h of reperfusion resulted in numerous TUNEL-positive cells in the intestinal epithelia of IL-17A WT mice (IL-17A WT I/R). Neutralization of IL-17A (IL-17A AB I/R) or deficiency in IL-17A (IL-17A KO I/R) markedly reduced the number of apoptotic cells in the small intestine after I/R. We believe that Paneth cell-derived IL-17A drives intestinal inflammation. We also propose that Paneth cell-derived IL-17A propagates to the liver via portal drainage with subsequent induction of IL-17A from other cell types, including Th17 T cells.

We implicate in this study a critical role for small intestinal Paneth cell-derived IL-17A in directly causing intestinal injury after I/R. Pharmacological Paneth cell depletion or genetic
deletion significantly reduced the induction of IL-17A and attenuated multiorgan injury after intestinal I/R. In this study, we not only detected increased plasma and tissue IL-17A after small intestinal I/R injury but also determined that the biggest IL-17A induction occurred in the intestine. Consistent with these findings, IL-17A mRNA expression was dramatically higher in the small intestine compared with liver or kidney after intestinal I/R. Furthermore, IL-17A immunoreactivity was localized to small intestinal Paneth cells in sham-operated mice. Finally, we observed profound increases in Paneth cell IL-17A expression after SMA I/R. Although T lymphocytes and neutrophils have been shown to express IL-17A (8), we failed to show significant IL-17A protein expression in CD3 or PMN infiltrating the small intestine after SMA I/R.

Supporting our findings, Takahashi et al. showed that IL-17A produced by Paneth cells mediates TNF-α-induced inflammation and shock (41). We also previously demonstrated that hepatic I/R acutely causes rapid release of small intestinal Paneth cell IL-17A, leading to intestinal injury and systemic inflammation (32). Paneth cell IL-17A generated by intestinal I/R may induce increased production of additional (e.g., TNF-α and IL-6) cytokines, further potentiating intestinal epithelial injury and remote organ injury. Because IL-17A regulates both innate and acquired immunity, IL-17A may serve as a crucial regulator of host defense systems. Taken together, our collective findings suggest that Paneth cell-derived IL-17A is critical for inducing intestinal injury as well as remote organ dysfunction after intestinal I/R.

The zinc chelator dithizone has been shown to deplete Paneth cell granules in mice and rats (37, 39). However, dithizone may be limited by systemic and nonspecific side effects (e.g., effects on lung vascular permeability), and Paneth cell depletion is transient. Therefore, we also performed studies in Sox9/Villin Cre−/− mice. These mice lack the Sox9 transcription factor in intestinal epithelial cells and show markedly reduced number of mature Paneth cells (2, 22). Both approaches of Paneth cell depletion allowed us to conclude that Paneth cells are critical in generating intestinal I/R injury and small intestinal IL-17A generation in mice after intestinal I/R.

Paneth cells provide important mucosal immunity against pathogens by producing and secreting several potent antimicrobial peptides (e.g., α-defensins and lysozymes) as well as several proinflammatory molecules (TNF-α, inducible nitric oxide synthase, as well as IL-17A) (16, 27, 28) (4, 39). α-Defensins are important regulators of small intestinal bacterial flora composition, and chronic deficiency in Paneth cell α-defensin expression could lead to chronic small intestinal inflammation (35, 44). Consistent with this, Paneth cell dysregulation or deficiency has been suggested to cause inflammatory bowel diseases such as Crohn’s disease (36). Therefore, although the Paneth cells (with ability to control bacterial growth) are important in providing mucosal protection against neighboring intestinal flora and avoiding chronic intestinal inflammation (25, 26), it appears that their acute dysregulation (with subsequent overproduction of IL-17A) after intestinal I/R...
may exacerbate intestinal and remote organ injury after intestinal I/R.

In contrast to our findings, Paneth cell depletion with dithizone increased bacterial translocation and systemic inflammation after hemorrhagic shock in rats (13). Therefore, the role of Paneth cells in systemic inflammation may differ according to the model of intestinal injury. Furthermore, because many cells can produce IL-17A, including CD4+ T cells, our study cannot rule out other sources of IL-17A in generating intestinal I/R injury. A previous study implicated IL-17A producing T cells infiltrating intestinal tissue after I/R and may serve as the source of cytotoxic IL-17A (10).

The mechanisms leading to Paneth cell degranulation as well as increased Paneth cell-derived IL-17A synthesis after intestinal I/R remain to be determined. SMA I/R-induced intestinal crypt inflammation and oxidative stress may induce Paneth cells to degranulate and increase cryptdin-1 synthesis, a mouse defensin and a specific marker for small intestinal Paneth cells. Moreover, because Toll signaling is upregulated and implicated in generating intestinal I/R, Toll receptor signaling may mediate Paneth cell degranulation. Indeed, Toll-like receptor (TLR) 9 receptor receptor-mediated Paneth cell degranulation has been described (11, 34). Because intestinal inflammation may lead to Paneth cell degranulation and activation, it may be possible to pharmacologically attenuate Paneth cell degranulation and subsequently reduce intestinal I/R injury via direct reduction in intestinal inflammatory response. For example, activation of A2b adenosine receptors and inhibition of equilibrative nucleoside transporters have been shown to powerfully decrease intestinal inflammation and injury via mechanisms involving hypoxia-inducible factor-1α (15, 23). These anti-inflammatory pharmacological approaches may downregu-
late intestinal TLR signaling-mediated Paneth cell degranulation.

We show in this study that kidney IL-17A levels were not modulated by Paneth cell depletion or with IL-17A antibody treatment. This could be due to a rather small rise in kidney IL-17A levels after intestinal I/R. However, renal injury after intestinal I/R was significantly attenuated by Paneth cell or IL-17A depletion/deletion. Therefore, IL-17A may not mediate intestinal I/R-induced AKI. Reduction in AKI due to IL-17A or Paneth cell modulation is most likely due to decreased intestinal IL-17A production and remote organ injury. Neutralization or depletion of IL-17A was significantly protective against intestinal I/R injury by altering enteric innate immunity in response to intestinal I/R. Modulation of Paneth cell dysregulation may have important therapeutic implications in reducing systemic complications arising from intestinal I/R injury. Future studies will address the mechanisms of intestinal I/R-induced Paneth cell degranulation and Paneth cell IL-17A induction.

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DISCLOSURES

The authors of this manuscript have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS


REFERENCES

I/R. Paneth cell-deficient Sox9flox/flox Villin Cre small intestine IL-17A levels after intestinal I/R were greater than the levels in (jejunum), hepatic, and kidney tissue IL-17A levels increased after intestinal IL-17A level detected in the systemic circulation. In addition, small intestine I/R. Again, the portal venous IL-17A increased significantly higher than


Fig. 11. Paneth cell depletion or deficiency reduces small intestinal apoptosis after I/R. A: representative fluorescent photomicrographs (of 4 experiments, magnifications of ×100) of TUNEL staining illustrating severe jejunal apoptosis after intestinal I/R injury. Thirty minutes of SMA ischemia and 5 h of reperfusion resulted in numerous TUNEL-positive cells in the intestinal epithelia of vehicle (LiCO₃)-treated mice (Vehicle I/R) and in Sox9flox/flox Villin Cre+/− mice (Sox9 WT I/R). Paneth cell depletion (Dithizone I/R) or deficiency (Sox9 KO I/R) markedly reduced the number of TUNEL-positive cells in the small intestine after I/R. B: quantification of apoptotic cells (in ×200 field) in jejunum confirmed significant reductions in the number of TUNEL-positive cells in Paneth cell-depleted and Paneth cell-deficient mice subjected to small intestinal I/R. *P < 0.05 vs. Vehicle I/R or Sox9 WT I/R group. Data are presented as means ± SE.
Fig. 12. Paneth cell depletion reduces proinflammatory gene expression and cryptdin-1 mRNA expression in the intestine, liver, and kidney after intestinal I/R. A and B: representative gel images (A) of RT-PCR and densitometric quantification of relative band intensities normalized to GAPDH (B) of proinflammatory markers TNF-α, ICAM-1, MCP-1, MIP-2, and IL-17A from jejunum of mice subjected to SMA I/R (n = 5/group). C–F: representative gel images (C and E) of RT-PCR and densitometric quantification of relative band intensities normalized to GAPDH (D and F) of proinflammatory markers TNF-α, ICAM-1, MCP-1, MIP-2, IL-17A, and IL-6 and cryptdin-1 mRNA from liver (C and D) and kidney (E and F) of mice subjected to SMA I/R (n = 4–5/group). Vehicle-treated mice subjected to intestinal I/R (Vehicle I/R) significantly increased the expression of all proinflammatory mRNAs examined as well as cryptdin-1 mRNA compared with the sham-operated mice. However, dithizone-treated Paneth cell-depleted mice (Dithizone I/R) had significantly reduced expression of proinflammatory mRNA expression and cryptdin-1 expression after intestinal I/R. *P < 0.05 vs. sham-operated mice. #P < 0.05 vs. Vehicle I/R. Error bars represent 1 SE.