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Paneth Cell–Mediated Multiorgan Dysfunction after Acute Kidney Injury

Sang Won Park,† Mihwa Kim,‡ Joo Yun Kim,§ Ahrom Ham,*, Kevin M. Brown,*, Yuko Mori-Akiyama,† André J. Ouellette,‡ Vivette D. D’Agati,§ and H. Thomas Lee*‡

Acute kidney injury (AKI) is frequently complicated by extrarenal multiorgan injury, including intestinal and hepatic dysfunction. In this study, we hypothesized that a discrete intestinal source of proinflammatory mediators drives multiorgan injury in response to AKI. After induction of AKI in mice by renal ischemia-reperfusion or bilateral nephrectomy, small intestinal Paneth cells increased the synthesis and release of IL-17A in conjunction with severe intestinal apoptosis and inflammation. We also detected significantly increased IL-17A in portal and systemic circulation after AKI. Intestinal macrophages appear to transport released Paneth cell granule constituents induced by AKI, away from the base of the crypts into the liver. Genetic or pharmacologic depletion of Paneth cells decreased small intestinal IL-17A secretion and plasma IL-17A levels significantly and attenuated intestinal, hepatic, and renal injury after AKI. Similarly, portal delivery of IL-17A in macrophage-depleted mice decreased markedly. In addition, intestinal, hepatic, and renal injury following AKI was attenuated without affecting intestinal IL-17A generation. In conclusion, AKI induces IL-17A synthesis and secretion by Paneth cells to initiate intestinal and hepatic injury by hepatic and systemic delivery of IL-17A by macrophages. Modulation of Paneth cell dysregulation may have therapeutic implications by reducing systemic complications arising from AKI. 

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cute kidney injury (AKI) is a major unresolved clinical problem costing more than 10 billion dollars per year in the United States (1, 2). Morbidity and mortality from AKI are high, in part because of the high incidence of extrarenal complications (2), leading to intestinal barrier disruption, liver dysfunction, respiratory failure, and a systemic inflammatory response syndrome that may progress to sepsis and multiorgan failure (3–6). Extrarenal systemic complications secondary to AKI are leading causes of mortality in the intensive care unit (3, 7), but the mechanisms that lead to extrarenal organ dysfunction remain unclear.

Profound hepatic injury and systemic inflammation occur after ischemic and nonischemic AKI with marked rises in plasma bilirubin, alanine aminotransferase (ALT), creatinine and blood urea nitrogen (BUN) (8–10). In previous studies, hepatic injury induced prominent periporal hepatocyte necrosis, apoptosis, and inflammation. In addition, rapid (<5 h) intestinal injury characterized by endothelial apoptosis, epithelial necrosis, and inflammation was evident (10). Significantly higher IL-17A levels were detected in the small intestine and in portal venous blood relative to levels in liver and the systemic circulation, respectively, implicating the small intestine as a major source of IL-17A generation after AKI.

Small intestinal Paneth cells produce and release IL-17A to mediate TNF-α–induced shock (11), and hepatic ischemia-reperfusion (IR) caused Paneth cell degranulation and elevated secretion of Paneth cell–derived IL-17A (12). These findings suggest that Paneth cells function as a source and reservoir of proinflammatory IL-17A to contribute to intestinal and hepatic injury as well as systemic inflammation after AKI. In contrast, intestinal IR injury causes rapid Paneth cell apoptosis with subsequent bacterial translocation to the liver and spleen, and Paneth cell granule depletion by zinc chelation exacerbated bacterial translocation and systemic inflammation after hemorrhagic shock, implicating the lineage as protective against systemic inflammation (13).

Therefore, the role of Paneth cells in systemic inflammation and multiorgan dysfunction after AKI remains unresolved, and the mechanisms of extraintestinal delivery of Paneth cell–derived IL-17A are not known. In this study, we report on the role of small intestinal Paneth cell–derived IL-17A and subsequent mechanisms involved in the pathogenesis of remote organ injury and systemic inflammation after AKI.

Materials and Methods

Murine model of renal IR or bilateral nephrectomy

C57BL/6 mice (20–25 g) were obtained from Harlan (Indianapolis, IN). Paneth cell–deficient mice (Sox9 flox/flox/ Villin Cre−/−) were generated as described (14). Sox9 flox/flox/ Villin Cre−/− mice were used as wild type controls. After Columbia University Institutional Animal Care and Use Committee approval, male mice under pentobarbital anesthesia were subjected to sham surgery (30 min), renal IR, or bilateral nephrectomy as described previously (15, 16). Sham-operated mice underwent identical abdominal manipulations as mice subjected to renal ischemia or bilateral nephrectomy (laparotomy, bilateral intestinal retraction and positioning). In a separate cohort of mice, recombinant murine IL-17A (0.3 or 1 μg per
mouse, i.v.) or vehicle (saline) was injected instead of subjecting to AKI to determine whether IL-17A can recapitulate hepatic, renal, and intestinal injury in mice. We also injected recombinant murine IL-17A (1 μg per mouse, i.v.) after Paneth cell granule depletion with dithizone treatment (100 mg/kg, i.v. 6 h prior to IL-17A injection). In some mice, we measured systolic blood pressure after sham surgery or AKI via indwelling carotid artery catheter. We ruled out blood pressure changes as a cause of systemic injury after AKI because neither renal IR nor nephrectomy resulted in postsurgical hypotension (Supplemental Fig. 1).

Assessment of hepatic and renal injury

Plasma ALT activity, bilirubin, creatinine, and BUN levels were determined as described previously (17, 18). Markers of liver and kidney injury was sampled at 5 h after bilateral nephrectomy and 24 h after renal IR. Plasma ALT activity and bilirubin level were measured using the Infinity ALT assay kit (Thermo Fisher Scientific, Waltham, MA) and the QuantiChrom Bilirubin Assay kit (BioAssay Systems, Hayward, CA). Plasma creatinine was measured by an enzymatic creatinine reagent kit (Thermo Fisher Scientific). BUN was measured by the Infinity BUN assay kit according to the manufacturer’s instructions (Thermo Fisher Scientific).

Macrophage depletion

Macrophages were depleted by i.p. injection of liposome-encapsulated dichloromethylene bisphosphonate (1 mg clodronate liposome per mouse, 200 μl) as described previously (19, 20) 48 h prior to induction of AKI. Clodronate liposome was purchased from Encapsula NanoSciences (Nashville, TN). Control mice received the same volume of PBS-containing liposome (PBS liposome). We determined the efficiency of macrophage depletion by performing spleen macrophage immunohistochemistry.

ELISA for plasma IL-17A

Five hours after induction of AKI, plasma, jejunum and isolated crypt IL-17A levels were measured with mouse-specific IL-17A ELISA kit according to the manufacturer’s instructions (eBiosciences, San Diego, CA) as described (10).

Portal vein plasma cryptdin-1 immunoblotting

Five hours after sham-operation or induction of AKI, portal vein plasma were obtained and processed for cryptdin-1 immunoblotting with Anti-(6C/ A)-Crp1 Ab as described previously (21, 22).

Pharmacological Paneth cell depletion

To deplete Paneth cell granules, mice were treated with dithizone (100 mg/ kg, i.v.) 6 h prior to renal ischemia or nephrectomy as described (23, 24). Dithizone is dissolved as 10 mg/ml of final concentration in the saturated lithium carbonate (1 g/100 ml).

Small intestine immunohistochemistry

Immunohistochemistry for macrophages (F4/80, 1:200 dilution, T-2006; Bachem, Torrance, CA), lysosome (1:100 dilution, PAI-29680; Thermo Fisher Scientific) or cryptdin-1 (25) was performed as described previously in paraffin-embedded tissue sections (10, 18).

Immunofluorescence staining for small intestine macrophage/ cryptdin-5 and macrophage/IL-17A

Small intestines were embedded in Tissue-Tek cryotryptic compound (Fisher Scientific, Pittsburgh, PA) and cut into 5-μm sections. After fixation with 3.7% paraformaldehyde, the sections were blocked in 10% BSA dissolved in PBS for 1 h at room temperature. The sections were then incubated with CD68 primary macrophage Ab (1:100 dilution, MCA1957; Serotec, Raleigh, NC) in a humidified chamber for 16 h at 4 °C. Secondary Ab incubation, using red fluorescent goat anti-rat IgG (1:200 dilution), was performed at room temperature for 30 min. Subsequently, cryptdin-5 primary Ab (1:200 dilution, generated in house) or IL-17A primary Ab (1:100 dilution, sc-7927, Santa Cruz Biotechnology) was applied in a humidified chamber for 16 h at 4 °C. The sections were incubated with green fluorescent secondary Abs with donkey anti-goat Ig G for cryptdin-5 or donkey anti-rabbit Ig G for IL-17A. Finally, sections were then washed twice in PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA).

RT-PCR

Conventional RT-PCR was performed to analyze the expression of IL-17A and cryptdin-1 as a quantitative marker for mature murine Paneth cells as described (16, 26, 27). We also performed quantitative real-time PCR (QRT-PCR) with the MyiQ Real Time Detection System (Bio-Rad, Hercules, CA) using SYBR Green I Brilliant Mastermix (Stratagene, La Jolla, CA). The Ct values were determined by using Mx3000P software. Values were normalized for GAPDH mRNA, and relative expression of proinflammatory mRNA was calculated with the ΔΔCt method.

Vascular permeability of liver and intestine tissues

Changes in liver, kidney, and small intestine vascular permeability were assessed by quantitating extravasation of Evans blue dye (EBD) into the tissue as described by Awad et al. (28) with some modifications. Two percent EBD (Sigma Bioreagents, St. Louis, MO) was administered (20 mg/kg) after ischemic AKI or bilateral nephrectomy. One hour later, mice were killed and perfused through the heart with 5 ml EDTA in 10 ml cold saline with heparin (100 U/ml). Liver and small intestine tissues were then removed and allowed to dry overnight at 60 °C, and the dry weights were determined. EBD was extracted in formamide (20 ml/g dry tissue; Sigma Biochemicals, St. Louis, MO), homogenized, and incubated at 60 °C overnight. Homogenized samples were centrifuged at 12,000 × g for 30 min, and the supernatants were measured at 620 and 740 nm in a spectrophotometer. The extravasated EBD concentration was calculated against a standard curve, and the data were expressed as micrograms of EBD per gram of dry tissue weight.

Detection of small intestine apoptosis

In situ TUNEL staining was used for detecting DNA fragmentation in apoptosis using a commercially available in situ cell death detection kit (Roche, Nutley, NJ) according to the manufacturer’s instructions. We further confirmed the TUNEL positivity in small intestine crypts by staining serial small intestine (jejenum) sections with TUNEL and CD34 (an endothelial cell marker; Abcam, Cambridge, MA). For DNA ladder, apoptotic DNA fragments were extracted according to the methods of Herrmann et al. (29) and was electrophoresed at 70 V in a 2.0% agarose gel in Tris-acetate-EDTA buffer. This method of DNA extraction selectively isolates apoptotic, fragmented DNA and leaves behind the intact chromatin. The gel was stained with ethidium bromide and photographed under ultraviolet illumination. DNA ladder markers (100 bp) were added to a lane of each gel as a reference for the analysis of internucleosomal DNA fragmentation.

Detection of small intestine apoptosis

Laser capture microdissection of Paneth cells

Laser capture microdissection (LCM) of individual Paneth cells was performed with the PixCell I LCM System (Arcturus Engineering, Mountain View, CA) as described (30). Small intestines were embedded in OCT compound (Sakura, Torrance, CA), sectioned at a thickness of 10 μm and mounted on 1.0 PEN Membrane Slides (Carl Zeiss, Thornwood, NY) according to the manufacturer’s instructions. We further confirmed the LCM staining kit (Ambion, Austin, TX) through a graded alcohol series (95%, 75%, 50%) followed by cresyl violet staining. After destaining via a 2 ml differentiated ethanol series (95% 75% and 50%) the tissue was dehydrated with ethidium bromide and photographed under ultraviolet illumination. Nucleus and chromatin patterns were visualized using a 20X objective of a microscope equipped with PALM RoboSoftware (Carl Zeiss, Thornwood, NY), and the total area of tissue collected per slide was tracked and recorded. RNA was isolated from the dissected tissue by following the protocol provided by the RNAPure Micro kit (Ambion, Austin, TX) via column purification.

Electron microscopy

Small intestines were fixed in 4% paraformaldehyde/3% glutaraldehyde in 10 mM sodium phosphate buffer (pH 7.4) for 48 h. All samples were postfixed with 1% osmium tetroxide in 100 mM cacodylate buffer (pH 7.4) on ice for 1 h. Samples were then treated with 0.5% aqueous uranyl acetate, dehydrated in graded alcohol, treated with propylene oxide, and embedded in Embed 812 (Electron Microscopy Sciences). The resin was polymerized in a 60°C oven for 2–3 d. Sections were cut with a DuPont diamond knife in Reichert-Jung UltraCut E ultracratome, collected on copper grids, and double stained with saturated aqueous uranyl acetate and lead citrate. Ultrathin sections were imaged for Paneth cells using a JEM-1200EX electron microscope manufactured by JEOL.

Isolation of intestinal crypts

Intact small intestinal crypts were isolated with the distended intestinal sac method as described by Taber et al. (31) with slight modifications. Small intestine from the duodenum to the ileum was removed and rinsed thoroughly with intestinal wash solution (0.15 M NaCl, 1 mM DTT, and 40 g/ml PMSF) and then filled with buffer A (10 mM NaCl, 27 sodium citrate, 1.5 KCl, 8 KH2PO4, 5.6 NaHPO4, and 40 g/ml PMSF (pH 7.4)). The ends were clamped with microclips, and the intestine was filled to a pressure of 50 cm H2O. The filled intestine was submerged in oxygenated 0.15 M NaCl at 37°C for 40 min and drained, and the solution was dis-
cared. The intestine was then filled with buffer B (109 mM NaCl, 2.4 mM KCl, 1.5 mM KH₂PO₄, 4.3 mM Na₂HPO₄, 1.5 mM EDTA, 10 mM glucose, 5 mM glutamine, 0.5 mM DTT, and 40 pg/ml PMSF [pH 7.4]) and incubated at 37˚C for another 20 min, and the intestinal contents were drained and collected. The cells from the 40–60-min fraction containing intact and isolated crypts were collected by pelleting at 100 × g for 5 min at 4˚C and washed once with PBS.

**Transfer of wild type IL-17A splenocytes to wild type or IL-17A–deficient mice**

Spleens from wild type (C57BL/6) mice were crushed, and splenocytes were passed through a nylon cell strainer (BD Biosciences, San Jose, CA) and collected in PBS. RBCs were lysed, and single-cell splenocyte suspensions were transferred intravenously (6 × 10⁶ to 1 × 10⁷ splenocytes per transfer; 200 μl) to wild type (C57BL/6), or to IL-17A–deficient mice 24 h before AKI.

**Statistical analysis**

The data were analyzed with two-tailed t test when means between two groups were compared or with one-way (e.g., plasma creatinine or ALT) ANOVA plus Tukey post hoc multiple comparison test to compare mean values across multiple treatment groups. In all cases, p < 0.05 was taken to indicate significance. All data are expressed as mean ± SEM.

**Results**

**Paneth cells degranulate after AKI**

Histologic examination of small intestine from sham-operated mice showed Paneth cells containing densely packed eosinophilic secretory granules (Fig. 1A). In contrast, renal IR and bilateral nephrectomy induced extensive Paneth cell degranulation within 5 h (Fig. 1B, 1C). Further evidence of Paneth cell degranulation was apparent by electron microscopy of small intestines following bilateral nephrectomy (Fig. 2B, 2C) and renal IR (Fig. 2D, 2E) compared with sham-operated mice (Fig. 2A). The crypt lumen from sham-operated mice was devoid of Paneth cell granules, whereas the crypt lumen from mice subjected to AKI showed granules being released into the lumen.

**Portal delivery of Paneth cell granule products by intestinal macrophages after AKI**

Detection of elevated Paneth cell α-defensin (cryptdin-1) protein levels in portal vein plasma after AKI induced by bilateral nephrectomy or renal IR (Fig. 3A) showed that Paneth cell secretory products were delivered to the liver. Because Paneth cells are the only enteric cell type that expresses α-defensins in mice, this epithelial cell lineage is unambiguously the source for their presence in the portal circulation.

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**FIGURE 1.** Paneth cell degranulation after AKI. Representative H&E staining images of small intestinal Paneth cells (ileum shown) containing dense eosinophilic granules within their apical cytoplasm (original magnification ×400). Compared with sham-operated animals (A), both renal IR and bilateral nephrectomy resulted in small intestinal Paneth cell degranulation (B, C) in 5 h. Insets show enlarged images (original magnification ×2000) of Paneth cells showing degranulation into the crypt lumen. Representative of five independent experiments.

**FIGURE 2.** Paneth cell degranulation after AKI observed with electron microscopy. (A) Sham-operated mice. (B and C) Representative electron micrograph images (original magnification ×3000) of small intestinal Paneth cell degranulation (indicated by asterisk) 5 h after bilateral nephrectomy or (D) after renal IR. The crypt lumen from sham-operated mice was devoid of Paneth cell granules. Representative of five experiments. N, Nucleus of Paneth cells; SC, stem cells located above the Paneth cells; SG, secretory granules of Paneth cells.
Macrophages mediate, at least in part, the removal of Paneth cell secretions from the lumen of small intestinal crypts of mice with AKI. Uptake of eosinophilic Paneth cell granules and granular secretions by phagocytes and their removal from the base of the small intestine was apparent in mice subjected to AKI (Fig. 3B, inset, arrows). Costaining for cryptdin-1 and F4/80 confirmed that macrophages also stain for cryptdin-1 in addition to F4/80 after induction of AKI (Fig. 3C, inset). In addition, we performed immunofluorescence colocalization of cryptdin-5 and macrophage CD68 in the small intestine. AKI induced by renal IR or bilateral nephrectomy resulted in Paneth cell degranulation as indicated by localization of cryptdin-5 away from the crypt base (Fig. 3D), and colocalization of cryptdin-5 and macrophages demonstrated that secreted cryptdin-5 was taken up by macrophages.

We also show that mice subjected to AKI after macrophage depletion, achieved with clodronate liposome injection 48 h prior, showed drastically less detectable cryptdin-5 protein localizing away from the crypt base and marked depletion of cryptdin-5 colocalization with macrophages (Fig. 3E) relative to control mice. Finally, macrophage-depleted mice had reduced portal venous cryptdin-1 (Fig. 3F).
nous cryptdin-1 protein compared with control liposome-injected mice after AKI (Fig. 3F). Taken together, these studies suggest that small intestinal macrophages mediate, at least in part, portal delivery of Paneth cell products after AKI.

**Paneth cells release IL-17A to cause multiorgan dysfunction after AKI**

To test whether Paneth cells are the small intestinal source of increased IL-17A after AKI, Paneth cell IL-17A mRNA levels were measured in laser capture microdissected crypts (Supplemental Fig. 2A). Table I lists the primer sequences utilized in this study. RNA recoveries from LCM crypts were sufficient for both conventional (Supplemental Fig. 2B) and QRTPCR and showed that IL-17A mRNA levels increased 8 ± 2-fold after bilateral nephrectomy (n = 4; p < 0.01). IL-17A ELISA performed on isolated crypts (Supplemental Fig. 3A, inset) showed that IL-17A protein levels were increased 8- to 10-fold after bilateral nephrectomy and 30 min of renal IR (Supplemental Fig. 3B). Measurements of IL-17A mRNA levels in isolated crypts by QRTPCR were consistent with IL-17A protein levels, increasing ~8-fold 5 h after bilateral nephrectomy or 30 min of renal IR (Supplemental Fig. 3C).

We also performed small intestine immunofluorescence staining with IL-17A (green) and macrophage (CD68, red) Abs. Five hours after AKI (renal IR or bilateral nephrectomy), IL-17A immuno-

![FIGURE 4. Intestinal macrophages take up Paneth cell-derived IL-17A to cause multiorgan dysfunction after AKI.](http://www.jimmunol.org/)

(A) Representative photographs (of four independent experiments; original magnification ×200; inset, original magnification ×600) of immunofluorescence stain for IL-17A (green) and CD68 (a macrophage-specific marker, red) in the small intestine of mice subjected to sham operation (Sham), bilateral nephrectomy (BNx), or renal IR (RIR). Insets in (A) and (B) show enlarged images (arrows). Five hours after AKI, IL-17A immunoreactivity increased in small intestinal crypts, and we show that IL-17A and CD68 co-stain (yellow, arrowheads in (A) and (B)] demonstrating that macrophages take up IL-17A released from the Paneth cells. (B) Representative immunofluorescence staining (of four independent experiments; original magnification ×200; inset, original magnification ×600) for IL-17A (green) and CD68 (red) in the small intestine of mice subjected to BNx or RIR with control liposome-injected or clodronate liposome-injected macrophage depletion. Mice were injected with liposomes i.p. 48 h before sham operation or AKI. Note that although Paneth cell IL-17A is increased after AKI in crypts after macrophage depletion, IL-17A no longer migrates away from the crypt base. (C and D) Recombinant murine IL-17A (0.3 or 1 μg per mouse i.v.; n = 4 each) recapitulates hepatic, renal, and intestinal injury in mice. (C) IL-17A injection causes dose-dependent liver (ALT) and renal (creatinine) injury. IL-17A (1 μg per mouse) also caused a similar degree of liver and kidney injury in mice treated with dithizone (100 mg/kg, i.v., 6 h prior to IL-17A injection). (D) IL-17A injection (1 μg) mimics plasma IL-17A levels achieved after AKI. Plasma samples were analyzed 5 h after IL-17A injection. *p < 0.05 versus vehicle-treated mice.
reactivity increased in small intestinal crypts, and we observed that IL-17A and CD68 costain (yellow, arrowheads), demonstrating that macrophages takes up IL-17A released from the Paneth cells (Fig. 4A). Mice subjected to AKI after macrophage depletion (with clodronate liposome injection) showed increased IL-17A expression in intestinal crypts compared with sham-operated mice. (Fig. 4A).

Table I. Primers used to amplify mRNAs encoding mouse GAPDH, IL-17A, and cryptdin-1 based on published GenBank sequences for mice

<table>
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<tr>
<th>Primer</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>Mouse cryptdin-1</td>
<td>NM_010031</td>
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<td>M32599</td>
<td>5′-ACACCCACGATCTTTCTC-3′</td>
<td>450</td>
<td>15</td>
<td>65</td>
</tr>
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</table>

Respective anticipated RT-PCR product size, PCR cycle number for linear amplification, and annealing temperatures used for each primer are provided.

FIGURE 5. Paneth cell deficiency with intestinal-specific Sox9 deletion. Sox9 flox/flox Villin Cre+/- (Wild Type) Mice are deficient in Paneth cell marker [cryptdin-1 mRNA (A) and in Paneth cells (B) original magnification ×1000] compared with wild type (Sox9 flox/flox Villin Cre+/- (Wild Type) Mice). Arrowheads indicate a complete lack of Paneth cells in Sox9 flox/flox Villin Cre+/- (Wild Type) Mice. (C) Paneth cell deficiency in Sox9 flox/flox Villin Cre+/- (KO) mice protects against AKI (creatinine and BUN) after renal IR (RIR). (D) Paneth cell deficiency also protects against hepatic injury (ALT and total bilirubin) after ischemic AKI (i.e., RIR) or bilateral nephrectomy (BNx) compared with Sox9 flox/flox Villin Cre+/- (wild type [WT]) mice. Mice were subjected to sham operation (Sham, n = 4), BNx (n = 5), or 30 min RIR (n = 5). Plasma was collected 5 h after BNx and 24 h after RIR. (E) Paneth cell deficiency in Sox9 flox/flox Villin Cre+/- (KO) mice reduces plasma (n = 4), small intestine (ileum shown; n = 4), and isolated crypts (n = 4) IL-17A levels in mice subjected to AKI (BNx and RIR). Plasma, small intestine, and isolated crypt samples were analyzed 5 h after AKI. (F) Paneth cell deficiency in Sox9 flox/flox Villin Cre+/- (KO) mice reduces IL-17A mRNA levels in isolated crypts after AKI. Small intestinal crypts were isolated 5 h after sham operation (Sham, n = 4), 5 h after BNx (n = 4), or 24 h after 30 min RIR (n = 4). *p < 0.05 versus sham-operated mice, †p < 0.05 versus WT mice subjected to AKI. (G) Paneth cell deficiency in Sox9 flox/flox Villin Cre+/- (KO) mice reduces proinflammatory mRNA expression (IL-17A, TNF-α, MCP-1, MIP-2, and ICAM-1) in the liver and jejunum after AKI. Tissues were extracted 5 h after BNx (n = 4) or 24 h after 30 min RIR (n = 4). Error bars represent 1 SEM. *p < 0.05 versus WT mice subjected to AKI.
FIGURE 6. Paneth cell granule depletion with dithizone treatment. (A) Representative H&E staining (of six experiments; original magnification ×400) of ileum from mice treated with vehicle (Li₂CO₃) or with dithizone 6 h prior. Note complete depletion of Paneth cell granules (arrows) after dithizone treatment (asterisks). (B) Representative ileum lysozyme immunostaining (of five experiments; original magnification ×400). Note heavy lysozyme stain in Paneth cells (arrowheads) of mice treated with vehicle (Li₂CO₃). Paneth cell depletion with dithizone treatment decreased lysozyme staining in Paneth cells. In addition, lysozyme staining in Paneth cells was reduced after bilateral nephrectomy (BNx) compared with sham-operated mice (Sham). We were able to detect lysozyme staining in crypt lumen (arrows and inset, original magnification ×2000). Villous lysozyme staining was also evident (asterisk). (C) Representative immunofluorescence stain (of three experiments; original magnification ×200; inset, original magnification ×600) for IL-17A (green) and CD68 (red) in small intestine of mice treated with dithizone (100 mg/kg, i.v., 6 h prior to renal ischemia or nephrectomy) and subjected to sham-operation, BNx, or renal IR. Paneth cell granule depletion reduces crypt IL-17A production after AKI. (D) Dithizone treatment reduces (Figure legend continues)
mice. However, macrophage-depleted mice had markedly less cryptdin-5 protein expression in intestinal epithelia away from base (green) and near complete loss of colocalization of IL-17A and macrophages (Fig 4B, yellow, arrowheads) compared with control liposome-injected mice.

Recombinant murine IL-17A recapitulates multiorgan injury observed after AKI. Administration of recombinant murine IL-17A at i.v. dosages of 0.3 or 1 μg per mouse induced dose-dependent hepatic and renal injury within 5 h after injection (Fig. 4C). In mice receiving 1 μg of IL-17A by i.v. injection, plasma IL-17A levels were similar to those induced by AKI (Fig. 4D), showing that circulating IL-17A was the same whether of enteric origin or administered parenterally. In addition, when IL-17A–deficient mice were subjected to renal IR or bilateral nephrectomy, the extent of Paneth cell degranulation was similar to wild type mice, showing that activation of secretion was independent of the cytokine (Supplemental Fig. 4A). Histologically, administration of exogenous recombinant murine IL-17A recapitulated the hepatic, renal, and small intestinal injuries of the bilateral nephrectomy-induced renal failure (Supplemental Fig. 4B–D), thus supporting the conclusion that Paneth cell–derived IL-17A mediates extra-renal remote organ injury after AKI. Furthermore, IL-17A continues to recapitulate hepatic and renal injury in dithizone-treated Paneth cell granule-depleted mice (Fig. 4C), supporting the conclusion that IL-17A causes liver and kidney injury independently of Paneth cells.

Paneth cell–deficient mice are protected from remote organ injury after renal IR or bilateral nephrectomy

To test whether Paneth cell secretory products are required for hepatic injury induced by AKI, responses in mice genetically deficient in the Paneth cell lineage (14) were investigated. Paneth cell deficiency characteristic of Sox9 flox/flox/ Villin Cre+/2 mice is apparent from their lack of cryptdin-1 mRNA (Table I, Fig. 5A) and from the absence of characteristic Paneth cell secretory granules crypts of these knockout mice (Fig. 5B). Renal IR injury led to a significant and graded rise in serum BUN and creatinine in Sox9 flox/flox/ Villin Cre+/2 control mice relative to sham-operated animals at 24 h, as did bilateral nephrectomy 5 h after injury (Fig. 5C). The plasma creatinine 24 h after renal IR increased.

plasma IL-17A levels (analyzed 5 h after AKI) in mice subjected to AKI (BNx or 30 min RIR; n = 4 each). Dithizone treatment also reduced IL-17A protein upregulation in small intestine (n = 4) and in isolated crypts (n = 4) 5 h after AKI. (E) Paneth cell granule depletion with dithizone treatment protects against hepatic injury after ischemic AKI (RIR) or bilateral nephrectomy (BNx). Mice were subjected to sham operation, vehicle (veh), or dithizone (Sham, n = 4), BNx (n = 6), or 30 min RIR (n = 6). Plasma was collected 5 h after BNx and 24 h after RIR. (F) Paneth cell granule depletion with dithizone treatment also protects against AKI (creatinine and BUN) after RIR. *p < 0.05 versus sham-operated mice, *p < 0.05 versus vehicle-treated animals subjected to AKI. Error bars represent 1 SEM.
higher than the plasma creatinine 5 h after bilateral nephrectomy. In addition, acute hepatic dysfunction also developed after renal IR or bilateral nephrectomy injury as shown by significantly elevated plasma ALT and bilirubin levels (Fig. 5D). However, Paneth cell–deficient mice were protected against both hepatic and renal injury caused by renal IR (Fig. 5C, 5D) and from hepatic injury after bilateral nephrectomy as evident from the lower plasma ALT levels (Fig. 5D). Because of the complete absence of renal function in the anephric state, plasma creatinine remained high in the Sox9-deficient state. Sox9-deficient mice had 10–20-fold lower IL-17A protein levels in plasma, ~160-fold lower levels in ileum, and 4–5-fold lower levels in isolated crypts after bilateral nephrectomy or induction of renal IR (Fig. 5E). In addition, Paneth cell deficiency in Sox9-null intestine reduced IL-17A mRNA levels in isolated crypts in response to AKI relative to control mice (Fig. 5F). Finally, mice with conditional Sox9 deficiency had lower mRNA levels for TNF-α, IL-17A, MCP-1, MIP-2, and ICAM-1 in jejenum, ileum, and liver (Fig. 5G, jejunum and liver shown).

**Pharmacologic depletion of Paneth cells attenuates remote organ injury after renal IR or bilateral nephrectomy**

To complement the studies in mice genetically deficient in the Paneth cells, we depleted Paneth cell granules pharmacologically. Zinc depletion with dithizone treatment rapidly depletes mouse Paneth cells of their secretory granules (23, 24). Secretory granules are evident and abundant in ileal Paneth cells from vehicle (lithium carbonate)–treated mice (Fig. 6A, left panel, arrows). In contrast, dithizone administration to mice almost completely depleted ileal Paneth cells of their granules within 6 h of dithizone exposure (Fig. 6A, right panel, asterisk). Lysozyme immunoreactivity was strong in crypt Paneth cells (Fig. 6B, arrows) of mice treated with Li₂CO₃ vehicle, and dithizone treatment eliminated lysozyme staining in Paneth cells because of granule depletion (arrowheads). In addition, crypt lysozyme staining was reduced after bilateral nephrectomy compared with sham-operated mice. In mice subjected to bilateral nephrectomy after vehicle treatment, lysozyme was released by Paneth cells into the crypt lumen (enlarged insert) and villous lysozyme staining was evident (asterisk). Most importantly, dithizone-induced Paneth cell granule depletion profoundly reduced crypt IL-17A production after AKI (Fig. 6C).

Treating mice with dithizone reduced plasma IL-17A levels induced by renal IR or bilateral nephrectomy by ~10-fold (Fig. 6D). Furthermore, dithizone granule depletion drastically reduced IL-17A protein levels in the small intestine (ileum) and in isolated crypts 5 h after induction of renal IR or bilateral nephrectomy. Depletion of Paneth cell granules with dithizone improved liver function after bilateral nephrectomy or 30 min of renal IR. Dithizone treatment also improved renal function after renal IR (Fig. 6F).

**FIGURE 8.** Macrophage depletion with clodronate liposome. Mice were subjected to sham operation (n = 4), bilateral nephrectomy (BNx) (n = 5), or ischemic AKI (renal IR [RIR]; n = 5) after control liposome or clodronate liposome injection. Mice were injected with liposomes i.p. 48 h before sham operation or AKI. Plasma was collected 5 h after BNx and 24 h after RIR. (A) Macrophage depletion protects against hepatic injury (ALT and bilirubin) after RIR or BNx. (B) Macrophage depletion also protects against AKI (creatinine and BUN) after RIR. (C) Macrophage depletion reduces systemic and portal vein plasma IL-17A levels without decreasing ileum IL-17A levels in mice subjected to AKI (BNx or 30 min RIR; n = 4 each, analyzed 5 h after AKI). *p < 0.05 versus sham-operated mice, †p < 0.05 versus control liposome-injected mice subjected to AKI. Error bars represent 1 SEM.
Depletion of Paneth cell granules with dithizone attenuated liver and small intestine apoptosis (reflected in decreased DNA laddering) after bilateral nephrectomy or renal IR (Fig. 7A). This reduced intestinal apoptosis was confirmed by TUNEL assays. Both renal IR and bilateral nephrectomy resulted in rapid intestinal villous apoptosis, predominantly in endothelial cells within 5 h, and Paneth cell depletion reduced intestinal cell apoptosis as determined by this assay as well (Fig. 7B). We confirmed that the TUNEL-positive cells in intestinal vili are endothelial cells by staining jejunal serial sections with TUNEL and CD34 (an endothelial cell marker; Abcam, Cambridge, MA) and confirmed that TUNEL-positive cells also stained for CD34 (data not shown). Furthermore, depletion of Paneth cells with dithizone did not induce Paneth cell or small intestinal crypt apoptosis.

Macrophage depletion attenuates hepatic injury and portal and systemic IL-17A delivery after renal IR or bilateral nephrectomy

Mice injected with clodronate liposome 48 h prior and subjected to renal IR or bilateral nephrectomy were protected against hepatic injury after AKI (Fig. 8A). Macrophage depletion also protected against renal IR injury (Fig. 8B). In addition, macrophage-depleted mice also had significantly less systemic and portal plasma IL-17A levels compared with control liposome-treated mice after AKI (Fig. 8C). However, IL-17A in the ileum did not change significantly in clodronate liposome-treated mice, suggesting that macrophage depletion reduces portal and systemic delivery without affecting the synthesis of Paneth cell–derived IL-17A.

Leukocyte IL-17A is not required for extrarenal remote organ injury after AKI

We initially determined whether IL-17A wild type splenocytes injected into IL-17A–deficient mice released IL-17A. We were able to detect IL-17A mRNA by RT-PCR in the liver, kidney, and small intestines of IL-17A–deficient mice after IL-17A wild type splenocyte injection (Fig. 9A). Furthermore, we were able to measure IL-17A protein (with ELISA) in plasma (152 ± 41 pg/ml; n = 5) and tissues (Fig. 9B) of IL-17A–deficient mice subjected to renal IR after splenocyte injection. Importantly, IL-17A–deficient mice transfused with wild type splenocytes 24 h before renal IR or bilateral nephrectomy were still protected against hepatic and renal injury after AKI (Fig. 9C). Wild type mice transfused with wild type splenocytes did not alter liver and kidney injury after AKI. These findings suggest that leukocyte IL-17A does not contribute to hepatic and renal injury after AKI. Rather, our data suggest that Paneth cell–derived IL-17A contributes to remote multiorgan injury after AKI (Fig. 10).

Discussion

AKI is a common complication during the perioperative period and is a strong, independent predictor of mortality (2). Unfortunately, current clinical management of AKI is limited to supportive...
Paneth cells to show that these crypts show increased IL-17A

Acute loss of renal function

Paneth cell IL-17A overproduction and degranulation

- Small intestinal inflammation
- Endothelial and epithelial cell injury
- Impaired intestinal barrier function

Macrophage uptake of Paneth cell granules

- Macrophage-mediated IL-17A delivery to liver

Hepatic inflammation, necrosis and apoptosis

Hepatic IL-6 and TNF-α overproduction and release into systemic inflammation

Increase in renal, hepatic and intestinal injury

FIGURE 10. Proposed mechanisms of AKI-induced liver dysfunction and systemic inflammation. Acute loss of renal function causes small intestinal Paneth cell generation of IL-17A and Paneth cell degranulation. We propose that IL-17A released by Paneth cells directly causes intestinal injury. Our data suggest that intestinal macrophages’ uptake of released Paneth cell granules promotes portal delivery of IL-17A; this leads to hepatic injury (necrosis, inflammation, and apoptosis) and increased generation and systemic release of TNF-α and IL-6, propagating multiorgan injury and systemic inflammation. The mechanisms that cause Paneth cells to produce increased IL-17A and degranulate after AKI remain to be determined.

measures (e.g., hemodialysis). One of the major reasons for difficulties in developing treatments for AKI is that patients with AKI frequently develop extrarenal organ dysfunction (4). For example, patients who develop AKI in the intensive care unit frequently suffer from respiratory, hepatic, and intestinal barrier dysfunction (7, 32, 33). Indeed, initiation or exacerbation of remote organ injury in patients suffering from AKI leads to a vicious cycle of organ injury and contributes significantly to mortality and morbidity (32). Therefore, a better understanding of the mechanisms of remote organ injury owing to AKI would lead to improved therapy for patients suffering from AKI.

Our current studies suggest that small intestinal Paneth cells generate proinflammatory cytokine IL-17A after ischemic AKI or bilateral nephrectomy. IL-17A release was originally characterized from Th17 CD4+ T cells (34, 35). Subsequent studies have demonstrated that other cell types including CD3ε invariant NK T cells, myeloid cells, neutrophils, and Paneth cells can produce IL-17A in response to pathogenic stimuli (36). Our previous (10) and current studies enabled us to implicate Paneth cell–derived proinflammatory IL-17A to directly cause multiorgan injury after ischemic AKI or bilateral nephrectomy. We previously demonstrated that ischemic AKI or bilateral nephrectomy caused a rapid release of small intestinal IL-17A and led to intestinal injury with subsequent hepatic dysfunction and systemic release of TNF-α and IL-6 (10). We also demonstrated the critical role of IL-17A release in response to AKI, because neutralization of IL-17A or deficiency in IL-17A blocked systemic inflammation and remote organ injury after ischemic AKI or bilateral nephrectomy (10). In this study, we show that Paneth cell depletion via pharmacologic or genetic approaches significantly reduced induction of IL-17A and attenuated multiorgan injury after AKI.

We ruled out the leukocyte and myeloid source of IL-17A based on the following experimental data. Using a calcium chelation technique, we were able to isolate individual crypts containing Paneth cells to show that these crypts show increased IL-17A mRNA and protein after ischemic AKI and bilateral nephrectomy. These isolated crypts are free of leukocytes and cells of myeloid origin. Because isolated crypts also contain stem cells and transition cells in addition to Paneth cells, we performed LCM to specifically capture Paneth cells to confirm increased expression of IL-17A mRNA in these cells.

Our collective findings suggest that Paneth cell–derived IL-17A is critical for inducing remote organ injury after AKI. We further propose that both ischemic AKI and bilateral nephrectomy induce production of IL-17A in Paneth cells rapidly promoting the increased production of additional (TNF-α and IL-6) cytokine generation, intestinal apoptosis, and hepatic injury. The unique position of IL-17A as a regulator of both innate and acquired immunity makes this cytokine a crucial signal for the reinforcement and crosstalk of host defense systems. The mechanisms leading to Paneth cell degranulation and increased Paneth cell–derived IL-17A after renal IR or bilateral nephrectomy remain to be determined. AKI resulting from IR or nephrectomy causes systemic inflammation, increased oxidant stress and TLR signaling (8, 32, 37). Moreover, TLR-mediated Paneth cell degranulation has been described (38, 39).

We were able to deplete small intestinal Paneth cell granules with pharmacologic (with dithizone treatment) or genetic (with Sox9/Villin Cre+/- mice) approaches. Dithizone, a zinc chelator, has been shown to deplete Paneth cell granules in adult mice and rats (23, 24). Although our TUNEL data demonstrate that dithizone did not induce small intestinal Paneth cell apoptosis, the use of dithizone may be limited by systemic and nonspecific side effects (e.g., effects on β-cells, pulmonary toxicity) especially at high doses, and Paneth cell depletion is transient (with complete repopulation of Paneth cell granules within 12–24 h after injection). Therefore, we complemented the dithizone studies with studies in Sox9/Villin Cre+/− mice. These mice selectively lack the Sox9 transcription factor in intestinal epithelial cells and as a result show absent or significantly reduced number of mature Paneth cells in adult mice (14, 40). These approaches of Paneth cell depletion allowed us to conclude that Paneth cells are critical in generating hepatic injury and small intestinal IL-17A generation in mice after AKI. Furthermore, we propose that reduction in Paneth cell IL-17A removed the key cytokine involved in causing multiorgan vascular impairment. Improved vascular integrity would lead to less leukocyte infiltration and cytokine generation and subsequently reduced epithelial necrosis, apoptosis, and inflammation.

Paneth cells are critical in regulating the precarious balance between intestinal bacteria and crypt stability required for the host (41–45). Indeed, small intestinal Paneth cells provide critical mucosal innate immunity against pathogens and can actively secrete several antimicrobial peptides (e.g., lysozyme, α-defensins, cryptidin-related peptides) (46–48). Paneth cells are also known to express transcripts for several proinflammatory molecules such as TNF-α, inducible NO synthase, GM-CSF, and IL-17A (24, 49). α-Defensins are important regulators of small intestinal bacterial flora composition and intestinal bacterial flora modulate intestinal Th17 cell differentiation (50, 51). Therefore, although the Paneth cells (with ability to kill bacteria and release proinflammatory mediators) are essential barriers providing mucosal and innate immunity (44, 52), we propose that their dysregulation and overproduction of IL-17A after AKI leads to a systemic inflammatory syndrome and causes extrarenal organ dysfunction. In contrast, previous studies suggest that chronic loss of Paneth cell α-defensin expression could skew mucosal responses toward a proinflammatory phenotype (51). Indeed, disruption of Paneth cells homeostasis has been implicated in the pathogenesis of
several inflammatory bowel diseases including Crohn disease and necrotizing enterocolitis (53). In small intestinal Crohn disease, Paneth cell products are significantly reduced, and ileal extracts collected from patients with Crohn disease have reduced capacity to clear bacteria (54). We propose that acute depletion of Paneth cells (with acute reduction of Paneth cell-derived IL-17A after AKI leading to multiorgan protection) have a very different effect compared with chronic depletion (bacterial floral changes, IL-17A induction, and increased chronic intestinal inflammation).

Our studies provide a potential mechanism for systemic absorption of Paneth cell granules released after AKI. We show in this study that intestinal macrophages ingest Paneth cell granules and transport them toward the intestinal epithelial villi promoting portal and systemic absorption of Paneth cell granules. Our studies further expand and are consistent with the findings demonstrated previously (6). Kramer et al. demonstrated that renal IR injury in rats cause lung injury and alter pulmonary vascular permeability via macrophage-derived inflammatory products. We propose that macrophages have a major role in mediating remote organ injury after renal IR. Furthermore, we propose that protective effects of macrophage depletion in renal IR (55) most likely involve direct (attenuation of renal inflammation by macrophages) and indirect (reduced intestinal uptake of Paneth cell-derived IL-17A by macrophages) mechanisms. However, our current data cannot rule out other potential mechanisms of portal delivery of Paneth cell products, including basolateral paracellular transcytosis.

A previous study by Li et al. (56) proposes that innate immune component of kidney IR requires neutrophil-derived IL-17A activation of IL-12/IFN-γ signaling pathways in mice. Their bone marrow chimera studies indicate that myeloid cell-derived IL-17A play a major role in generating renal IR injury. However, we find that IL-17A wild type mouse splenocyte transfusion did not exacerbate renal and hepatic injury after AKI in IL-17A KO mice. Therefore, our studies suggest that remote organ injury to the liver and intestine is dependent on Paneth cell-derived IL-17A production. However, although our studies suggest that splenocytes are not an important source of IL-17A, we cannot exclude a role for neutrophil-derived IL-17A as proposed by Li et al. (56).

Although pharmacologic or genetic Paneth cell granule depletion resulted in significant hepatic protection after bilateral nephrectomy and hepatic and renal protection after renal IR, the protective responses observed were partial. Paneth cell–depleted or –deleted mice still had increased liver and kidney injury compared with sham-operated mice. It is possible that Paneth cell depletion or deletion was incomplete in our study. It is also possible that in addition to the Paneth cell–derived IL-17A, other cell types including leukocytes or epithelial cells may generate IL-17A and additional cytokines in response to AKI (35, 36). Our previous study suggests that increased IL-17A after AKI originates in the small intestine with subsequent hepatic and systemic induction of IL-6 and TNF-α (10). We also previously determined that blockade of one cytokine was sufficient to attenuate hepatic and small intestinal injury and circulating levels of other cytokines after AKI. These findings suggest that the generation of cytokines after injury is not a redundant process, rather individual cytokine (e.g., IL-17A) may generate another cytokine (e.g., TNF-α).

In summary, we propose that the small intestinal Paneth cell generation of IL-17A leads to intestinal and hepatic injury and subsequent generation of TNF-α and IL-6, further potentiating renal injury and systemic inflammatory responses. Small intestinal Paneth cells may initiate the cascade of multiorgan injury by altering enteric innate immunity in response to ischemic AKI or bilateral nephrectomy. Modulation of Paneth cell dysregulation may have important therapeutic implications in reducing systemic complications arising from AKI. Future studies will address the mechanisms of AKI-induced Paneth cell degranulation and Paneth cell IL-17A induction.

Disclosures
The authors have no financial conflicts of interest.

References


