CXCL1/KC and CXCL2/MIP-2 Are Critical Effectors and Potential Targets for Therapy of *Escherichia coli* O157:H7-Associated Renal Inflammation

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Neutrophilia is a characteristic of hemolytic uremic syndrome caused by Shiga toxin (Stx2)-producing *Escherichia coli*. However, the role of neutrophils in the toxin-induced renal injury occurring in enterohemorrhagic *E. coli* infection remains undefined. We report the trafficking of neutrophils to the kidney of C57BL/6 mice throughout a 72-hour time course after challenge with purified *E. coli* Stx2 and lipopolysaccharide (LPS). Increased neutrophils were observed in the renal cortex, particularly within the glomeruli where a more than fourfold increase in neutrophils was noted within 2 hours after challenge. Using microarray analysis, an increased number of transcripts for chemoattractants CXCL1/KC (69-fold at 2 hours) and CXCL2/MIP-2 (29-fold at 2 hours) were detected. Ribonuclease protection assays, Northern blotting, enzyme-linked immunosorbent assay, and immunohistochemistry confirmed microarray results, showing that both chemokines were expressed only on the immediate periglomerular epithelium and that these events coincided with neutrophil invasion of glomeruli. Co-administration of Stx2 with LPS enhanced and prolonged the KC and MIP-2 host response (RNA and protein) induced by LPS alone. Immunoneutralization *in vivo* of CXCL1/KC and CXCL2/MIP-2 abrogated neutrophil migration into glomeruli by 85%. These data define the molecular basis for neutrophil migration into the kidney after exposure to virulence factors of Shiga toxin-producing *E. coli* O157:H7. (Am J Pathol 2007, 170:526–537; DOI: 10.2353/ajpath.2007.060366)

chemokines responsible for neutrophil migration and subsequent tissue injury have been a recent focus of investigation.^{1–4} Bacteria-induced renal inflammatory disease has been infrequently studied in this regard, and the molecular basis for neutrophil invasion of the kidney in this setting is not clear. Success in identifying chemotactic molecules that account for renal neutrophil invasion *in vivo* could define therapeutic strategies to limit unnecessary host cell injury caused by infiltrating activated neutrophils.

Shiga toxin-producing *Escherichia coli* are associated with hemolytic uremic syndrome (HUS), which is the leading cause of acute renal failure in young children.⁵ It is widely accepted that Shiga toxin types 1 or 2 (Stx1, Stx2) together with lipopolysaccharide (LPS) cause the vascular disease that often accompanies enterohemorrhagic *E. coli* infection, particularly in the kidney.^{6,7} Neutrophils are an early and important leukocyte present in histopathological examination of patients with HUS and in mouse models of this disease.^{8–10} Neutrophils, capable of transporting Stx to target organs,¹¹ produce several proinflammatory mediators (oxygen-free radicals, neutrophil-specific proteases, products of lipid peroxidation), many of which are injurious to cells.¹² However, important aspects of the biology of neutrophil recruitment remain unresolved, especially the functional role played by the many neutrophil-active chemokines capable of directing migration of cells to specific host sites *in vivo*. Further, the cellular source of these host-derived chemokines and the relative contribution of LPS and Stx2 to induce these chemokines are not clear. Our studies show 1) a rapid up-regulation by these two *E. coli* macromolecules of a selected subset of chemokines and adhesion factors; 2) the importance of Stx2 in the enhancement and prolongation of chemokine gene activation; 3) a focal expression of the protein gene products (chemokines: periglo-

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Neutrophil influx is a hallmark of many inflammatory diseases including those of the kidney, and the putative

merular tubular epithelial cells, and VCAM-1: glomerular capillary tufts); and 4) a marked reduction (>85%) in renal neutrophil infiltrate by *in vivo* immunoneutralization of CXCL1/KC and CXCL2/MIP-2. Follow-up studies showed that each of these chemokines contributed nearly equally to neutrophil migration into the kidney.

Materials and Methods

Materials

The following immunoglobulin reagents were used: rat anti-mouse neutrophil, clone 7/4, used at 1:20 (Caltag, Burlingame, CA); goat anti-mouse KC used at 1:200 (R&D Laboratories, Minneapolis, MN); rabbit anti-mouse MIP-2, used at 1:50 (Serotec, Raleigh, NC); and goat anti-mouse VCAM-1 used at 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA). All biotin-labeled secondary antibodies were from Vector Laboratories (Burlingame CA) and used according to the manufacturer's directions. Stx2 was isolated from a lysate of a clinical strain of *E. coli* (a gift from Dr. Allison O'Brien, Uniformed Services Medical Center, Bethesda, MD). The final product was purified by immunoaffinity column chromatography using the 11E10 monoclonal antibody (American Type Culture Collection, Manassas, VA). Endotoxin contaminants were removed using a LPS detoxification column (Pierce Chemical Co., Rockford, IL), and Stx2 was determined to have <0.06 EU of endotoxin per ml by the *Limulus* amebocyte lysate assay (Associates of Cape Cod, East Falmouth, MA). Purity of the toxin preparation was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with silver staining, demonstrating only two bands (subunits A and B of the holotoxin). Biological function was determined in dose-response experiments with Vero cells (American Type Culture Collection), where 50% cytotoxicity was found when Stx2 was present at 10 nmol/L.

Animal Experiments

C57BL/6 mice (male, 22 to 24 g) were purchased from Charles River Laboratories (Wilmington, MA). CXCR2 knockout mice and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were injected intraperitoneally with either 6 μ g of LPS per 20-g mouse (O55:B5; Sigma Chemical Co., St. Louis, MO), 4 to 12 ng of immunoaffinity-purified Stx2 per 20-g mouse, or both (Stx2/LPS). Mice were then euthanized at 0, 2, 4, 6, 8, 12, 24, 48, or 72 hours after injection. Kidneys removed from a phosphate-buffered saline (PBS)-treated mouse served as the control. All of the animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at the University of Virginia, Charlottesville, VA.

Microarray Analysis

One half of one kidney from each mouse was placed into 2 ml of stabilization buffer, RNA Later (Ambion, Austin, TX), for up to 2 weeks at 4°C until extraction. Total RNA

was extracted using the RNeasy midi kit (Qiagen, Santa Clarita, CA) following the manufacturer's protocol and was quantified by absorbance at 260 nm. Total RNA from the control and challenged mice were compared using GeneChip Expression analysis probe arrays, specifically the Mouse Genome 430A 2.0 (Affymetrix, Santa Clara, CA). In brief, the RNA was transcribed into cDNA via Superscript RT (reverse transcriptase from Invitrogen, Carlsbad, CA) and then used to make biotinylated cRNA using T7 RNA polymerase. The biotinylated cRNA was precipitated, fragmented, and analyzed on a 1% agarose gel. A hybridization solution was prepared, which contained the fragmented cRNA, herring sperm DNA, acetylated bovine serum albumin, and 2-(*N*-morpholino)ethanesulfonic acid hybridization buffer. After prewetting the array with hybridization buffer, the array was hybridized for 16 hours. The hybridization solution was then removed and the array washed and stained. Results were analyzed using GeneX Va Software (original version at the National Center for Genome Resources, <http://www.ncgr.org/>). Normalization was achieved by calculating the 50th percentile of all measurements for each sample, and each measurement for each gene was divided by this number. Normalized values below 0 were set to 0. In the case of PAF and LTB₄, genes required for their synthesis were monitored because these molecules are lipids. Biological duplicate experiments were performed.

Northern Blotting

The pBC-KC plasmid in *E. coli* HB101 was purchased from American Type Culture Collection (item no. 35791, accession no. J04596). The plasmid was grown, harvested, and digested with *Pst*I to isolate the 0.8-kb probe fragment. The digest was separated first on a 0.8% agarose gel followed by a 0.6% low-melting point agarose gel, and the band was excised. The probe was isolated and concentrated using an Elutip-d column (Schleicher & Schuell, Keene, NH) and ethanol precipitation. The purified insert was labeled with [³²P]dATP (Perkin Elmer, Boston, MA) using a random primers DNA labeling system (Invitrogen).

Ten μ g of RNA (isolated and purified as described above) were separated on a 1% agarose, 2.2 mol/L formaldehyde gel and transferred by capillary action to a nylon membrane (Hybond-N; Amersham, Piscataway, NJ). RNA was crosslinked to a membrane using a UV Stratalinker (Stratagene, La Jolla, CA). Blots were prehybridized for at least 4 hours at 42°C in ULTRAhyb buffer solution (Ambion). Blots were then hybridized overnight at 42°C in the same solution containing the labeled probe. After hybridization, the blots were washed in increasingly stringent standard saline citrate/SDS buffer solutions (2× 5 minutes in 2× standard saline citrate, 0.1% SDS; 2× 15 minutes in 0.1× standard saline citrate, 0.1% SDS) at 42°C to remove background and then subjected to autoradiography using X-OMAT film (Eastman Kodak, Rochester, NY). The blot was stripped between probes by boiling in 0.1% SDS. Autoradiographs were scanned and quantitated with a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and Image

Quant 5.2 software. The method used was volume integration using the object average background. The volume of the target band was divided by the volume of the housekeeping gene band (18S or L32) and multiplied by 100; results were then graphed using Microsoft Excel.

Ribonuclease Protection Assay

The MIP-2 probe was part of the mCK-5b multiprobe template set from BD Biosciences Pharmingen (San Diego, CA). The protected size was 205 nucleotides. It was labeled with α -[32 P]UTP (Perkin Elmer) using a Pharmingen transcription kit. Using the RPA III kit (Ambion), 10 μ g of each sample was hybridized with an excess of probe overnight in solution at 56°C. Free probe and other single-stranded RNAs were digested with RNase, and the RNase-protected RNA was purified and resolved on a denaturing 5% acrylamide gel. Gels were developed by direct exposure to Kodak XAR film at -70°C for overnight. Results were normalized to mL32 RNA (protected size = 112 nucleotides) run simultaneously. Quantitation of bands detected by autoradiography was performed as described above.

Immunohistochemistry

One kidney from each mouse was bisected, placed in plastic cassettes, and fixed in 4% paraformaldehyde for 24 hours before processing and paraffin-embedding. Sections were cut at 3 μ m and placed on charged slides. Staining for immunolocalization of specific antigens was accomplished using the following immunoglobulins: a monoclonal rat anti-mouse neutrophil IgG (clone 7/4), a goat anti-mouse KC IgG, a goat anti-mouse MIP-2 IgG, and a goat anti-mouse VCAM-1 IgG (source and working dilutions described above), with incubations at 25°C for 1 hour. An avidin/biotin horseradish peroxidase system from Vector Laboratories was used with diaminobenzidine to give a brown-colored end product at the site of immunoreactive antigen. Finally, sections were counterstained in hematoxylin, mounted, and photographed. The scoring of samples for quantitation of neutrophils, KC, and MIP-2 is described below (see Statistics).

Cytokines in Renal Tissue Homogenates

One half of one kidney was placed into 1 ml of ice-cold lysis buffer [50 mmol/L HEPES, 1% Triton X-100, and 100 μ l/ml protease inhibitor solution (no. P8340; Sigma Chemical Co.)] and immediately homogenized by hand, using a glass tissue grinder followed by sonification (Branson Sonifier, model 250; output setting, 4; 10 seconds; Danbury, CT). After 30 minutes on ice, samples were centrifuged at 14,000 rpm for 10 minutes at 4°C. The resultant supernatant was aliquoted and stored at -70°C until analyzed. For measurement of cytokines, samples were diluted as needed, and VCAM-1, CXCL1/KC, and CXCL2/MIP-2 were measured by enzyme-linked immunosorbent assay using a matched mouse cytokine antibody pair (Duo Set; R&D Systems). A standard curve

was generated from each set of samples assayed and calibrated against purified *E. coli*-expressed recombinant mouse cytokine per the manufacturer's directions.

Neutralization Studies

Mice were injected with immunoglobulin intravenously via the lateral tail vein. The experimental group ($n = 6$) received IgG, protein G affinity chromatography-purified, with specificity for CXCL1/KC (100 μ g/mouse) as well as IgG for CXCL2/MIP-2 (100 μ g/mouse). The source and species of origin for each immunoglobulin was stated above (see Materials). Mice in the control group received either an equal quantity of irrelevant (preimmune) immunoglobulin (200 μ g/mouse) purified from the same species ($n = 6$) or saline in the same volume (100 μ l/mouse). Eighteen hours later, all mice received intraperitoneal Stx2 (4.5 ng/20-g mouse) and LPS (6.0 μ g/20-g mouse). Kidneys were removed at 2 and 6 hours after Stx2/LPS injection, fixed in 4% paraformaldehyde, and stained for neutrophils, as described above. Times for immunohistochemical examination were chosen based on prior experiments (Figure 1D; Figure 2, A and C), showing a significant accumulation of neutrophils in the kidney at these times after Stx2/LPS challenge. In subsequent experiments, identical immunoneutralization studies were performed, except that IgG specific for a single chemokine (CXCL1/KC or CXCL2/MIP-2) or saline was injected in a 100- μ l volume 18 hours before Stx2/LPS challenge.

Statistics

Data were expressed as the mean $\pm$ 1 SD. The Student's *t*-test was used to determine statistical significance, with $P < 0.01$ taken as significant. A semiquantitative scale was established to express results as an index, designated staining level intensity. For KC and MIP-2, intensity levels of staining of glomeruli and tubules were designated as follows: 1) <25%; 2) 25 to 50%; 3) 51 to 75%; and 4) >75%. For VCAM-1, this same scale was used, except endothelium of vessels containing red blood cells (and not glomeruli/tubules) was counted. In all cases, 0 indicated negligible staining anywhere in the section. For quantitation of neutrophils in glomeruli, three sets of 20 glomeruli (minimum of 60 total) in a kidney section were counted and results reported as percent positive glomeruli, eg, number of glomeruli containing one or more neutrophils divided by the total number of glomeruli counted. For the extraglomerular cortex, five or more nonoverlapping microscope fields, representing the entire kidney section, were counted for quantifying neutrophils and reported as the number of neutrophils per field.

Results

Renal Neutrophil Infiltration, Red Blood Cell Congestion, and Death in the Model

Renal parenchymal red blood cell congestion and neutrophil invasion were important pathological findings in

Figure 1. Renal injury with neutrophil invasion and death in a mouse model of renal inflammation. **A** and **B**: C57BL/6 mice were injected intraperitoneally with PBS (**A**) or Stx2/LPS (**B**), and their renal tissue was examined by H&E staining after 8 hours. Congestion of glomeruli with dilated capillary tufts filled with red blood cells (**arrowheads**) and invasion with neutrophils (**arrow**) was found in Stx2/LPS-challenged mice (**B**) but not in controls (**A**). **C**: Representative example of staining for immunoreactive neutrophils is shown at 8 hours for Stx2/LPS-challenged mice. **Arrows** indicate immunopositive neutrophils. **Inset** shows a high-powered view of an immunoreactive neutrophil. **D**: Survival as a function of time in mice after injection of Stx2 (4.5 ng/20-g mouse), LPS (6.0 μ g/20-g mouse), or Stx2/LPS. Five mice per group were used. No mice in the Stx2 alone or Stx2/LPS groups survived beyond 4.5 days. **E**: Neutrophil migration into glomeruli throughout time, as induced by Stx2 alone, LPS alone, or Stx2/LPS. Cells were positively identified as neutrophils by their staining with the mouse neutrophil-specific monoclonal immunoglobulin 7/4. A minimum of 60 glomeruli were counted per time point for each of the three treatments, and these data are representative of three independent biological replicate experiments (four mice total per time point). **F**: Neutrophil entry into the extraglomerular cortex as induced by Stx2, LPS, or Stx2/LPS. Mice were euthanized, and all immunoreactive neutrophils were counted at nine time intervals as described in Materials and Methods. Data represent three independent experiments, ie, four mice total per time point. Glomeruli were counted as positive when they contained one or more neutrophils; for the extraglomerular cortex, nonoverlapping fields were evaluated and expressed as the number of neutrophils counted per field. $P < 0.031$ for points at 2 to 6 hours in Stx2/LPS- and LPS-challenged mice, compared with control value at time = 0 hours. Original magnifications: $\times 400$ (**A**, **B**); $\times 100$ (**C**); $\times 1000$ (**C**, **inset**).

mice exposed to Stx2/LPS. Eight hours after intraperitoneal challenge with Stx2/LPS, glomeruli were markedly congested with red blood cells, and neutrophils could be identified nearby (Figure 1B). In contrast, the cortex and glomeruli in control mice showed an occa-

sional and modest number of red blood cells in glomerular capillary tufts (Figure 1A). Stx2/LPS-challenged mice became lethargic, and by day 3.5 all were dead (Figure 1D). No mice died after the sublethal dose of LPS (6 μ g/20-g mouse), but all mice died by

day 4.5 after Stx2 alone (4.5 ng/20-g mouse is approximately two times the LD₅₀).

To determine the time course of, and principal initiating stimulus for, neutrophil appearance in renal glomeruli in our model, mice were exposed to Stx2, LPS, or both for 0 to 72 hours. The animals were euthanized, the kidneys were removed, and sections were examined for the number of glomeruli containing one or more neutrophils (Figure 1C). LPS was more potent than Stx2 in inducing the glomerular accumulation of neutrophils, with the highest amount of neutrophil influx occurring at 2 to 8 hours after challenge ($P < 0.01$, comparing the number of neutrophil-positive glomeruli at 0 versus 2 hours) (Figure 1E). Mice injected with Stx2/LPS and with LPS had comparable numbers of neutrophil-positive glomeruli and neutrophil-positive fields of extraglomerular cortex at 2 hours (Figure 1, E and F). At 2 and 4 hours, 42 to 54% of glomeruli from Stx2/LPS-challenged mice contained neutrophils. Specifically, there were two or more neutrophils in 30 to 42% of glomeruli, three or more in 12%, and four or more in 2 to 6%. Subsequently, greater than 18% of all glomeruli had a persistent neutrophil accumulation at 48 hours in two of the three treatment groups (LPS and Stx2). These data indicate renal neutrophil infiltration, red cell congestion, and death are important and coincident findings in our model when mice are exposed to two *E. coli*-associated macromolecules, Stx2 and LPS.

Intrarenal Localization of Neutrophils Induced by LPS or Stx2

To determine whether other areas of the kidney, in addition to glomeruli, were a primary site of neutrophil invasion in mice, immunoreactive neutrophils were counted in individual renal compartments throughout entire tissue sections of kidneys from two series of mice receiving one of two challenge regimens, either LPS alone or Stx2 alone. The medullary and papillary compartments showed only minimal neutrophil infiltrates (less than two cells/field). In glomeruli, changes at 2 to 8 hours were statistically increased from the 0-hour control, when LPS (but not Stx2) was used for disease induction (Figure 1E, $P < 0.03$). In the extraglomerular cortex, the number of neutrophils (expressed as the number per field of cortex) was also increased compared to the 0-hour control value from 2 to 12 hours after injection of LPS but not after Stx2 (Figure 1F, $P < 0.025$). Representative results in tissue at 8 hours from a Stx2/LPS-challenged mouse are shown in Figure 1C. These data suggest that chemotactic stimuli for neutrophil migration are present within 2 to 4 hours after exposure to Stx2/LPS and to LPS and that they direct infiltrating cells primarily to glomeruli and to the extraglomerular cortex of the kidney.

Renal Gene Activation after Induction with Stx2 and/or LPS

To determine the chemotactic and adhesion molecules likely responsible for neutrophil entry into the kidney in

Table 1. Microarray Analysis of Kidney RNA to Identify Genes Up-Regulated after *in Vivo* Challenge with LPS and/or Stx2

Induction agent	Gene	Fold-increase over control*	
		2 Hours	24 Hours
LPS	CXCL1/KC	69.5	1.4
	CXCL2/MIP-2	29.7	1
	VCAM-1	14.6	1.7
Stx2	CXCL1/KC	1	1.8
	CXCL2/MIP-2	1	1.4
	VCAM-1	1	1.1
Stx2/LPS	CXCL1/KC	82.3	2.8
	CXCL2/MIP-2	40.9	1.4
	VCAM-1	15.7	1.4

*Duplicate biological replicate experiments were performed and representative data are shown.

our model, microarray analyses of renal tissue are reported for 0, 2, and 24 hours after challenge with each of the two inducing agents (LPS, Stx2) as well as both together. Gene array data analyzed using GeneX Va software revealed three genes associated with neutrophil migration were expressed at a high level (>10 -fold) and were tabulated in terms of fold-increase over values in control (0 hour, PBS-injected) kidneys (Table 1). Among neutrophil chemoattractants, CXCL1/KC and CXCL2/MIP-2 gene expression was markedly increased (69- and 29-fold, respectively) at 2 hours after challenge with LPS. Although Stx2 alone did not induce genes for either chemokine, Stx2/LPS resulted in an $\geq 25\%$ additional increase in CXCL1/KC and CXCL2/MIP-2 gene expression (Table 1). Interestingly, genes for other chemokines able to initiate neutrophil migration (C5a, PAF, LTB₄, NAP2, and NCFA) were not induced (less than or equal to two-fold increase) throughout 72 hours by any of the three challenge regimens, LPS, Stx2, or Stx2/LPS (data not shown). Only CXCL1/KC remained modestly elevated at 24 hours after challenge (Table 1).

Among adhesion molecules, VCAM-1 showed a robust and nearly equivalent LPS or Stx2/LPS activation of its gene 2 hours after injection, returning to normal by 24 hours (Table 1). Other adhesion molecules associated with (but not specific for) neutrophil migration (E-selectin, P-selectin) were not up-regulated except for E-selectin at 2 hours (eightfold to ninefold increase after LPS or Stx2/LPS). In subsequent studies, we chose to focus on those molecules (CXCL1/KC, CXCL2/MIP-2, and VCAM-1) pertinent to neutrophil migration and adhesion in which gene expression increased >10 -fold after challenge with LPS or Stx2/LPS.

Renal CXCL1/KC mRNA and Protein in Vivo

To further elucidate the mechanism for entry of neutrophils into the kidney in our model, Northern blots of temporally collected mouse renal RNA were analyzed after *in vivo* challenge with *E. coli*-associated LPS and/or Stx2. CXCL1/KC gene expression was rapidly and markedly up-regulated 2 hours after LPS (but not Stx2) challenge (Figure 2A). Quantification by densitometry and normalization against an 18S rRNA confirmed enhanced early

Figure 2. Time course and localization of the neutrophil chemotactic factor CXCL1/KC in the mouse kidney. **A:** Northern blot using a CXCL1/KC-specific probe together with total renal RNA isolated at timed intervals (0 to 72 hours) from mice after intraperitoneal injection of LPS alone, Stx2 alone, or Stx2/LPS. Ribosomal RNA (18S) is shown as a control for RNA loading in each well. **Lane C,** buffer control; **lane 72C,** 72-hour PBS-injected mouse; **lane B,** blank lane; **lane L,** normal liver; and **lane S,** normal spleen. **B:** Ratio of CXCL1/KC to 18S ribosomal RNA, obtained by densitometric analysis of chemokine-specific and 18S-specific mRNA shown in **A**. The results of each treatment regimen (LPS alone, Stx2 alone, and Stx2/LPS) are included for comparison. **D:** CXCL1/KC protein in homogenates after exposure to Stx2, LPS, or Stx2/LPS. Shown is the mean $\pm$ 1 SD, determined by enzyme-linked immunosorbent assay, using recombinant CXCL1/KC as the standard. * $P < 0.05$, comparing CXCL1/KC protein concentration after LPS versus Stx2/LPS challenge. **C** and **E–G:** Time course and immunochemical localization of CXCL1/KC protein in C57BL/6 renal tissue after intraperitoneal injection of LPS. **C:** Summary of cell staining throughout time. **E–G:** Representative staining of CXCL1/KC protein at 0 hours (**E**), 2 hours (**F**), and 4 hours (**G**). **Arrows** indicate positively stained periglomerular epithelium. Original magnifications, $\times 400$.

expression (at 2 and 4 hours) as well as a second induction (at 8 hours) of the CXCL1/KC gene when mice were challenged with Stx2/LPS (Figure 2B). This was confirmed at the protein level, in which the combination of Stx2/LPS elicited more CXCL1/KC protein at 6 to 12 hours after challenge, compared with LPS induction alone ($P < 0.05$) (Figure 2D). Histochemical results for CXCL1/KC-immunoreactive protein after LPS challenge showed that changes were limited to the first 2 to 4 hours (Figure 2C), consistent with the Northern blot findings. CXCL1/KC-

specific immunoglobulin demonstrated localization to the epithelium adjacent to glomeruli at 2 and 4 hours after LPS, involving the initial portion of the proximal tubule (Figure 2, F and G; arrows). No CXCL1/KC chemokine was evident in control mice (Figure 2E). Together, these data suggest that the CXCL1/KC protein is induced rapidly by LPS *in vivo* at a site appropriate to promote glomerular neutrophil invasion in the model and that this chemokine-specific protein response is enhanced in the presence of Stx2 and LPS (Figure 2, A and B).

Figure 3. Characterization of neutrophil chemotactic factor CXCL2/MIP-2 in C57BL/6 mouse kidney. **A:** Appearance of renal CXCL2/MIP-2-specific RNA throughout time after intraperitoneal injection of LPS, Stx2, or both. At pre-established time intervals, kidneys were removed and the presence of CXCL2/MIP-2-specific RNA determined by ribonuclease protection assay analysis. **Lane C**, buffer only; **lane 72C**, 72-hour RNA from PBS-injected mice; **lane +**, positive control. **B:** CXCL2/L32 mRNA ratios throughout time determined by densitometry. Comparison among the three induction modes (Stx2, LPS, and Stx2/LPS) is shown. L32 was used for normalization among samples. **C:** Immunoreactive CXCL2/MIP-2 protein in renal homogenates throughout time after *in vivo* exposure to LPS or Stx2/LPS. The results were determined by enzyme-linked immunosorbent assay and shown as the mean $\pm$ 1 SD. * $P < 0.05$, comparing CXCL2/MIP-2 protein concentration after LPS versus Stx2/LPS challenge. **D–I:** Localization of immunoreactive CXCL2/MIP-2 in renal tissue of mice injected with LPS; time after challenge as follows: **E**, 0 hour (PBS) control; **F**, 2 hours; **G**, 2 hours; **H**, 4 hours; **I**, 8 hours. **Arrows** in **G–I** indicate CXCL2/MIP-2 staining of periglomerular tubular epithelium; **arrowheads** in **F**, examples of stained periglomerular epithelium at low power. **Insets** in **E** and **F** are high-power views of cortical areas (original location shown by small rectangles) from control (**E**) and LPS-challenged (**F**) mice. Original magnifications: $\times 100$ (**E**, **F**); $\times 400$ (**G–I**); $\times 1000$ (**E**, **F**, **insets**).

CXCL2/MIP-2 Gene Activation and in Vivo Expression in the Mouse Kidney

mRNA for a second molecule, well established for its neutrophil chemotactic properties, CXCL2/MIP-2, was elevated 29- to 40-fold in mouse kidney after LPS or Stx2/LPS challenge *in vivo* (Table 1). To define further the presence and time course of this molecule in the mouse renal inflammation model, temporally collected RNA was analyzed by the ribonuclease protection assay using a

MIP-2-specific probe (Figure 3, A and B). The data showed that LPS alone induced expression of the CXCL2/MIP-2 gene and that the induction of CXCL2 RNA appeared to be enhanced at 2 and 8 hours by the co-administration of Stx2 and LPS (Figure 3B).

At the protein level, CXCL2/MIP-2 showed an early peak at 2 hours, with substantial levels remaining for 24 hours (Figure 3C). The combination of Stx2/LPS elicited more immunoreactive CXCL2 protein than LPS alone at 2, 4, 6, 12, and 24 hours ($P < 0.05$). Histochemically, ex-

pression was short-lived (Figure 3D), where low-powered ($\times 100$) views showed widely dispersed immunoreactive CXCL2/MIP-2 throughout the cortex at 2 hours (arrowheads in Figure 3F, with inset demonstrating the finely stippled pattern of staining) compared with the 0-hour control (Figure 3E). At higher power ($\times 400$), CXCL2/MIP-2 was periglomerular, most markedly at 2 and 4 hours (Figure 3, G and H) with gradually diminishing expression at 8 hours (Figure 3I). Taken together, these experiments suggest that Stx2/LPS are potent and rapid inducers of CXCL2/MIP-2 mRNA and protein in tubular epithelium adjacent to glomeruli. Importantly, the appearance of CXCL2/MIP-2 expression at 2 hours is coincident with neutrophil migration into the kidney in our model (Figure 1, E and F).

Renal Neutrophil Accumulation after Immunoglobulin Neutralization of Chemokines

To determine whether the local renal up-regulation of CXCL1/KC and CXCL2/MIP-2 was responsible for neutrophil invasion of the kidney in our model of renal injury, mice were injected with 100 $\mu\text{g}/\text{mouse}$ of affinity chromatography-purified IgG to each chemokine (CXCL1 and CXCL2), an equal quantity of irrelevant IgG matched for species of origin, or an equal volume of saline. Compared with those receiving saline or irrelevant IgG, mice given cytokine-specific immunoglobulin demonstrated a remarkable decrease in neutrophil invasion of the renal extraglomerular cortex (Figure 4A) as well as of the glomeruli themselves (Figure 4B) ($P < 0.01$, comparing mice receiving immune IgG with those given saline or control IgG). This was true at both 2 and 6 hours after Stx2/LPS injection (Figure 4, A and B), and the results were confirmed with an identical experiment (data not shown). These data strongly suggest that CXCL1/KC and CXCL2/MIP-2 play a functionally dominant role in *E. coli* Stx2/LPS-induced neutrophil accumulation in the kidney.

To define the relative contribution of CXCL1/KC and CXCL2/MIP-2 to neutrophil migration into the kidney in Stx2/LPS-challenged mice, *in vivo* immunoneutralization was performed to target each chemokine individually (Table 2). In the extraglomerular cortex, neutrophil migration was significantly decreased in mice given only IgG to CXCL1/KC and in those injected only with IgG to CXCL2/MIP-2 at both 2 and 6 hours ($P < 0.04$). In glomeruli, neutrophil accumulation was reduced substantially at 6 hours by immunoglobulin of each specificity ($P < 0.036$). For comparison, Table 2 lists outcomes (from Figure 4) when both immunoglobulins, eg, to CXCL1/KC and CXCL2/MIP-2, were injected into mice. Data displayed in Table 2 on single immunoglobulin injections are representative of triplicate biological replicate experiments.

CXCR2 is the receptor for both CXCL1/KC and CXCL2/MIP-2 in mice. Thus, a study was also conducted with Stx2/LPS-injected CXCR2 knockout mice, available only on a BALB/c background (all other experiments performed in C57BL/6 mice). The results demonstrated an 80% reduction of neutrophil accumulation in the cortex of these mice when compared with Stx2/LPS-injected con-

Figure 4. Effect on neutrophil accumulation by *in vivo* neutralization of CXCL1/KC and CXCL2/MIP-2 and in a CXCR2 knockout mouse. **A:** Neutrophils in nonoverlapping fields of extraglomerular renal cortex in mice 2 and 6 hours after intraperitoneal challenge with Stx2/LPS. Intravenous pretreatment with Ig-exp (anti-KC and anti-MIP-2), Ig-ctrl (irrelevant immunoglobulin matched to Ig-exp in quantity and species of origin), or saline was given 18 hours before challenge. Baseline values are those in nonchallenged (normal) mice. $*P < 0.001$, comparing Ig-exp mice with those receiving control immunoglobulin or saline. The data are representative of two biological replicate experiments, eg, four mice per time point. **B:** Neutrophils in glomeruli, reported as percent glomeruli with one or more neutrophils at 2 or 6 hours after Stx2/LPS challenge. A minimum of 80 glomeruli were assessed per tissue section. The experimental conditions and statistical analysis are precisely the same as in **A**. **C:** Two BALB/c CXCR2 knockout and two BALB/c control mice were injected with Stx2/LPS and euthanized at 4 hours, and their kidneys were stained for neutrophils. Shown are neutrophil counts in the nonglomerular cortex from experimental and control mice. $*P < 0.05$, comparing mice with and without the CXCR2 receptor.

Table 2. Effects of Chemokine-Specific Immunoglobulin on Renal Neutrophil Infiltration after Challenge with Stx2/LPS

Site	Pre-treatment infusion (hours)	Study time (% of saline control)	Number of neutrophils	P value*
Extra-glomerular cortex	Ig(CXCL1/KC)	2	64 ± 19	0.012
	Ig(CXCL2/MIP-2)	2	68 ± 11	0.015
	Ig(CXCL1/KC and CXCL2/MIP-2)	2	13 ± 10	<0.001
	Ig(CXCL1/KC)	6	45 ± 8	0.026
	Ig(CXCL2/MIP-2)	6	63 ± 20	0.039
	Ig(CXCL1/KC and CXCL2/MIP-2)	6	17 ± 17	<0.001
Glomeruli	Ig(CXCL1/KC)	2	78 ± 11	0.043
	Ig(CXCL2/MIP-2)	2	103 ± 19	
	Ig(CXCL1/KC and CXCL2/MIP-2)	2	5 ± 5	<0.001
	Ig(CXCL1/KC)	6	48 ± 17	0.012
	Ig(CXCL2/MIP-2)	6	56 ± 25	0.035
	Ig(CXCL1/KC and CXCL2/MIP-2)	6	11 ± 10	<0.001

* Comparing Ig_{exp1} mice with those receiving control immunoglobulin.

trol BALB/c wild-type mice (Figure 4C). Together with prior results (Figures 2 and 3) showing local juxtaglomerular chemokine expression, these results suggest that CXCL1/KC and CXCL2/MIP-2 equally contribute to induction of neutrophil migration into the renal cortex after *in vivo* exposure to *E. coli* Stx2/LPS.

Early Expression of VCAM-1 in Renal Glomeruli

Because the VCAM-1 gene was up-regulated in the kidney early after LPS and Stx2/LPS exposure (Table 1) and because the gene product is important for egress of leukocytes across the endothelium, we studied the presence and time course of immunoreactive VCAM-1 in glomerular vessels after challenge with *E. coli* macromolecules. VCAM-1 was evident in renal glomeruli within 2 hours of challenge with LPS or Stx2/LPS (Figure 5A). That is, at 2, 8, 12, and 24 hours after Stx2/LPS, the number of VCAM-1-positive glomeruli was 20% greater than with LPS alone. Further, VCAM-1 protein was easily identified immunohistochemically on capillary endothelium (Figure 5C). Overall VCAM-1-positive glomeruli were most abundant at 4 to 6 hours and gradually declined thereafter (Figure 5A). Staining was also seen in the cortex at 2 to 24 hours (Figure 5C) but was absent in the 0-hour control (Figure 5B). The presence and time course of VCAM-1 protein in kidney of mice injected with LPS or Stx2/LPS was confirmed using enzyme-linked immunosorbent assay, and most VCAM-1 protein was found at 4 to 6 hours (data not shown). In the Stx2/LPS model, VCAM-1 expression and neutrophil migration into glomeruli were almost coincident, both being remarkably elevated between 2 to 8 hours after Stx2/LPS challenge (Figure 5D). The data indicate that VCAM-1 is expressed on endothelial surfaces early in our model, and its time course suggests it is integral to neutrophil egress into kidney after challenge with *E. coli*-derived molecules Stx2/LPS.

Discussion

The identification of chemokines has been an important step toward a better understanding of cell trafficking that initiates inflammation. Their critical role in neutrophil mi-

gration from the circulation has been demonstrated *in vivo* in animals that lack one or more members of the superfamily of G protein-coupled chemokine receptors such as in the CXCR2-deficient mouse.¹³ In fully constituted animals, chemokine receptor-bearing cells such as neutrophils migrate toward the cellular source of the chemokine, where subsequent interaction with endothelial adhesion molecules initiates the process leading to firm adhesion and diapedesis into tissue. The current study defines the source, timing, identity, and functional significance of chemokines and adhesion factors expressed in the kidney, the primary target organ in *E. coli* O157:H7-induced disease.

Redundancy among soluble peptides/chemotactic factors able to effect migration of leukocytes to a particular organ is well established. Further, peptides resulting from transcription of the chemokine gene superfamily show homologous sequences and a highly conserved cysteine motif. Four families of chemokines are described depending on whether the second two cysteines are separated (CXC) or not (CC) by an intervening amino acid, whether the second cysteine is missing (C), or whether the first pair of cysteines is separated by three intervening amino acids (CX3C).¹⁴ Only the Gly-Leu-Arg-containing CXC cytokines are regarded as chemotactic for neutrophils.¹⁵ In addition to CXCL1/KC and CXCL2/MIP-2, other peptides or lipids including C5a, PAF, LTB₄, NAP2, and NCFA have been shown to be chemotactic for neutrophils.¹⁶ Given this array of factors of potential interest, we turned to an approach that involved an initial discovery phase using microarray analysis of control and experimental tissue to identify those chemokine-related genes that are significantly up-regulated in a time course consistent with neutrophil invasion in the model. Among the seven established neutrophil chemoattractants in mice, we found that mRNA for only two, CXCL1/KC and CXCL2/MIP-2, were elevated by >10-fold and thus focused the majority of our studies on these peptides.

The CXC chemokine CXCL1/KC was originally identified in platelet-derived growth factor-stimulated Balb/C 3T3 fibroblasts.¹⁷ Although KC shows 65% amino acid sequence identity with human gro- α and shares functional properties with human interleukin (IL)-8, no struc-

Figure 5. Time course and localization of VCAM-1 in renal tissue of C57BL/6 mice exposed to LPS alone or a combination of Stx2/LPS. **A:** Comparison of two challenge regimens for induction of VCAM-1 in glomeruli *in vivo*. Both LPS alone and Stx2/LPS significantly up-regulated VCAM-1 expression in glomeruli. * $P < 0.05$, comparing Stx2/LPS for expression of VCAM-1 in glomeruli at 4, 8, 12, 24, and 48 hours. Data are shown as the mean $\pm$ 1 SD. **B:** Absence of VCAM-1 in the renal glomeruli and cortex before challenge with Stx2 or Stx2/LPS. **C:** Immunolocalization VCAM-1 in kidneys of mice at 12 hours (LPS alone) after challenge. **Arrow**, VCAM-1-positive glomerular capillary endothelium; **arrowhead**, VCAM-1 on vascular endothelium associated with a glomerulus. **D:** Simultaneous VCAM-1 expression and neutrophil invasion of glomeruli throughout time in mice challenged with Stx2/LPS. At each time point, glomeruli were evaluated for VCAM-1 expression and for the presence of neutrophils. Data are shown as the mean $\pm$ 1 SD. Values for neutrophils and VCAM-1 at 2 to 12 hours were significantly different from those of the 0-hour (PBS-injected) control ($P \leq 0.02$). Original magnifications, $\times 400$ (**B**, **C**).

tural homologue of IL-8 has been identified in mice.¹⁷ A remarkable set of macromolecules and peptides can induce CXCL1/KC, including LPS, thrombin, double-stranded RNA (poly IC), interferon- γ , tumor necrosis factor (TNF)- α , and IL-1 α , produced by a number of cell types including macrophages, fibroblasts, and endothelial cells.¹⁸ Furthermore, KC transgenic mice show a marked infiltration of neutrophils at the site of transgene expression.¹⁹ Our data show an early localized expression of CXCL1/KC by renal tubular epithelium in the immediate periglomerular area (Figure 2). Evidence that chemokine in this periglomerular location may be relevant includes the near simultaneous appearance of this chemokine and neutrophils in kidney (2 hours; Figure 1E and Figure 2, B–G) and the notion that vectorial flow of blood in the kidney (glomerulus to cortex) likely causes leukocytes from the bloodstream to first enter the glomerulus in response to adjacent chemotactic stimuli, allowing some to egress into tissue. Further movement of neutrophils did not seem to occur beyond Bowman's capsule, which may be a soft barrier to migration. Likewise, prior studies^{1,3} as well as our own data suggest that the CXCL2 chemokine MIP-2 follows a time course nearly identical to that of CXCL1/KC, after induction. Whether

CXCL1/KC and CXCL2/MIP-2 are the functionally relevant molecules involved in neutrophil recruitment to the kidney in response to bacterial products has not been previously clarified.

The current work is the first report of neutrophil chemotaxis in a model of renal inflammation and injury after challenge with *E. coli* LPS and Shiga toxin 2. First, two chemokines (CXCL1/KC and CXCL2/MIP-2) were up-regulated in the kidney and were shown to be essential for attraction of neutrophils that accumulate in renal tissue. Second, mice immunoneutralized *in vivo* to these two chemokines showed reduced renal inflammation (Figure 4, A and B) and prolonged survival after Stx2/LPS challenge (data not shown). Furthermore, studies using knockout mice for CXCR2 (the primary receptor for CXCL1/KC and CXCL2/MIP-2) confirmed these findings. The narrow selection of only two proteins, the rapid rate of their appearance, and the localized expression of these factors in the periglomerular tubular epithelium suggests a highly regulated process for their induction. Third, the presence of Stx2, in addition to LPS, in the induction regimen actively enhanced the expression of CXCL1/KC, CXCL2/MIP-2, and VCAM-1 at the mRNA and protein levels. Cell types other than neutrophils may be important

also in HUS as we have recently reported. Peripheral blood monocytes, as a percentage of all leukocytes, are increased at 8 hours after Stx2/LPS challenge in our mouse model and are activated as indicated by increased cell size, linearized chromatin, and pseudopod formation. In the renal cortex, the number of monocytes per field is elevated greater than twofold at 8, 24, 48, and 72 hours.¹⁰ We believe these findings define important events leading to renal inflammation in HUS, some of which may present targets for therapeutic intervention.

Little is known about the early changes in the histopathology of the kidney in patients with HUS. Prior reports have usually involved pathological material obtained weeks to months after presentation.^{20,21} More recently, Inward and colleagues²² studied 15 children who died in the acute phase of their illness (average of 8 days from presentation) and reported a uniform postmortem pathological appearance. Elastase⁺ and CD15⁺ intraglomerular neutrophils were significantly greater in number than in controls, and glomerular capillary thrombosis was a cardinal finding. Other studies indicate renal neutrophils are activated in HUS, as shown by neutrophil degranulation and raised plasma concentrations of elastase, IL-8, and soluble adhesion molecules.^{23–25} These pathological findings are parallel to those in our study of intraglomerular neutrophils and red blood cell congestion, indicating that a renal neutrophil-rich inflammatory response with associated vascular phenomenon is likely to be important in HUS. Consistent with this, staining of renal sections from Stx2/LPS-challenged mice demonstrated that the percentage of glomeruli positive for fibrin rose from 23% at 0 hour in controls to 75% at 8 hours and to 93% at 48 hours, when thrombi of red blood cells and fibrin were observed in glomerular arterioles.¹⁰ Furthermore, recent functional data in our mouse model show diminished renal function (creatinine and blood urea nitrogen) beginning at 12 hours after challenge with Stx2/LPS and that these parameters continued to deteriorate until death (creatinine was 0.93 mg/dl and blood urea nitrogen was 114.3 mg/dl at 72 hours) versus 0-hour control values of 0.38 mg/dl creatinine and 20.2 mg/dl blood urea nitrogen.¹⁰ In addition, Fernandez and colleagues²⁶ demonstrated a 40% increase in survival in Stx2-challenged mice when neutrophil depleted. We believe, therefore, that pathophysiological findings discovered in the current model may have relevance for *E. coli*-induced renal inflammation in patients.

LPS has enhanced other models of renal injury, resulting in a similar degree of neutrophil influx. Huugen and colleagues²⁷ found that LPS administration dose-dependently increased (by twofold to fourfold) anti-MPO Ig-induced glomerular crescent formation and glomerular necrosis, as well as glomerular neutrophil accumulation (by sixfold). Concomitantly in their model, the number of neutrophils per glomerulus increased from 0.9 to 1.9 when LPS was added to their standard induction regimen of anti-MPO Ig. This degree of neutrophil influx is similar to our model, in which there were 1.8 neutrophils per glomerulus 2 hours after Stx2/LPS challenge. TNF- α was thought to play a role in their model, because *in vivo* immunoneutralization of TNF- α attenuated the

Figure 6. Proposed schema of pathophysiological events leading to the occurrence of kidney injury in *E. coli*-induced inflammatory disease. Intrarenal events were suggested by data in the current study. (Modified from Harel et al, *J Clin Invest* 92:218, 1993.)

LPS-mediated aggravation of anti-MPO Ig-induced glomerulonephritis.²⁷

It is also notable that we were unable to detect an early (2- to 4-hour) increase in ICAM in renal tissue in our model, given previous studies of adhesion molecules in other models of renal injury. For example, immunoglobulin neutralization of ICAM-1 during reperfusion of the ischemic kidney reduced tissue injury.^{28,29} Furthermore, ischemia/reperfusion injury as well as neutrophil infiltration were decreased in ICAM-1^{-/-} (genetically deficient) mice, compared with wild-type controls.³⁰ Immunohistochemical studies in the current model, however, showed no LPS-induced ICAM-1 expression in glomeruli or in the cortex at the time of neutrophil invasion 2 to 4 hours after challenge (data not shown), although microarrays detected late (48 to 72 hours) expression of ICAM mRNA in kidney. In contrast, we found early up-regulation of the VCAM-1 gene, with expression of VCAM-1 protein on glomerular endothelial tufts by 2 hours after exposure to LPS and Stx2. This would suggest that VCAM-1 may facilitate egress of neutrophils into parenchyma as reported by others in hosts with high cytokine levels.³¹ Although VCAM-1 is known to interact with monocytes, for which it is an established adhesion molecule,³² we propose it to be involved in neutrophil migration in our model.

From prior work^{5,6,9,11,12} and the current studies, a unified pathway for renal inflammation after intestinal infection with *E. coli* O157:H7 can be suggested (Figure 6). LPS and Shiga toxin 2 are the primary *E. coli* products inducing renal inflammation, because injection of these in purified form results in kidney histopathology including changes similar to human HUS (Figure 1B). With breakdown in the colonic mucosal epithelial barrier, LPS and

Stx2 gain access to the blood stream and contact renal vascular endothelium, perhaps transported on the surface of formed elements of the blood (neutrophils) as indicated by Te Loo and colleagues.¹¹ LPS with enhancement by Stx2 (Figure 2, B and C; Figure 3, B and C; and Figure 5A) induces expression of chemokines CXCL1/KC and CXCL2/MIP-2 mRNA and protein on periglomerular proximal tubular epithelium as well as VCAM-1 on glomerular capillary tufts (Figure 2, A, E–G; Figure 3, A, E–I; and Figure 5C) to effect neutrophil migration from the blood stream into renal tissue 2 to 6 hours after challenge. These two chemokines are functional in the model and are each central to renal neutrophil infiltration as shown by *in vivo* immunoneutralization experiments with factor-specific immunoglobulin used separately or together (Figure 4, A and B; Table 2). Products released by neutrophils activated in kidney may lead to irreversible renal damage and death in the model. Although exposure to pathogenic *E. coli* may not be preventable in the human population, our study suggests that reduction of local chemokine expression or function in target organs such as the kidney limits neutrophil egress from the circulation and provides a potential strategy for therapeutic intervention in *E. coli* O157:H7-induced renal disease.

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